Figure 2 Repertoire complexity and antigen binding capacity of camel IgG_1, IgG_2 and IgG_3 analysed by radioimmunoprecipitation (A) or Western blotting (B & C).

(A) Serum or purified IgG fractions from healthy or *Trypanoma evansi* infected *C. dromedarius* (CATT titer 1/160 (7)) were incubated with labelled trypanosome lysate, recovered with Protein A Sepharose and analysed by SDS-PAGE. The relative counts recovered are inscribed below each lane. No trypanosome proteins bind to the Protein A or to the healthy camel immunoglobulins.

(B) 20 µg of IgG_1, IgG_2 and IgG_3 from healthy and trypanosome infected animals were separated by SDS-PAGE without prior reduction or heating. The electroblotted proteins were incubated with the labelled trypanosome lysate. The IgG_2 shows a single antigen binding component corresponding to the heavy chain immunoglobulin whereas the IgG_3 fraction appears to contain in addition two larger antigen binding components barely detectable by Ponceau Red staining (C). These are possibly Ig classes copurified as immunocomplexes present in the serum of the infected animals.

METHODS. (^{35}S)-methionine labelled *Trypanosoma evansi* lysate (500,000 counts) (22) was incubated (4°C, 1 hour) with 10 µl of serum or, 20 µg of IgG_1, IgG_2 or IgG_3 in 200 µl of 0.4 M NaCl, 10 mM EDTA, 10 mM Tris (pH 8.3), containing 0.1 M TLCK. 10 mg of Protein A SeDharose suspended in 200 µl of the same buffer was added (4°C, 1 hour). After washing and centrifugation, each pellet was resuspended in 75 µl SDS PAGE sample solution containing DTT, and heated for 3 min. at 100°C. After centrifugation, 5 µl of the supernatant was saved for radioactivity counting and the remainder analysed by SDS PAGE and fluorography.
The nitrocellullose filter of the Western blot of purified fractions IgG_1, IgG_2 and IgG_3 was stained with Ponceau Red (C) or incubated with 1% ovalbumin in TST buffer (Tris 10 mM, NaCl 150 mM, Tween 0,05%) (B). The membrane was extensively washed with TST buffer and incubated for 2 hours with (^{35}S)-labelled trypanosome antigen. To avoid unspecific binding, the labelled trypanosome antigen

lysate was filtered (45 μ) and incubated with healthy camel immunoglobulin and ovalbumin adsorbed on a nitrocellulose membrane.

Figure 3 Amino acid sequences of the V_{H} framework, and hinge/$C_{H}2$ of *Camelus dromedarius* heavy chain immunoglobulins, compared to human (italic) V_{H} framework (subgroup III) and hinges of human IgG (14).

METHODS. Total RNA was isolated from a dromedary spleen (23). mRNA was purified with oligo T-paramagnetic beads (PolyATract-Promega). 1 μg mRNA was used for preparing double-strand cDNA (23) after an oligo-dT priming using enzymes provided by Boehringer Mannheim. 5 μg of cDNA was amplified by PCR in a 100 μl reaction mixture (10mM Tris-HCl pH 8.3, 50 mM KC1,15 mM $MgCl_2$, 0.01% (w/v) gelatine, 200 μM of each dNTP). 25 pmoles of each oligonucleotide of the mouse V_{H} (24), containing a XhoI site, and 5'-CGCCATCAAGGTACCAGT-TGA-3' (see SEQ. ID. NO: 3) were used as primers. The 3' end primer was deduced from partial sequences corresponding to γ chain amino acid 296 to 288 (T.Atarhouch, C. Hamers-Casterman, G. Robinson, private communication) in which one mismatch was introduced to create a KDnI restriction site. After a round of denaturing annealing (94°C for 5 min. and 54°C for 5 min.), 2 U of Taq DNA polymerase were added, to the reaction mixture before subjecting it to 35 cycles of amplification (5). The PCR products were purified by phenol-chloroform extraction followed by HPLC (Genpak-fax column, Waters) and finally by MERMAID (BIO 101, Inc.). After these purification steps, the amplified cDNA was digested with XhoI and KpnI, and ligated into pBluescript.

The clones were sequenced by the dideoxy chain termination method (25). The sequences were translated into amino acids which allowed their assignment to well defined domains of the Ig molecule (14); see SEQ. ID. NO: 4-12

Figure 4 Schematic representation of the structural organisation of the camel immunoglobulins (adapted from 26).

On the basis of size consideration, the IgG_1 fraction possess probably the normal antibody assembly of two light and two heavy chains. IgG_3 would have a hinge comparable in size to the human IgG_1, IgG_2 and IgG_4. The two antigen binding sites

are much closer to each other as this camel IgG lacks the C_H1 domain. In the camel IgG_2 the long hinge, being formed of Pro-X repeats (X= Glu, Gln or Lys), most likely adopt a rigid structure (19,20). This long hinge could therefore substitute the C_H1 domain and bring the two antigen binding sites of IgG_2 to normal positions.

--- End of Draft publication ---

Background of the invention

Already at a very early stage during evolution antibodies have been developed to protect the host organisms against invading molecules or organisms. Most likely one of the earliest forms of antibodies must have been developed in *Agnatha*. In these primitive fishes antibodies of the IgM type consisting of heavy and lights chains have been detected. Also in many other forms of life ranging from amphibians to mammals antibodies are characterized by the feature that they consist of two heavy and two light chains, although the heavy chains of the various classes of immunoglobulins are quite different. These heavy and light chains interact with each other by a number of different physical forces, but interactions between hydrophobic patches present on both the heavy and light chain are always important. The interaction between heavy and light chains exposes the complementarity determining regions (CDRs) of both chains in such a way that the immunoglobulin can bind the antigen optimally. Although individual heavy or light chains have also the capability to bind antigens (Ward *et al.*, Nature 341 (1989) 544-546 = ref. 5 of the above given draft publication) this binding is in general much less strong than that of combined heavy and light chains.

Heavy and light chains are composed of constant and variable domains. In the organisms producing immunoglobulins in their natural state the constant domains are very important for a number of functions, but for many applications of antibodies in industrial processes and products their variable domains are sufficient. Consequently many methods have been described to produce antibody fragments.

One of these methods is characterized by cleavage of the antibodies with proteolytic enzymes like papain and pepsin resulting in (a) antibody fragment comprising a light

chain bound via an S-S bridge to part of a corresponding heavy chain formed by proteolytic cleavage of the heavy chain (Fab), or (b) a larger fragment of the antibody comprising two of these Fabs still connected to each other via an S-S bridge in enlargements of the heavy chain parts, indicated with $F(ab)_2$, respectively (see patent applications EP-A-0125023 (GENENTECH / Cabilly *et al.*, 1984) and WO-A-93/02198 (TECH. RES. CENT. FINLAND / Teeri *et al.*, 1993) for definitions of these abbreviations). The disadvantage of the enzymatic route is that the production of whole antibodies is expensive and the enzymatic processing increases the costs of these fragments even more. The high costs of antibody fragments block the application of these fragments in processes and products outside the pharmaceutical industry.

Another method is based on linkage on DNA level of the genes encoding (parts of) the heavy chain and the light chain. This linkage and the subsequent production of these chimeric immunoglobulins in microorganisms have been described (for Fab fragments see e.g. Better *et al.*, Science **240** (1988) 1041-1043, for F_v fragments (combination of variable fragments of the heavy chain (V_H) and light chain (V_L) still connected to each other by non-covalent binding interactions) see e.g. Skerra *et al.*, Science **240** (1988) 1938, and for single chain F_v fragments (ScF_v; an F_v fragment in which the two variable fragments are linked to each other by a linker peptide) see e.g. Bird *et al.*, Science **242** (1988) 423-426. Provided that an appropriate signal sequence has been placed in front of the single chain V_H and V_L antibody fragment (ScF_v), these products are translocated in *E. coli* into the periplasmic space and can be isolated and activated using quite elaborate and costly procedures. Moreover the application of antibody fragments produced by *E. coli* in consumer products requires extensive purification processes to remove pyrogenic factors originating from *E. coli*. For this and other reasons the production of ScF_v in microorganisms that are normally used in the fermentation industry, like prokaryotes as *Streptomyces* or *Bacillus* (see e.g. Wu *et al.* Bio/Technology **11** (1993) 71) or yeasts belonging to the genera *Saccharomyces* (Teeri *et al.*, 1993, *supra*), *Kluyveromyces*, *Hansenula*, or *Pichia* or moulds belonging to the genera *Aspergillus* or *Trichoderma* is preferred. However with a very few exceptions the production of ScF_v antibodies using these systems

proved to be impossible or quite poor. Although the exact reasons for the poor production are not well known, the use of linkers between the V_H and V_L chains not designed for secretion (Teeri *et al.*, 1993, *supra*) may be a reason.

Another reason may be incorrect folding of ScF_v. The frameworks and to a limited extend the CDRs of variable domains of light and heavy chains interact with each other. It has been described by Chothia *et al.* (J. Mol. Biol. 186 (1985) 651-663 = ref. 13 of the above given draft publication) that this interaction involves amino acids at the following positions of the variable region of the heavy chain: 35, 37, 39, 44-45, 47, 100-103 and 105 (numbering according to Kabat *et al.*, In "*Sequences of Proteins of Immunological Interest*, Public Health Service, NIH, Washington DC, 1983 = ref. 14 of the above given draft publication). Especially leucine at position 45 is strongly conserved and the whole apolar side chain of this amino acid seems to be involved in the interaction with the light chain. These strong interactions may fold the ScF_v into a structure that can not be translocated in certain types of lower eukaryotes.

Thus the use of a linker in the production of ScF_v for connecting a V_H chain to a V_L chain, might negatively influence either the translocation, or the folding of such ScF_v or both.

Not prior-published European patent application 92402326.0 filed 21.08.92 (C. Casterman & R. Hamers) discloses the isolation of new animal-derived immunoglobulins devoid of light chains (also indicated as heavy chain immunoglobulins), which can especially originate from animals of the camelid family (*Camelidae*). This European patent specification, now publicly available as EP-A1-0 584 421, is incorporated herein by reference. These heavy chain immunoglobulins are characterized in that they comprise two heavy polypeptide chains sufficient for the formation of one or more complete antigen binding sites, whereby a complete antigen binding site means a site which will alone allow the recognition and complete binding of an antigen, which can be verified by any known method regarding the testing of the binding affinity. The European patent specification further discloses methods for

isolating these heavy chain immunoglobulins from the serum of *Camelidae* and details of the chemical structure of these heavy chain immunoglobulins. It also indicates that these heavy chain immunoglobulins and derivatives thereof can be made by using recombinant DNA technology in both prokaryotes and eukaryotes. The present invention relates to a further development of the work disclosed in that prior-filed but not prior-published European specification.

Due to the absence of light chains in most of the immunoglobulins of *Camelidae* such linkers are not necessary, thereby avoiding the above-mentioned potential problems.

As described above in the draft publication for Nature, now publicly available as Nature 363 (3 June 1993) 446-448, and in the not prior-published European patent application 92402326.0 (*supra*) it was surprisingly found that the majority of the protein A-binding immunoglobulins of *Camelidae* consists just of two heavy chains and that these heavy chains are quite different from common forms of heavy chains, as the C_H1 domain is replaced by a long or short hinge (indicated for IgG_2 and IgG_3, respectively, in Figure 4 of the above given draft publication for Nature). Moreover these heavy chains have a number of other features that make them remarkably different from the heavy chains of common immunoglobulins. One of the most significant features is that they contain quite different amino acid residues at those positions involved in binding to the light chain, which amino acids are highly conserved in common immunoglobulins consisting of two heavy and two light chains (see Table 1 and SEQ. ID. NO: 13-31).

Table 1 **Comparison af amino acid sequences of various immunoglobulins**

Alignment of a number of V_H regions of Camel heavy chain antibodies compared with those of mouse (M, top line) and human (H, second line). Framework fragments are indicated in capitals, CDR fragments in small print; see SEQ. ID. NO: 13-31 for sequences indicated by M, H, 1, 2, 3, 7, 9, 11, 13, 16, 17, 18, 19, 20, 21, 24, 25, 27, 29, respectively.

	1				50
m	EVKLVESGGG	LVQPGGSLRL	SCATSGFTFS	dfyme..WVR	QPPGKRLEWI
h	EVQLVESGGG	LVQPGGSLRL	SCAASGFTFS	syams..WVR	QAPGKGLEWV
cam1	........GG	SVQAGGSLRL	SCAASGYSNC	pltws..WYR	QFPGTEREFV
cam2	DVQLVASGGG	SVQAGGSLRL	SCTASGDSFS	rfams..WFR	QAPGKECELV
cam3	........GG	SVQTGGSLRL	SCAVSGFSFS	tscma..WFR	QASGKQREGV
cam7	........GG	SVQGGGSLRL	SCAISGYTYG	sfcmg..WFR	EGPGKEREGI
cam9	........GG	SVQAGGSLTL	SCVYTNDTGT	...mg..WFR	QAPGKECERV
cam11	........GG	SVQAGGSLRL	SCNVSGSPSS	tyclg..WFR	QAPGREREGV
cam13	........GG	SVEAGGSLRL	SCTASGYVSS	...ma..WFR	QVPGQEREGV
cam16	........GG	SAQAGGSLRL	SCAAHGIPLN	gyyia..WFR	QAPGKGREGV
cam17	........GG	SVQPGGSLTL	SCTVSGATYS	dysig..WIR	QAPGKDREVV
cam18	........GG	SVQAGGSLRL	SCTGSGFPYS	tfclg..WFR	QAPGKEREGV
cam19	........GG	SVQAGGSLRL	SCAASDYTIT	dycma..WFR	QAPGKERELV
cam20	........GG	SVQVGGSLRL	SCVASTHTDS	stcig..WFR	QAPGKEREGV
cam21	........GG	SVQVGGSLKL	SCKISGGTPD	rvpkslaWFR	QAPEKEREGI
cam24	........GG	SVQAGGSLRL	SCNVSGSPSS	tyclg..WFR	QAPGKEREGV
cam25	........GG	SVQTGGSLRL	SCEISGLTFD	dsdvg..WYR	QAPGDECKLV
cam27	........GG	SVQAGGSLRL	SCASSSKYMP	ctydmt.WYR	QAPGKEREFV
cam29	.....exxGG	SVQAGGSLRL	SCVASGFNFE	tsrma..WYR	QTPGNVCELV

	51				100
m	A..asrnkan	dytteysasv	kgRFIVSRDT	SQSILYLQMN	ALRAEDTAIY
h	S..xisxktd	ggxtyyadsv	kgRFTISRDN	SKNTLYLQMN	SLRAEDTAVY
cam1	S..smd...p	dgntkytysv	kgRFTMSRGS	TEYTVFLQMD	NLKPEDTAMY
cam2	S..siq...s	ngrtteadsv	qgRFTISRDN	SRNTVYLQMN	SLKPEDTAVY
cam3	Aainsgggrt	yyntyvaesv	kgRFAISQDN	AKTTVYLDMN	NLTPEDTATY
cam7	A..tiln..g	gtntyyadsv	kgRFTISQDS	TLKTMYLLMN	NLKPEDTGTY
cam9	A..hit...p	dgmtfidepv	kgRFTISRDN	AQKTLSLRMN	SLRPEDTAVY
cam11	T..aint..d	gsiiyaadsv	kgRFTISQDT	AKETVHLQMN	NLQPEDTATY
cam13	A..fvqt..a	dnsalygdsv	kgRFTISHDN	AKNTLYLQMR	NLQPDDTGVY
cam16	A..ting..g	rdvtyyadsv	tgRFTISRDS	PKNTVYLQMN	SLKPEDTAIY
cam17	A..aant..g	atskfyvdfv	kgRFTISQDN	AKNTVYLQMS	FLKPEDTAIY
cam18	A..gins..a	ggntyyadav	kgRFTISQGN	AKNTVFLQMD	NLKPEDTAIY
cam19	A.aiqvvrsd	trltdyadsv	kgRFTISQGN	TKNTVNLQMN	SLTPEDTAIY
cam20	A..siyf..g	dggtnyrdsv	kgRFTISQLN	AQNTVYLQMN	SLKPEDSAMY
cam21	A..vlst..k	dgktfyadsv	kgRFTIFLDN	DKTTFSLQLD	RLNPEDTADY
cam24	T..aint..d	gsviyaadsv	kgRFTISQDT	AKKTVYLQMN	NLQPEDTATY
cam25	Sgilsdgtpy	tksgdyaesv	rgRVTISRDN	AKNMIYLQMN	DLKPEDTAMY
cam27	S..sin...i	dgkttyadsv	kgRFTISQDS	AKNTVYLQMN	SLKPEDTAMY
cam29	S..siy...s	dgktyyvdrm	kgRFTISREN	AKNTLYLQLS	GLKPEDTAMY

Table 1 (Cont.) Comparison af amino acid sequences of various immunoglobulins

Alignment of a number of V_H regions of Camel heavy chain antibodies compared with those of mouse (M, top line) and human (H, second line). Framework fragments are indicated in capitals, CDR fragments in small print; see SEQ. ID. NO: 13-31 for sequences indicated by M, H, 1, 2, 3, 7, 9, 11, 13, 16, 17, 18, 19, 20, 21, 24, 25, 27, 29, respectively.

	101			139
m	YCARdyygss	.....y..	f.....dvWG	AGTTVTVSS
h	YCARxxxxxx	xxxxxyyyyh	x....fdyWG	QGTLVTVSS
cam1	YCKTalqpgg	ycgygx....	......clWG	QGTQVTVSS
cam2	YCGAvslmdr	isqh.....	......gcRG	QGTQVTVSL
cam3	YCAAvpahlg	pgaildlkky	......kyWG	QGTQVTVSS
cam7	YCAAelsggs	celpllf...	......dyWG	QGTQVTVSS
cam9	YCAAdwkywt	cgaqtggyf.	......gqWG	QGAQVTVSS
cam11	YCAArltemg	acdarwatla	trtfaynyWG	QGTQVTVSS
cam13	YCAAqkkdrt	rwaeprew..	......nnWG	QGTQVTASS
cam16	FCAAgsrfss	pvgstsrles	.sdy..nyWG	QGIQVTASS
cam17	YCAAadpsiy	ysilxiey..	......kyWG	QGTQVTVSS
cam18	YCAAdspcym	ptmpappird	sfgw..ddFG	QGTQVTVSS
cam19	SCAAtssfyw	ycttapy...	......nvWG	QGTQVTVSS
cam20	YCAIteiewy	gcnlrttf..	......trWG	QGTQVTVSS
cam21	YCAAnqlagg	wyldpnywls	vgay..aiWG	QGTHVTVSS
cam24	YCAArltemg	acdarwatla	trtfaynyWG	RGTQVTVSS
cam25	YCAVdgwtrk	eggiglpwsv	qcedgynyWG	QGTQVTVSS
cam27	YCKIdsypch	ll.....	......dvWG	QGTQVTVSS
cam29	YCAPveypia	dmcs.....	......ryGD	PGTQVTVSS

For example, according to Pessi *et al.* (1993) a subdomain portion of a V_H region of common antibodies (containing both heavy chains and light chains) is sufficient to direct its folding, provided that a cognate V_L moiety is present. Thus it might be expected from literature on the common antibodies that without V_L chains proper folding of heavy chains cannot be achieved. A striking difference between the common antibodies and the *Camelidae*-derived heavy chain antibodies is, that the highly conserved apolar amino acid leucine (L) at place 45 present in common antibodies is replaced in most of the *Camelidae*-derived heavy chain antibodies by the charged amino acid arginine (R), thereby preventing binding of the variable region of the heavy chain to that of the light chains.

Another remarkable feature is that one of the CDRs of the heavy chains of this type of immunoglobulins from *Camelidae*, CDR3, is often much longer than the

corresponding CDR3 of common heavy chains. Besides the two conserved cysteines forming a disulphide bridge in common V_H fragments, the *Camelidae* V_H fragments often contain two additional cysteine residues, one of which often is present in CDR3.

According to the present inventors these features indicate that CDR3 may play an important role in the binding of antigens by these heavy chain antibodies and can compensate for the absence of light chains (also containing CDRs) in binding of antigens by immunoglobulins in *Camelidae*.

Thus, as the heavy chains of *Camelidae* do not have special features for interacting with corresponding light chains (which are absent), these heavy chains are very different from common heavy chains of immunoglobulins and seem intrinsically more suitable for secretion by prokaryotic and lower eukaryotic cells.

The present inventors realized that these features make both intact heavy chain immunoglobulins of *Camelidae* and fragments thereof very attractive for their production by microorganisms. The same holds for derivatives thereof including functionalized fragments. In this specification the term "functionalized fragment" is used for indicating an antibody or fragment thereof to which one or more functional groups, including enzymes and other binding polypeptides, are attached resulting in fusion products of such antibody fragment with another biofunctional molecule.

Summary of the invention

In a broad sense the invention provides a process for the production of an antibody or a fragment or functionalized fragment thereof using a transformed lower eukaryotic host containing an expressible DNA sequence encoding the antibody or (functionalized) fragment thereof, wherein the antibody or (functionalized) fragment thereof is derived from a heavy chain immunoglobulin of *Camelidae* and is devoid of light chains, and wherein the lower eukaryotic host is a mould or a yeast. Thus the lower eukaryotic host can be a mould, e.g. belonging to the genera *Aspergillus* or *Trichoderma*, or a yeast, preferably belonging to the yeast genera *Saccharomyces*, *Kluyveromcyes*, *Hansenula*, or *Pichia*. Preferably the fragments still contain the whole variable domain of these heavy chains.

The invention also provides methods to produce such heavy chain immunoglobulins or (functionalized) fragments thereof in which methods the framework or the CDRs of these heavy chains are modified by random or directed mutagenesis in such a way that the mutated heavy chain is optimized for secretion by the host microorganism into the fermentation medium.

Another embodiment of the invention is that CDRs can be grafted on these optimized frameworks (compare grafting of CDRs on human immunoglobulins as described by e.g. Jones *et al.*, Nature 321 (1986) 522). These CDRs can be obtained from common antibodies or they may originate from heavy chain immunoglobulins of *Camelidae*. The binding properties may be optimized by random or directed mutagenesis. Thus in a process according to the invention an antibody or (functionalized) fragment thereof derived from a heavy chain immunoglobulin of *Camelidae* can be produced which comprises a CDR different from the CDR belonging to the natural antibody ex *Camelidae* which is grafted on the framework of the variable domain of the heavy chain immunoglobulin ex *Camelidae*.

The invention also provides a method for the microbiological production of catalytic antibodies. These antibodies are preferably raised in *Camelidae* against transition state molecules following procedures similar to the one described by Lerner *et al.*, Science 252 (1991) 659-667. Using random or site-directed mutagenesis such catalytic antibodies or fragments thereof can be modified in such a way that the catalytic activity of these (functionalized) antibodies or fragments can be further improved.

For preparing modified heavy chain antibodies a process according to the invention is provided, in which the DNA sequence encodes a modified heavy chain immunoglobulin or a (functionalized) fragment thereof derived from *Camelidae* and being devoid of light chains, and is made by random or directed mutagenesis or both. Thus the resulting immunoglobulin or (functionalized) fragment thereof is modified such that

- it is better adapted for production by the host cell, or
- it is optimized for secretion by the lower eukaryotic host into the fermentation medium, or
- its binding properties (k_{on} and k_{off}) are optimized, or

- its catalytic activity is improved, or
- it has acquired a metal chelating activity, or
- its physical stability is improved.

Another particular embodiment of the present invention relates to genes encoding fusion proteins consisting of both a heavy chain immunoglobulin from *Camelidae* or part thereof and a second protein or another polypeptide, e.g. an enzyme, in particular an oxido-reductase, and to expression products of such genes. By means of the heavy chain immunoglobulin (fragment) the protein or enzyme can be guided to a target thereby increasing the local efficiency of the protein or enzyme significantly. Thus according to this embodiment of the invention a process is provided, in which the functionalized antibody or fragment thereof comprises a fusion protein of both a heavy chain immunoglobulin from *Camelidae* or a fragment thereof and another polypeptide, e.g. an enzyme, preferably an oxido-reductase.

As a result of a process according to the invention known products may be produced, e.g. antibodies also produced by *Camelidae*, but many of the possible products will be new products, thus the invention also provides new products obtainable by a process according to the invention.

The products so produced can be used in compositions for various applications. Therefore, the invention also relates to compositions containing a product produced by a process according to the invention. This holds for both old products and new products.

Brief Description of the Figures

Figures 1-4 were already described above in the draft publication.

Figure 1 Characterisation and purification of camel IgG classes on Protein A, Protein G and gel filtration.

Figure 2 Repertoire complexity and antigen binding capacity of camel IgG_1, IgG_2 and IgG_3 analysed by radioimmunoprecipitation (A) or Western blotting (B & C).

Figure 3 Amino acid sequences of the V_H framework, and hinge/C_H2 of *Camelus dromedarius* heavy chain immunoglobulins, compared to human (italic) V_H framework (subgroup III) and hinges of human IgG (14); see SEQ. ID. NO: 4-12.

Figure 4 Schematic representation of the structural organisation of the camel immunoglobulins (adapted from 26).

Figure 5 DNA and amino acid sequences of the Camel V_H fragments followed by the Flag sequence as present in pB03 (Figure 5A), pB09 (Figure 5B) and pB24 (Figure 5C); see SEQ. ID. NO: 32-37.

Figure 6 Nucleotide sequence of synthetic DNA fragment cloned into pEMBL9 (Example 1); see SEQ. ID. NO: 38-41.

Figure 7 Schematic drawing of plasmid pUR4423

Figure 8 Schematic drawing of plasmid pUR4426

Figure 9 Schematic drawing of plasmid pUR2778

Figure 10 Schematic drawing of plasmid pUR4429

Figure 11 Schematic drawing of plasmid pUR4430

Figure 12 Schematic drawing of plasmid pUR4445

Figure 13 Schematic drawing of plasmid pUR4446

Figure 14 Schematic drawing of plasmid pUR4447

Figure 15 Schematic drawing of plasmid pUR4451

Figure 16 Schematic drawing of plasmid pUR4453

Figure 17 Schematic drawings of plasmids pUR4437 and pUR4438

Figure 18 Schematic drawings of plasmids pUR4439 and pUR4440

Figure 19 Nucleotide sequence of synthetic DNA fragment cloned into pEMBL9 (Example 6); see SEQ. ID. NO: 42-45.

Figure 20 Schematic drawing of plasmid pAW14B.

Figure 21 Western blot analysis of culture medium of *S. cerevisiae* transformants containing pUR4423M (see A) or pUR4425M (see B). Samples were taken after 24 (see 1) or 48 hours (see 2). For pUR4425M two bands were found due to glycosylation of the antibody fragment.

Detailed description of the invention

The present invention relates to the production of antibodies or (functionalized) fragments thereof derived from heavy chain immunoglobulins of *Camelidae* by eukaryotes, more in particular by lower eukaryotes such as yeasts and fungi.

Therefore, mRNA encoding immunoglobulins of *Camelidae* was isolated and transcribed into cDNA according to the procedures described in the above given draft publication and not prior-published European patent application 92402326.0. In each case primers for the PCR reaction directed to the N-terminus of the V_H domain and PCR primers that either hybridize with the C-terminal regions of the V_H domain or with the short or large hinge regions as described in the above given draft publication, or with the C-terminal region of the C_H2 or C_H3 domains can be used. In this way structural genes can be obtained encoding the following fragments of heavy chain immunoglobulins of *Camelidae* (Table 2).

Table 2. The various forms of immunoglobulins of *Camelidae* that can be expressed in microorganisms.

a.	the variable domain of a heavy chain;
b.	the variable domain and the short hinge of a heavy chain;
c.	the variable domain and the long hinge of a heavy chain;
d.	the variable domain, the C_H2 domain, and either the short or long hinge of a heavy chain;
e.	a complete heavy chain, including either the short or long hinge.

According to procedures described in detail in the Examples these cDNAs can be integrated into expression vectors.

Known expression vectors for *Saccharomyces, Kluyveromcyes, Hansenula, Pichia* and *Aspergillus* can be used for incorporating a cDNA or a recombinant DNA according to the invention. The resulting vectors contain the following sequences that are required for expression: (a) a constitutive, or preferably an inducible, promoter; (b) a leader or signal sequence; (c) one of the structural genes as described in Table 2

and (d) a terminator. If the vector is an episomal vector, it preferably comprises an origin of replication as well as a selection marker, preferably a food grade selection marker, (EP-A-487159, UNILEVER / Leenhouts *et al.*). If the vector is an integration vector, then it preferably comprises sequences that ensure integration and a selection marker in addition to the sequences required for expression of the structural gene encoding a form of the heavy chain immunoglobulin of *Camelidae* or derivatives thereof. The preferred sequences for integration are sequences encoding ribosomal DNA (WO 91/00920, 1991, UNILEVER / Giuseppin *et al.*) whereas the selection marker will be preferably a food grade marker.

For *Saccharomyces* the preferred inducible promoter is the GAL7 promoter (EP-A-0255153, UNILEVER / Fellinger *et al.*); for *Kluyveromyces* the preferred inducible promoter is the inulinase promoter (not yet published EP application 92203932.6, UNILEVER / Toschka & Verbakel, which is incorporated herein by reference); for *Hansenula* or *Pichia* the preferred inducible promoter is the methanol-oxidase promoter (Sierkstra *et al.*, Current Genetics 19 (1991) 81-87) and for *Aspergillus* the preferred inducible promoter is the endo-xylanase promoter (not prior-published PCT application PCT/EP 92/02896, UNILEVER / Gouka *et al.*, now publicly available as WO-A-93/12237, which is incorporated herein by reference).

To achieve efficient secretion of the heavy chain immunoglobulin or parts thereof the leader (secretion) sequences of the following proteins are preferred: invertase and α-factor for *Saccharomyces*, inulinase for *Kluyveromyces*, invertase for *Hansenula* or *Pichia* (Sierkstra *et al.*, 1991 *supra*) and either glucoamylase or xylanase for *Aspergillus* (not prior-published PCT application WO-A-93/12237, *supra*). As food-grade selection markers, genes encoding anabolic functions like the leucine2 and tryptophan3 are preferred (Giuseppin *et al.* 1991, *supra*). The present invention describes the heterologous production of (functionalized) derivatives or fragments of immunoglobulins in a microorganism, which immunoglobulins in nature occur not as a composite of heavy chains and light chains, but only as a composite of heavy chains. Although the secretion mechanism of mammals and microorganisms is quite similar, in details there are differences that are important for developing industrial processes.

To obtain frameworks of the heavy chain immunoglobulins, that are optimally secreted by lower eukaryotes, genes encoding several different heavy chains can be cloned into the coat protein of bacteriophages and subsequently the frameworks of these heavy chain immunoglobulins can be mutated using known PCR technology, e.g. Zhou *et al.*, (1991). Subsequently the mutated genes can be been cloned in *Saccharomyces* and *Aspergillus* and the secretion of the mutated genes can be compared with the wild type genes. In this way frameworks optimized for secretion may be selected.

Alternatively these structural genes can be linked to the cell wall anchoring part of cell wall proteins, preferably GPI-linked cell wall proteins of lower eukaryotes, which result in the expression of a chimeric protein on the cell wall of these lower eukaryotes (not prior-published EP application 92202080.5, UNILEVER / Klis *et al.*, now publicly available as International (PCT) patent application WO-A-94/01567, which is incorporated herein by reference).

Both methods have the advantage that the binding parts of the immunoglobulins are well exposed to the surrounding of the cell, microorganism, or phage and therefore can bind antigens optimally. By changing the external conditions the binding rates and dissociation rates of this binding reaction can be influenced. Therefore, these systems are very suitable to select for mutated immunoglobulins that have different binding properties. The mutation of the immunoglobulins can either be obtained by random mutagenesis, or directed mutagenesis based on extensive molecular modelling and molecular dynamical studies.

mRNAs encoding heavy chains of immunoglobulins raised in *Camelidae* against transition state molecules (Lerner *et al.*, 1991 *supra*) can be obtained using standard techniques. The structural genes encoding various forms of immunoglobulins according to the invention as summarized in Table 2 can be cloned into the coat protein of bacteriophages or as fusion with the anchoring part of cell wall proteins and can be tested on the catalytic property. In this way immunoglobulins or parts thereof having catalytic properties can be determined and selected. Genes encoding these selected immunoglobulins or parts thereof can be mutated as described before and recloned in bacteriophages, but preferably cloned as chimeric cell wall bound catalysts in lower eukaryotes. By performing appropriate catalytic assays, catalytic

immunoglobulins or parts thereof with improved catalytic properties can be determined and selected using standard techniques.

An important application of antibodies, especially outside the pharmaceutical industry, will be chimeric proteins consisting of the binding part of antibodies and enzymes. In this way catalytic biomolecules can be designed that have two binding properties, one of the enzyme and the other of the antibody. This can result in enzymes that have superior activity. This can be illustrated with the following examples:

a. If the substrate of the enzymic reaction is produced by an organism or an enzyme is recognized by the binding domain of the antibody, the local concentration of the substrate will be much higher than for enzymes lacking this binding domain and consequently the enzymic reaction will be improved. In fact this is a mimic of vectorial metabolism in cells (compare e.g. Mitchell, (1979) Science **206** 1148-1159);

b. If the substrate of the enzymic reaction is converted into a molecule that kills organisms, then the efficiency and specificity of killing can be increased significantly if the enzyme is equipped with an antibody binding domain that recognizes the target organism (e.g. compare Takahashi *et al.*, (1993) Science **259** 1460-1463);

The invention will be illustrated by the following Examples without being limited thereto. In previously filed Unilever patent specifications several expression vectors were described, e.g. for the yeasts *S. cerevisiae*, *Kluyveromyces*, and *Hansenula*, and the mould *Aspergillus*. Examples of these publications are EP-A-0173378 (UNILEVER / Ledeboer *et al.*), EP-A-0255153, *supra*, and PCT applications WO-A-91/19782 (UNILEVER / van Gorcom *et al.*) and (not prior-published) WO-A-93/12237, *supra*. The genes encoding antibodies or (functionalized) fragments thereof according to the invention can be incorporated into the earlier described expression vectors or derivatives thereof using procedures well known to a skilled person in the art. All techniques used for the manipulation and analysis of nucleic acid materials were performed essentially as described in Sambrook *et al.* (1989)

(see also ref. 23 of the above given draft publication), except where indicated otherwise.

In the description of the Examples the following endonuclease restriction sites are used:

5	*Afl*II	C↓TTAAG	*Mlu*I	A↓CGCGT
	*Bsp*HI	T↓CATGA	*Nco*I	C↓CATGG
	*Bsp*HI	T↓CATGA	*Not*	GC↓GGCCGC
	*Bst*EII	G↓GTNACC	*Nru*I	TCG↓CGA
	*Eag*I	C↓GGCCG	*Sal*I	G↓TCGAC
10	*Eco*RI	G↓AATTC	*Xho*I	C↓TCGAG
	*Hin*dIII	A↓AGCTT	*Bbs*I	$GAAGAC(N)_2$↓ $CTTCTG(N')_6$↓

Example 1 Construction of cassettes encoding V_H fragments originating from *Camelidae*.

For the production of V_H fragments originating from *Camelidae*, the antibody gene fragments were isolated and cloned as described above in the draft publication. The thus obtained gene fragments encode the V_H region, a short or a long hinge region and about 14 amino acids of the C_H2 region. By using standard molecular biological techniques (e.g. PCR technology), the V_H gene fragments could be subcloned and equipped at their 5'-ends with a gene fragment encoding the *pelB* signal sequence and at their 3'-ends with a gene fragment encoding the Flag tail (13 amino acids). Three of these clones were named pB3, pB9 and pB24 and were deposited at the Centraal Bureau voor Schimmelcultures, Baarn on 20 April 1993 with deposition numbers: CBS 270.93, CBS 271.93 and CBS 272.93, respectively. The DNA and amino acid sequences of the *Camelidae*-V_H fragments followed by the Flag sequence are presented in Figure 5(A-C); see SEQ. ID. NO: 32-37.

1.1 Construction of pUR4421

For the construction of yeast expression plasmids encoding the V_H fragments preceded by the invertase (=SUC2) signal sequence, the α-mating factor prepro-

sequence, or the inulinase signal sequence and followed by either nothing, or a Myc tail or Flag tail, the constructs described below can be prepared.

The multiple cloning site of plasmid pEMBL9 (Denthe *et al.*, 1983) (ranging from the *Eco*RI to the *Hin*dIII site) was replaced by a synthetic DNA fragment having the nucleotide sequence as indicated in Figure 6; see SEQ. ID. NO: 38-41. The 5'-part of this nucleotide sequence comprises an *Eag*I site, the first 4 codons of the *Camelidae* V_H gene fragment and a *Xho*I site coinciding with codons 5 and 6. The 3'-part comprises the last 5 codons of the *Camelidae* V_H gene (encoding VTVSS; see SEQ. ID. NO: 47) part of which coincides partially with a *Bst*EII site), eleven codons of the Myc tail, and an *Eco*RI site. The *Eco*RI site, originally present in pEMBL9, is not functional any more, because the 5'- end of the nucleotide sequence contains AATTT instead of AATTC, indicated in Figure 6 as "(*Eco*RI)". The resulting plasmid is called pUR4421.

1.2 Constructs with Flag tail.

After digesting the plasmid pB3 with *Xho*I and *Eco*RI, a DNA fragment of approximately 425 bp was isolated from agarose gel. This fragment codes for a truncated V_H-Flag fragment, missing the first 5 amino acids of the *Camelidae* V_H. The obtained fragment can be cloned into pUR4421. To this end plasmid pUR4421 can be digested with *Xho*I and *Eco*RI, after which the about 4 kb vector fragment can be isolated from an agarose gel. Ligation with the about 425 bp fragment will result in plasmid pUR4421-03F.

1.3 Constructs with Myc tail.

After digesting the plasmid pB3 with *Xho*I and *Bst*EII, a DNA fragment of approximately 365 bp was isolated from agarose gel. This fragment codes for a truncated V_H fragment, missing both the first 4 (QVKL; see SEQ. ID. NO: 46) and the last 5 (VTVSS; see SEQ. ID. NO: 47) amino acids of the *Camelidae* V_H fragment.

The obtained fragment was cloned into pUR4421. To this end plasmid pUR4421 was digested with *Xho*I and *Bst*EII, after which the about 4 kb vector fragment was isolated from an agarose gel. Ligation with the about 365 bp fragment resulted in

plasmid pUR4421-03M, in which the gene encoding the V_H fragment is reconstituted.

1.4 Constructs encoding V_H only.

Upon digesting pUR4421-03M or pUR4421-03F with *Bst*EII and *Hin*dIII, the vector fragments of about 4.4 kb can be isolated from agarose gel and religated in the presence of a synthetic linker peptide having the following sequence:

```
BstEII                  HindIII
GTCACCGTCTCCTCATAATGA
       GCAGAGGAGTATTACTTCGA
```

(see SEQ. ID. NO: 48-49).

In the thus obtained plasmid, pUR4421-03, the Myc tail or Flag tail sequences are removed and the V_H gene fragment is directly followed by a stop codon.

1.5 Other constructs.

After isolating the gene fragments encoding V_H-hinge-C_H2 fragments as described above in the draft publication, or encoding the intact heavy chain immunoglobulin, it is possible, e.g. by using PCR technology, to introduce an appropriate restriction enzyme recognition site (e.g. *Eco*RI or *Hin*dIII) downstream of the hinge region, downstream of the C_H2 region, or downstream of the total gene. Upon isolating a *Xho*I-*Eco*RI or *Xho*I-*Hin*dIII fragment encoding the V_H fragment with a C-terminal extension, the fragment can be cloned into pUR4421 digested with the same restriction enzymes.

In analogy with the construction of pUR4421-03, a number of other constructs can be produced encoding functionalized heavy chain fragments in which a second polypeptide is fused to the C-terminal part of the V_H fragment. Optionally, the V_H fragment and the second polypeptide, e.g. an enzyme, might be connected to each other by a peptide linker.

To this end either the *Bst*EII-*Hin*dIII fragment or the *Bst*EII-*Eco*RI fragment of either pUR4421-03F or pUR4421-03M has to be replaced by another *Bst*EII-*Hin*dIII or *Bst*EII-*Eco*RI fragment. The latter new fragment should code for the last amino acids (VTVSS, see SEQ.ID. NO: 47) of the V_H fragment, optionally for a linker peptide, and for the polypeptide of interest e.g. an enzyme. Obviously, the introduction of the DNA fragment should result in an in frame fusion between the

V_H gene fragment and the other DNA sequence encoding the polypeptide of interest.

Alternatively, it is possible to replace the *Eag*I-*Xho*I fragment of pUR4421-03 with another DNA fragment, coding for a polypeptide of interest, optionally for a peptide linker, and for the first 4 (QVKL, see SEQ.ID. NO: 46) amino acids of the V_H fragment, resulting in an in frame fusion with the remaining part of the V_H fragment. In this way, it is possible to construct genes encoding functionalized V_H fragments in which the second polypeptide is fused at the N-terminal part of the V_H fragment, optionally via a peptide linker.
Obviously, it is also possible to construct genes encoding functionalized V_H fragments having a polypeptide fused to the N-terminal as well as fused to the C-terminal end, by combining the above described construction routes.
The polypeptides used to functionalize the V_H fragments might be small, like the Myc and the Flag tails, or intact enzymes, like glucose oxidase, or both.

From all the above described constructs, derived from pUR4421, an appropriate *Eag*I-*Hin*dIII fragment, encoding the functionalized V_H fragment, can be isolated and cloned into a number of different expression plasmids. Several are exemplified in more detail in the following Examples. Although only the V_H fragments are exemplified, similar constructs can be prepared for the production of larger heavy chain fragments (e.g. V_H-hinge or V_H-hinge-C_H2) or intact heavy chains. The *Eag*I site is introduced before the first codon of the V_H fragment, facilitating an in frame fusion with different yeast signal sequences.
In particular cases, were additional *Eag*I and/or *Hin*dIII sites are present in the cloned fragments, it is necessary to perform partial digestions with one or both restriction enzymes.

Although the above and following constructions only consider the V_H fragment cloned in pB3, a comparable construction route can be used for the construction of expression plasmids for the production of V_H fragments like V_H-09 and V_H-24, or other V_H fragments.

Example 2 Construction of *S. cerevisiae* episomal expression plasmids for *Camelidae* V_H.

For the secretion of recombinant protein from *S. cerevisiae* it is worthwhile to test in parallel the two most frequently applied homologous signal sequences, the SUC2 invertase signal sequence and the prepro-α mating factor sequence.

The episomal plasmid pSY1 and pSY16 (Harmsen *et al.*, 1993) contain expression cassettes for the α-galactosidase gene. Both plasmids contain the GAL7 promoter and PGK terminator sequences. pSY1 contains the invertase (SUC2) signal sequence and pSY16 contains a slightly modified (Harmsen *et al.*, 1993) prepro-α-mating factor signal sequence.

Both plasmids, pSY1 and pSY16 can be digested with *Eag*I and *Hin*dIII, the about 6500 bp long vector backbone of both plasmids can be isolated and subsequently ligated with the *Eag*I/*Hin*dIII fragments from pUR4421-03F (~465 bp), pUR4421-03M (~455 bp) or pUR4421-03 (~405 bp) (See above).

This results in a series of 6 different episomal plasmids for expression in *S. cerevisiae*, containing behind the SUC2- and the α mating factor prepro-sequence the V_H-Flag coding sequence (designated pUR4423F and pUR4426F), the V_H-Myc coding sequence (designated pUR4423M and pUR4426M) or the coding sequence of V_H followed by a stop codon (designated pUR4423, Figure 7 and pUR4426, Figure 8).

Obviously, it is possible to use promoter systems different from the inducible GAL7 promoter, e.g. the constitutive GAPDH promoter.

2.1 Production of V_H-03-myc and V_H-24-myc.

After introducing the expression plasmids pUR4423M (coding for V_H-03-myc, preceded by the SUC2-signal sequence) and pUR4425M (coding for V_H-24-myc, preceded by the SUC2-signal sequence) into *S. cerevisiae* via electroporation, transformants were selected from minimal medium agar plates (comprising 0.7 % yeast nitrogen base, 2 % glucose and 2 % agar, supplemented with the essential amino acids and bases).

For the production of antibody fragments the transformants were grown overnight in selective minimal medium (comprising 0.7 % yeast nitrogen base, 2 % glucose,

supplemented with the essential amino acids and bases) and subsequently diluted ten times in YPGal medium (comprising 1 % yeast extract, 2 % bacto pepton and 5 % galactose). After 24 and 48 hours of growth, samples were taken for Western blot analysis (Figure 21). For the immuno detection of the produced V_H-myc fragments monoclonal anti-myc antibodies were used.

In essentially the same way comparable results were obtained with a yeast transformed with pUR4424M containing a DNA sequence encoding the V_H-09-myc protein.

Example 3 Construction of *S. cerevisiae* multicopy integration vectors for the expression of *Camelidae* V_H.

To combine the benefits of high copy number and mitotically stable expression, the concept of a multicopy integration system into the rDNA locus of lower eukaryotes has already been successfully proven (Giuseppin *et al. supra*).

One of these vectors is pUR2778, a derivative of pUR2774 (Giuseppin *et al. supra*) from which the pol1-S.O. reporter gene sequence was removed (Figure 9).

This integrating plasmid, pUR2778, can be used for integration of *Camelidae* V_H coding sequences, hence the vector can be digested with *Sac*I and *Hin*dIII after which the ~7.3 kb vector fragment can be isolated.

From the in example 2 described pUR4423 or pUR4426 types of plasmids, *Sac*I-*Hin*dIII fragments can be isolated encoding a V_H fragment preceded by a signal sequence (SUC2 or α mating factor prepro) and followed by nothing or a Myc or Flag tail.

Ligation of these *Sac*I-*Hin*dIII fragments with the ~7.3 kb vector fragment will result in integration plasmids, encoding the (functionalized) V_H fragments under the regulation of the strong and inducible GAL7 promoter.

In this way the following expression plasmids were obtained:

pUR4429	P_{gal7} - SUC2 sig.seq. - V_{II}-03
pUR4429F	P_{gal7} - SUC2 sig.seq. - V_{II}-03 - Flag tail
pUR4429M	P_{gal7} - SUC2 sig.seq. - V_{II}-03 - Myc tail
pUR4430	P_{gal7} - α mat.fac. prepro. - V_{II}-03
pUR4430F	P_{gal7} - α mat.fac. prepro. - V_{II}-03 - Flag tail
pUR4430M	P_{gal7} - α mat.fac. prepro. - V_{II}-03 - Myc tail

For schematic drawings see Figure 10 for pUR4429 and Figure 11 for pUR4430. Obviously, comparable constructs can be prepared for other heavy chain antibodies or fragments thereof.

As mentioned before, different promoters might be used, for example, the constitutive GAPDH promoter.

Example 4 Construction of expression plasmids for the production of (functionalized) V_{II} fragments from *Camelidae* by *Kluyveromyces*

4.1. Construction of *Kluyveromyces lactis* episomal expression plasmids *Camelidae*.

Yeast strains of the genus *Kluyveromyces* have been used for the production of enzymes, such as ß-galactosidase for many years, and the growth of the strains has been extensively studied. *Kluyveromyces lactis* is well known for the ability to utilize a large variety of compounds as carbon and energy sources for growth. Since these strains are able to grow at high temperatures and exhibit high growth rates, they are promising hosts for industrial production of heterologous proteins (Hollenberg, C. *et al.*, EP-A-0096430, GIST-BROCADES N.V., 1983).

The plasmids pUR2427 and pUR2428 are pTZ19R derivatives with the promoter and the DNA sequence encoding either the signal peptide (=pre-sequence) (in pUR2428), or the natural prepro-sequence (in pUR2427), of inulinase (inu) from *Kluyveromyces marxianus*. Both plasmids contain a unique *Bsp*MI site suitable to create a perfect joint with *Eag*I or *Not*I digested DNA-fragments (not yet published European patent application 92203932.6, *supra*). In both plasmids a unique *Hin*dIII site is located a bit further downstream of the *Bsp*MI-site, so that *Eag*I-*Hin*dIII cut DNA-fragments encoding V_{II} from *Camelidae* either solely or with Myc- or Flag- tail

can be easily ligated into *Bsp*MI-*Hin*dIII digested pUR2427 or pUR2428. Thereby a set of six plasmids can be created containing the promoter and secretion signals of the *Kluyveromyces marxianus* inulinase gene, joint in frame to *Camelidae* Vh encoding sequences, all on a *Eco*RI-*Hin*dIII restriction fragment:

pUR4445 P_{inu} - Inu prepro seq. - V_H - 03
pUR4445M P_{inu} - Inu prepro seq. - V_H - 03 - Myc
pUR4445F P_{inu} - Inu prepro seq. - V_H - 03 - Flag
pUR4446 P_{inu} - Inu pre seq. - V_H - 03
pUR4446M P_{inu} - Inu pre seq. - V_H - 03 - Myc
pUR4446F P_{inu} - Inu pre seq. - V_H - 03 - Flag .

Maps of pUR4445 and pUR4446 are shown in Figure 12 and Figure 13.

The *Eco*RI-*Hin*dIII fragments of these plasmids can be ligated into the expression vector pSK1 (not yet published European patent application 92203932.6, *supra*), from which the α-galactosidase expression cassette including the *GAL7*-promoter is removed with a *Eco*RI(partial) and *Hin*dIII digestion. The resulting plasmids can then be transformed for example in *K. lactis* strain MSK110 (a, *uraA*, *trp1*::*URA3*), as they contain the trp1 marker and the pKD1 episomal plasmid sequences:

pUR4447 P_{inu} - Inu prepro seq. - V_H - 03
pUR4447M P_{inu} - Inu prepro seq. - V_H - 03 - Myc
pUR4447F P_{inu} - Inu prepro seq. - V_H - 03 - Flag
pUR4448 P_{inu} - Inu pre seq. - V_H - 03
pUR4448M P_{inu} - Inu pre seq. - V_H - 03 - Myc
pUR4448F P_{inu} - Inu pre seq. - V_H - 03 - Flag .

A map of pUR4447 is shown in Figure 14.

Transformation can be performed by standard techniques such as the methods of Beggs (1978) or electroporation, using 0.67% Yeast Nitrogen Base (without amino acids) and 2% glucose as the selection medium for transformants.

4.2. Construction of *Kluyveromyces lactis* multicopy integration vectors.

Alternatively, since all tailed and non-tailed versions of the Vh fragments, joined to the inulinase promoter and secretion signals, are located on *Eco*RI-*Hin*dIII fragments, the rDNA multicopy integration plasmid pMIRKGAL-TΔ1 (Bergkamp *et al.*, 1992) can be used in a similar way as the pSK1 plasmid. In order to replace the α-gal expression cassette present in this plasmid, by a antibody fragment cassette, these plasmids have to be digested with *Eco*RI(partial) and *Hin*dIII. After isolating the vector fragments, they can be ligated with the about 1.2 kb *Eco*RI-*Hin*dIII fragments which can be obtained from the plasmids described in example 4.1. The resulting plasmids can be linearized with *Sac*II and transformed to MSK110, resulting in *K. lactis* strains with potentially high and stable expression of single chain V_H fragments.

pUR4449	P_{inu} - Inu prepro seq. - V_H - 03
pUR4449M	P_{inu} - Inu prepro seq. - V_H - 03 - Myc
pUR4449F	P_{inu} - Inu prepro seq. - V_H - 03 - Flag
pUR4450	P_{inu} - Inu pre seq. - V_H - 03
pUR4450M	P_{inu} - Inu pre seq. - V_H - 03 - Myc
pUR4450F	P_{inu} - Inu pre seq. - V_H - 03 - Flag .

4.3. Construction of *Kluyveromyces marxianus* episomal plasmids.

Kluyveromyces marxianus is a yeast which is perhaps even more attractive than *K. lactis* for industrial biotechnology, due to its short generation time on glucose (about 45 minutes) and its ability to grow on a wide range of substrates, and its growth at elevated temperatures (Rouwenhorst *et al.*, 1988).

The shuttle vector pUR2434, containing the leu2 marker and the pKD1 plasmid sequences (not yet published European patent application 92203932.6, *supra*), located on a pUC19 based vector, can be cut with *Eco*RI(partial) and *Hin*dIII to remove the α-galactosidase expression cassette. In this vector the *Eco*RI-*Hin*dIII fragments containing the Vh expression cassettes as described in example 4.1, can be ligated. The resulting plasmids can then be transformed into KMS3, the neat leu2-auxotroph CBS6556 *K. marxianus* strain (Bergkamp, 1993) using the method of Meilhoc *et al.* (1990).

pUR4451 P_{inu} - Inu prepro seq. - V_H - 03
pUR4451M P_{inu} - Inu prepro seq. - V_H - 03 - Myc
pUR4451F P_{inu} - Inu prepro seq. - V_H - 03 - Flag
pUR4452 P_{inu} - Inu pre seq. - V_H - 03
pUR4452M P_{inu} - Inu pre seq. - V_H - 03 - Myc
pUR4452F P_{inu} - Inu pre seq. - V_H - 03 - Flag .

A map of pUR4451 is shown in Figure 15.

4.4 Construction of *Kluyveromyces marxianus* multicopy integration vectors.

For high and stable expression in *Kluyveromyces marxianus*, the multicopy integration system as described by Bergkamp (1993), can be used. The following cloning route, based on the route for constructing pMIRKM-GAL5 (Bergkamp, 1993), results in suitable expression vectors for production of Vh fragments from *Camelidae*. The *Eco*RI-*Nhe*I(Klenow filled) fragments of pUR4447,-M,-F and pUR4448,-M,-F containing the Vh fragment expression cassettes as described in example 4.1, can be isolated and ligated in *Eco*RI-*Eco*RV digested pIC-20H. From the plasmids obtained in this way, and which are equivalents of the pIC-αgal plasmid, the *Bam*HI-*Nru*I fragment can be isolated and ligated with *Bam*HI-*Sma*I digested pMIRKM4. The result of this will be expression vectors which are equivalent to pMIRKM-GAL5, and contain a tailed or non-tailed Vh fragment from camel under control of inulinase promoter and secretion signals, in a vector which also contains the *K. marxianus LEU2*-gene with defective promoter, and *K. marxianus* rDNA sequences for targeted integration into the genome. These vectors can be used to transform for example KMS3.

pUR4453 P_{inu} - Inu prepro seq. - V_H - 03
pUR4453M P_{inu} - Inu prepro seq. - V_H - 03 - Myc
pUR4453M P_{inu} - Inu prepro seq. - V_H - 03 - Flag
pUR4454 P_{inu} - Inu pre seq. - V_H - 03
pUR4454M P_{inu} - Inu pre seq. - V_H - 03 - Myc
pUR4454F P_{inu} - Inu pre seq. - V_H - 03 - Flag .

A map of pUR4453 is shown in Figure 16.

Example 5. Construction of Hansenula polymorpha integrating vectors for the expression of (functionalized) V_H fragments from *Camelidae*.

In search for productive systems able to carry out authentic posttranscriptional processing and overcoming the limitation of higher eukaryotic expression systems, such as high costs, low productivity and the need for stringent control procedures for the detection of contaminating agents could be overcome by the methylotrophic yeast *H. polymorpha*. This strain is able to grow on methanol as its sole carbon and energy source, so the presence of methanol in the growth medium rapidly induces the enzymes of the methanol pathway, such as the key enzymes methanol oxidase (MOX) and dihydroxyacetone synthase (DHAS).

While experiments to express foreign genetic information from an episomal plasmid resulted a low plasmid stability, chromosomal integration is the method of choice (Sierkstra *et al.*, 1991). By utilizing the DNA of the *mox* gene as integration locus the latter were able to express and secrete α-galactosidase regulated by *mox* promoter and -terminator. Here, the *S. cerevisiae* SUC2 signal sequence was proven to be efficiently functional for secretion.

The same approach can be used for expression and secretion of *Camelidae* V_H antibody fragments. Plasmids analogous to pUR3515 (without an origin of replication functional in yeast) and pUR3517 (containing the HARS2 sequence as origin of replication) can be used as expression vectors (Sierkstra *et al.*, 1991). As a starting vector pUR3501 can be used (Sierkstra *et al.*, 1991) in which by means of site directed mutagenesis (e.g. via PCR technology), an *Eag*I restriction site is introduced at the junction between the invertase (=SUC2) signal sequence and the α-galactosidase. From the resulting plasmid, pUR3501*Eag*, it is possible to replace the *Eag*I-*Hin*dIII fragment comprising the α-galactosidase gene by an *Eag*I-*Hin*dIII fragment encoding a (functionalized) antibody fragment, obtained as described in example 1. In case of using the *Eag*I-*Hin*dIII fragments of the pUR4421-03 series (example 1), this would result in plasmids pUR4437 (Figure 17), pUR4437M and pUR4437F. In these plasmids the nucleotide sequence encoding the (functionalized) V_H is preceded by a nucleotide sequence encoding the invertase signal sequence and the *mox* promoter sequence. The obtained plasmids can be digested with *Bam*HI and *Hin*dIII and after filling in the sticky ends with Klenow polymerase, the about

2.6 kb fragments can be ligated into plasmid pUR3511 which was digested with *Sma*I (Sierkstra *et al.*, 1991). In this way the terminator sequence of the *mox* gene can by fused downstream of the V_H encoding sequences. From the thus obtained plasmids, pUR4438 (Figure 17) *Eco*RI-*Hin*dIII fragments of about 3 kb can be isolated, containing the *mox* promoter, the invertase signal sequence, the (functionalized) V_H fragment and the *mox* transcription terminator. Subsequently these fragments can be cloned into plasmid pUR3513 (no yeast origin of replication) or in pUR3514 (HARS origin of replication) as described by Sierkstra *et al.* (1991), resulting in two sets of plasmids:

pUR4439	P_{mox} - SUC2 sig. seq. - V_H - *mox* term. -- no origin
pUR4439M	P_{mox} - SUC2 sig. seq. - V_H - *mox* term. -- no origin
pUR4439F	P_{mox} - SUC2 sig. seq. - V_H - *mox* term. -- no origin
pUR4440	P_{mox} - SUC2 sig. seq. - V_H - *mox* term. -- HARS origin
pUR4440M	P_{mox} - SUC2 sig. seq. - V_H - *mox* term. -- HARS origin
pUR4440F	P_{mox} - SUC2 sig. seq. - V_H - *mox* term. -- HARS origin .

Maps of pUR4439 and pUR4440 are shown in Figure 18.

Essentially the same can be done with other *Eag*I-*Hin*dIII fragment, obtained as described in example 1.

The newly obtained plasmids can be transformed by electroporation of *H. polymorpha* A16 (CBS4732, *leu-*) and can be selected by growing on selective medium containing 0.68% YNB and 2% glucose. Induction medium should contain 0.5% methanol instead of the glucose.

Example 6 **Construction *Aspergillus niger* var. *awamori* integration vectors for the production of V_H fragments from *Camelidae*.**

The multiple cloning site of plasmid pEMBL9 (ranging from the *Eco*RI to the *Hin*dIII site) was replaced by a synthetic DNA fragment having the nucleotide sequence as indicated in Figure 19; see SEQ. ID. NO: 42-45. The 5'- part of the nucleotide sequence contains a *Nru*I restriction site followed by the first codons of the *Camelidae* V_H gene fragment and a *Xho*I restriction site. The 3'-part encodes for

a *Bst*EII restriction site, the last codons of the *Camelidae* V_H gene, eleven codons of the Myc tail and finally a *Eco*RI and a *Afl*II site. The resulting plasmid is pUR4432.

After digesting plasmid pB3 with *Xho*I and *Eco*RI, a DNA fragment of approximately 425 bp can be isolated from agarose gel. This fragment codes for a truncated V_H-Flag fragment, missing the first 5 amino acids of the *Camelidae* V_H.
The obtained fragment can be cloned into pUR4432. To this end plasmid pUR4432 can be digested with *Xho*I and *Eco*RI, after which the about 4 kb vector fragment was isolated from an agarose gel. Ligation with the about 425 bp fragment resulted in plasmid pUR4433F.

After digesting the plamids pB3 with *Xho*I and *Bst*EII, a DNA fragment of approximately 365 bp was isolated from agarose gel. This fragment codes for a truncated V_H fragments, missing the first and last 5 amino acids of the *Camelidae* V_H.
The obtained fragment was cloned into pUR4432. To this end plasmids pUR4432 can be digested with *Xho*I and *Bst*EII, after which the about 4 kb vector fragment was isolated from an agarose gel. Ligation with the about 365 bp fragments resulted in plasmids pUR4433M. In a similar way the *Xho*I-*Bst*EII fragments of pB9 and pB24 were cloned into the pUR4432 vector fragment, resulting in pUR4434M and pUR4435M, respectively.
Upon digesting pUR4433M or pUR4433F with *Bst*EII and *Hin*dIII, the vector fragments of about 4.4 kb can be isolated from agarose gel and religated in the presence of a synthetic linker peptide having the following sequence:

```
BstEII                    AflII      HindIII
GTCACCGTCTCCTCATAATGATCTTAAGGTGATA
      GCAGAGGAGTATTACTAGAATTCCACTATTCGA
```

(see SEQ. ID. NO: 50-51).

In the thus obtained plasmid, pUR4433, the Myc tail or Flag tail sequences are removed and the V_H gene fragment is directly followed by a stop codon.

Analogous as described in example 1.5, it is possible to clone nucleotide sequences encoding longer fragments of the heavy chain immunoglobulins into pUR4432 or to replace the *Bst*EII-*Afl*II fragments of the above mentioned plasmids pUR4433,

pUR4433F or pUR4433M with other *BstE*II-*Afl*II fragments, resulting in frame fusions encoding functionalized V_H fragments, having a C-terminal extension.
Upon replacing the *Nru*I-*Xho*I fragments of pUR4433, pUR4433F or pUR4433M, in frame fusions can be constructed encoding functionalized V_H fragments, having an N-terminal extension.
In the above described constructs an *Nru*I site was introduced before the first codon of the (functionalized) V_H fragment, facilitating an in frame fusion with the precursor-sequence of xylanase, see (not prior-published) WO-A-93/12237, *supra*.
For the construction of *Aspergillus* expression plasmids, from the plasmids pUR4433F, pUR4433M and pUR4433, respectively, an about 455, 445 and 405 bp *Nru*I-*Afl*II fragment has to be isolated encoding the V_H fragment with a Flag, a Myc or no tail.

Plasmid pAW14B was the starting vector for construction of a series of expression plasmids containing the *exlA* expression signals and the genes coding for (functionalized) V_H fragments of *Camelidae* heavy chain antibodies. The plasmid comprises an *Aspergillus niger* var. *awamori* chromosomal 5 kb *Sal*I fragment on which the 0.7 kb *exlA* gene is located, together with 2.5 kb of 5'-flanking sequences and 2.0 kb of 3'-flanking sequences (see Figure 20 and (not prior-published) WO-A-93/12237, *supra*).
Starting from pAW14B, pAW14B-10 was constructed by removing the *Eco*RI site originating from the pUC19 polylinker, and introducing a *Not*I site. This was achieved by digesting plasmid pAW14B with *Eco*RI and after dephosphorylation the linear 7.9 kb *Eco*RI fragment was isolated. The fragment was religated in the presence of the "*Eco*RI"-*Not*I linker:

5'- AATTGCGGCCGC -3' (see SEQ. ID. NO: 52).

Subsequently the *Afl*II site, located downstream of the *exlA* terminator was removed by partially cleaving plasmid pAW14B-10 and religating the isolated, linearized plasmid after filling in the sticky ends, resulting in plasmid pAW14B-11.
Finally, pAW14B-12 was constructed using pAW14B-11 as starting material. After digestion of pAW14B-11 with *Afl*II (overlapping with the *exlA* stop codon) and *Bgl*II

(located in the *exl* promoter) the ~2.4 kb *Afl*II-*Bgl*II fragment, containing part of the *exlA* promoter and the *exlA* gene was isolated as well as the ~5.5 kb *Afl*II-*Bgl*II vector fragment. After partial digestion of this ~2.4 kb fragment with *Bsp*HI (located in the *exl*A promoter and at the *exlA* start codon) an about 1.8 kb *Bgl*II-*Bsp*HI *exlA* promoter fragment (up to the ATG initiation codon) was isolated and ligated with the about 5.5 kb *Afl*II-*Bgl*II vector fragment of pAW14B-11 in the presence of the following adaptor:

```
(BspHI) BbsI      AflII
  CATGCAGTCTTCGGGC
      GTCAGAAGCCCGAATT
```

(see SEQ. ID. NO: 53-54).

For the construction of the V_H expression plasmids, pAW14B-11 can be partially digested with *Nru*I and digested with *Afl*II, after which the ~ 7 kb vector fragment can be isolated from agarose gel and contains the xylanase promoter, the DNA sequence encoding the xylanase signal sequence and the xylanase terminator. Upon ligation of the *Nru*I-*Afl*II fragments of pUR4433M, pUR4434M and pUR4435M with the pAW14B-11 vector, plasmids pUR4436M, pUR4437M and pUR4438M were obtained, respectively. In these plasmids the *Camelidae* V_H polypeptides are preceded by the 27 amino acid long precursor sequence of xylanase and followed by the myc-tail (of 11 amino acids; see Examples 1.3 en 2, Figures 6 and 19, and SEQ.ID. NO: 41 = 45).

In a similar way plasmids can be constructed encoding the V_H fragments followed by the FLAG-tail or without a tail.

After introducing the *amdS* and *pyrG* selection markers into the unique *Not*I site of pUR4436M, pUR4437M and pUR4438M using conventional techniques, e.g. as described in Examples 2 and 3 of (not prior-published) WO-A-93/12237, *supra*, the plasmids were transferred to *Aspergillus*.

Production of the Camel V_H fragments by the selected transformants was achieved by growing the strains in inducing medium essentially as described in example 2.2 of (not prior-published) WO-A-93/12237, *supra*. Western blot analysis of the culture medium was perforemd as described in Example 2.1 above and revealed the presence of the antibody fragments.

Obviously, expression vectors can be constructed in which different promoter systems, e.g. glucoamylase promoter, and/or different signal sequences, e.g. glucoamylase or glucose oxidase signal sequences, are used.

Example 7 Production of glucose oxidase - V_H fusion proteins

Glucose oxidase catalyses the oxidation of D-glucose to D-gluconate under the release of hydrogen peroxide. Glucose oxidase genes (*gox*) from *Aspergillus niger* have been cloned (Frederick *et al.* (1990) J. Biol. Chem. 265 3793, Kriechbaum *et al.*, 1989) and the nucleotide sequences are available from the EMBL data bank under accession numbers J05242 and X16061. The nucleotide sequence of the latter is used as a basis for the following construction route.

Upon cloning the *gox* gene from *A. niger* it is possible, by applying PCR technology, to introduce convenient restriction sites.

To introduce a *Bsp*HI restriction site, overlapping with the ATG initiation codon, the sequence ATC ATG CAG can be changed to ATC ATG AGG. In the same experiment an *Eco*RI restriction site can be introduced which is located upstream of the *Bsp*HI site. This can be achieved by using the following PCR primer:

*Eco*RI *Bsp*HI
5'-TCACTGAATTCGGGATC ATG AGG ACT CTC CTT GTG AGC TCG CTT-3'

(see SEQ. ID. NO: 55).

A second PCR primer, having the following sequence can be used:

*Afl*II *Bbs*I *Sal*I
5'-ATGTCACAAAGCTTAAGCACGAAGACA GTC GAC CGT GCG GCC GGA GAC-3'
*Hin*dIII

(see SEQ. ID. NO: 56)

in the same PCR experiment, in order to introduce a *Bbs*I site, a *Afl*II site and a *Hin*dIII site, downstream of the unique *Sal*I site present in the glucose oxidase gene. After digesting the DNA obtained from this PCR experiment with *Eco*RI and *Hin*dIII, an *Eco*RI - *Hin*dIII fragment of about 160 bp can be isolated and cloned into pEMBL9, which was digested with the same enzymes, resulting in plasmid pGOX1.

From pGOX1 an about 140 bp *Bsp*HI - *Afl*II fragment can be isolated and introduced into the 7.2 kb *Bbs*I-*Afl*II vector fragment of pAW14B-12, resulting in

pAW14B-GOX. In this plasmid, the 5'- part of the *gox* gene, encoding the first 43 amino acids, is fused in frame with the ATG initiation codon of the *exlA* gene.

In a second PCR experiment, a *Mlu*I restriction site can be introduced near the 3'-end of the *gox* by changing the sequence TAT GCT TCC to TAC GCG TCC. In the same experiment a *Hin*dIII site can be introduced downstream of the *Mlu*I site. As a second primer an oligo nucleotide should be used hybridizing upstream of the *Sal*I site. After digesting the DNA obtained from this PCR experiment with *Sal*I and *Hin*dIII, an *Sal*I - *Hin*dIII fragment of about 1.7 kb can be isolated and cloned into pEMBL9, which was digested with the same enzymes, resulting in plasmid pGOX2. Upon digesting pGOX2 with *Mlu*I and *Hin*dIII, an about 5.7 kb vector fragment can be isolated.

From the plasmids pUR4433, pUR4433F, pUR4433M and the like, *Xho*I-*Hin*dIII fragments can be isolated, encoding the truncated *Camelidae* V_H fragment with or without a tail sequence, and missing the first 4-6 N-terminal amino acids (see Example 1). These fragments can be ligated into the 5.7 kb pGOX2 vector fragment by using *Mlu*I-*Xho*I adaptors. These adaptors are designed in such a way that they result in an in frame fusion between the 3'-end of the *gox* gene and the restored V_H gene fragment, optionally intersected with a DNA sequence encoding a peptide linker sequence.

An example of these designed adaptors is:

```
MluI                                                    XhoI
 CGCGTCCATGCAGTCCTCAGGTGGATCATCCCAGGTGAAACTGC
      AGGTACGTCAGGAGTCCACCTAGTAGGGTCCACTTTGACGAGCT
      S  M  Q |S  S  G  G  S  S |Q  V  K  L  L  E
```

(see SEQ. ID. NO: 57-59)

which encodes for the last amino acids of GOX, an SSGGSS linker sequence (see SEQ. ID. NO: 62) and the N-terminal amino acids of the Camel V_H fragment of pB3. Instead of the SSGGSS linker (see SEQ. ID. NO: 62) it is possible to use other linkers such as the repeated sequence linkers described in the above indicated European patent application 92402326.0, e.g. a repeated sequence Pro-X, with X being any amino acid, but preferably Gln, Lys or Glu, the sequence containing

advantageously at least 3 repeats of Pro-X and especially a fragment composed of a 12-fold repeat of the sequence Pro-X.

In case the about 435 bp *Xho*I-*Hin*dIII fragment of pUR4433M is used in combination with the above described adaptor, this would result in pGOX2-03M. From this plasmid a *Sal*I-*Afl*II fragment of about 2.1 kb encoding the C-terminal part of glucose oxidase followed by the linker peptide, the Camel V_H fragment of pB3 and finally the Myc tail.

Upon digesting pAW14B-GOX partially with *Bbs*I, and with *Afl*II, the about 7.4 kb vector fragment can be isolated. This fragment contains the xylanase promoter, the DNA sequence encoding the N-terminal part of glucose oxidase and the xylanase promoter. Due to the digestion with *Bbs*I, a *Sal*I sticky end is created, corresponding with the *Sal*I restriction site originally present in the *gox* gene. Ligation of the *Sal*I-*Afl*II vector fragment with the about 2.1 kb *Sal*I-*Afl*II fragment of pGOX2-03M, resulting in pUR4441M. This expression plasmid encodes for a single chain polypeptide comprising the glucose oxidase enzyme, the (functionalized) Camel V_H fragment and the Myc tail.

Introduction of this type of expression plasmids in *Aspergillus* can be achieved essentially as described in example 6.

As the naturally occurring glucose oxidase is a homodimeric enzyme, it might be expected that a fusion protein, comprising glucose oxidase and an antibody fragment as a C-terminal extension, has an increased avidity for the antigen/antibody binding, if this fusion protein is produced as a homodimer. Alternatively, it is possible to produce heterodimers, consisting of one glucose oxidase molecule connected to a V_H fragment and one wild type glucose oxidase molecule. This can be achieved by producing with the same strain both wild type glucose oxidase and the fused glucose oxidase-V_H fragment, or by mixing the two different homodimers produced by different strains under conditions whereby the mixture of dimers are dissociated and subsequently associated.

Example 8 Engineering of *Camelidae* V_H fragments

8.1 Random and targeted random mutagenesis.

After expressing a number of different *Camelidae* V_H fragments in lower eukaryotic host organisms as described above, or in prokaryotes, fragments produced in relative higher amounts can be selected. Upon subjecting the *Xho*I-*Bst*EII gene fragments to a (targeted) random mutagenesis procedure, it might be possible to further improve special characteristics of the V_H fragment, e.g. further improvement of the production level, increased stability or increased affinity.

To this end the following procedure might be followed.

Upon replacing the polylinker of the phagemid vector pHEN1 (Hoogenboom *et al.*, 1991) located on a *Nco*I-*Not*I fragment by a new polylinker having the following sequence:

```
NcoI                XhoI                BstEII              NotI
 CATGGCCAGGTGAAACTGCTCGAGTAAGTGACTAAGGTCACCGTCTCCTCAGC
     CGGTCCACTTTGACGAGCTCATTCACTGATTCCAGTGGCAGAGGAGTCGCCGG
```

(see SEQ. ID. NO: 60-61) it becomes possible to introduce *Xho*I-*Bst*EII fragments encoding truncated *Camelidae* V_H fragments in the phagemid.

Following mutagenesis of the V_H encoding sequence (random mutagenesis) or a specific part thereof (targeted random mutagenesis), the mutated V_H fragments can be expressed and displayed on the phage surface in essentially the same way as described by Hoogenboom *et al.* (1991). Selecting phages displaying (mutant) V_H fragments, can be done in different ways, a number of which are described by Marks *et al.* (1992). Subsequently, the mutated *Xho*I-*Bst*EII fragments can be isolated from the phagemid and introduced into expression plasmids for yeast or fungi as described in previous examples.

Upon producing the mutant V_H fragments by these organisms, the effects of the mutations on production levels, V_H fragment stability or binding affinity can be evaluated easily and improved V_H fragments can be selected.

Obviously, a similar route can be followed for larger antibody fragments. With similar procedures the activity of catalytic antibodies can be improved.

8.2 Site-directed or designed mutagenesis

As an alternative to the methods described above in Example 8.1 it is possible to use the well-known technique of site-directed mutagenesis. Thus, designed mutations, preferably based on molecular modelling and molecular dynamics, can be introduced in the V_H fragments, e.g. in the framework or in the CDRs.

8.3 Construction V_H fragments with regulatable binding efficiencies.

For particular applications, the possibility to regulate the binding capacity of antibody fragments might be necessary. The introduction of metal ion binding sites in proteins is known from the literature e.g. Pessi *et al.* (1993). The present inventors envisage that the introduction of a metal binding site in an antibody fragment by rational design can result in a regulatable antibody fragment, when the metal binding site is introduced at a position such that the actual binding of the metal ion results in a conformational change in the antibody fragments due to which the binding of the antigen to the antibody fragment is influenced. Another possibility is that the presence of the metal prevents antigen binding due to steric hindrance.

8.4 Grafting of CDR regions on the framework fragments of a Camelidae V_H fragment.

Grafting of CDR fragments onto framework fragments of different antibodies or fragments thereof is known from the literature (see Jones *et al.* (1986), WO-A-92/15683, and WO-A-92/01059). In these cases the CDR fragments of murine antibody fragments were grafted onto framework fragments of human antibodies. The sole rationale behind the "humanization" was to increase the acceptability for therapeutic and/or diagnostic applications in human.

Essentially the same approach can however also be used for a totally different purpose. Although antibody fragments share some homology in the framework areas, the production levels vary considerably.

Once an antibody or an antibody fragment, e.g. a *Camelidae* V_H fragment, has been identified, which can be produced to high levels by an production organism of interest, this antibody (fragment) can be used as a starting point to construct "grafted" antibody (fragments), which can be produced in high levels and have an

other specificity as compared to the original antibody (fragment). In particular cases it might be necessary to introduce some modifications in the framework fragments as well in order to obtain optimal transitions between the framework fragments and the CDR fragments. For the determination of the optimal transitions molecular dynamics and molecular modelling can be used.

To this end a synthetic gene, encoding the "grafted V_H" fragment, can be constructed and introduced into an expression plasmid. Obviously it is possible to adapt the codon usage to the codons preferred by the host organism.

For optimization of the "grafted V_H" fragment, the procedure as described in example 8.1 can be followed.

Literature mentioned in the specification additional to that mentioned in the above given draft publication

- Adair, J.R. *et al.*, WO-A-92/01059 (CELLTECH Ltd, 1992)
- Beggs (1978) Nature **275** 104
- Bendig, M.M. *et al.* WO-A-92/15683 (MERCK PATENT GmbH, 1992)
- Bergkamp, R.J.M., Kool, I.M., Geerse, R.H., Planta, R.J. (1992) Multiple copy integration of the α-galactosidase gene from *Cyamopsis tetragonoloba* into the ribosomal DNA of *Kluyveromyces lactis*. Current Genetics **21** 365-370
- Bergkamp, R.J.M., PhD Thesis Free University of Amsterdam (1993), Heterologous gene expression in *Kluyveromyces* yeasts
- Better *et al.* (1988) Science **240** 1041-1043
- Bird et al., (1988) Science **242** 423-426
- Cabilly, S. *et al.*, EP-A-0125023 (GENENTECH, 1984)
- Denthe, *et al.* (1983) Nucl. Acids Res. **11** 1645
- Fellinger, A.J. *et al.*, EP-A-0255153 (UNILEVER, 1988)
- Frederick *et al.* (1990) J. Biol. Chem. **265** 3793
- Giuseppin, M.L.F., Lopes, M.T.S., Planta, R.J., Verbakel, J.M.A., Verrips, C.T. (1991) Process for preparing a protein by a yeast transformed by multicopy integration of an expression vector. PCT application WO 91/00920 (UNILEVER)
- Harmsen, M.M., Langedijk, A.C., van Tuinen, E., Geerse, R.H., Rauè, H.A., Maat, J., (1993) Effect of pmr1 disruption and different signal sequences on the

intracellular processing and secretion of *Cyamopsis tetragonoloba* α-galactosidase by *S. cerevisiae*. Gene **125** 115-123

- Hollenberg, C. *et al.*, EP-A-0096430 (GIST-BROCADES N.V., 1983))
- Hoogenboom H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P., and Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Research **15** 4133-4137
- Jones *et al.* (1986) Nature **321** 522
- Kriechbaum *et al.* (1989) FEBS Lett. **255** 63
- Ledeboer, A.M. *et al.*, EP-A-0173378 (UNILEVER, 1986)
- Leenhouts, C.J. *et al.*, EP-A-0487159 (UNILEVER, 1992)
- Lerner, Benkovic and Schultz, (1991) Science **252** 659-667
- Marks, J.D., Hoogenboom, H.R., Griffiths, A.D., and Winter, G. (1992) Molecular evolution of proteins on filamentous phage. J. Biol. Chem. **267** 16007-16010
- Meilhoc, E., Masson, J., Teissié, J. (1990) High efficiency transformation of intact yeast cells by electric pulses. Bio/Technology **8** 223-227
- Mitchell, P., (1979) Science **206** 1148-1159)
- Pessi et al. (1993) Nature **362** 367.
- Rouwenhorst, R.J., Visser, L.E., van der Baan, Scheffers, W.A., van Dijken, J.P. (1988) Production, distribution and kinetic properties of inulinase in continuous culture of *Kluyveromyces marxianus* CBS 6556. Appl. Environm. Microbiol. **54** 1131-1137.
- Sierkstra, L.N., Verbakel, J.M.A. and Verrips, C.T. (1991) Optimisation of a host/vector system for heterologous gene expression by *Hansenula polymorpha*. Current Genetics **19** 81-87.
- Skerra et al. (1988) Science **240** 1938
- Takahashi et al. (1993) Science **259** 1460-1463);
- Teeri et al., WO-A-93/02198 (TECH. RES. CENT. FINLAND, publ. 04.02.1993)
- Van Gorcom, R.F.M. *et al.*, WO-A-91/19782 (UNILEVER, 1991)
- Wu et al. (1993) Bio/Technology **11** 71
- Zhou *et al.* (1991) Nucleic Acids Research **19** 6052

Additional references to prior-filed but not prior-published patent applications, which are incorporated herein by reference:

- not prior-published PCT application EP 92/02896, filed 09.12.92 with priority date of 09.12.91 (UNILEVER / R.J. Gouka *et al.*), now publicly available as WO-A-93/12237
- not prior-published EP application 92202080.5, filed 08.07.92 (UNILEVER / F.M. Klis *et al.*), now publicly available as International (PCT) patent application WO-A-94/01567)
- not prior-published EP application 92402326.0, filed 21.08.92 (C. Casterman & R. Hamers), now publicly available as EP-A1-0 584 421
- not yet published EP application 92203932.6, filed 11.12.92 (UNILEVER / H.Y. Toschka & J.M.A. Verbakel).

Information on deposits of micro-organisms under the Budapest Treaty is given in Example 1 on page 23, lines 23-25 above. In agreement with Rule 28 (4) EPC, or a similar arrangement for a State not being a Contracting State of the EPC, it is hereby requested that a sample of such deposit, when requested, will be submitted to an expert only.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Unilever N.V.
(B) STREET: Weena 455
(C) CITY: Rotterdam
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): NL-3013 AL

(A) NAME: Unilever PLC
(B) STREET: Unilever House Blackfriars
(C) CITY: London
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): EC4P 4BQ

(A) NAME: Leon Gerardus Joseph FRENKEN
(B) STREET: Geldersestraat 90
(C) CITY: Rotterdam
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): NL-3011 MP

(A) NAME: Cornelis Theodorus VERRIPS
(B) STREET: Hagedoorn 18
(C) CITY: Maassluis
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): NL-3142 KB

(A) NAME: Raymond HAMERS
(B) STREET: Vijversweg 15
(C) CITY: Sint-Genesius-Rode
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-1640

(A) NAME: Cécile HAMERS-CASTERMAN
(B) STREET: Vijversweg 15
(C) CITY: Sint-Genesius-Rode
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-1640

(A) NAME: Serge Victor Marie MUYLDERMANS
(B) STREET: Brusselse Steenweg 55
(C) CITY: Hoeilaart
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-1560

(ii) TITLE OF INVENTION: Production of antibodies or (functionalized) fragments thereof derived from heavy chain immunoglobulins of Camelidae.

(iii) NUMBER OF SEQUENCES: 62

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Pro Glu Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ala Pro Glu Leu Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGCCATCAAG GTACCAGTTG A 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: human heavy chain framework (subgroup III)
(Xaa = CDR1, Xaa Xaa = CDR2 and Xaa Xaa Xaa = CDR3)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Xaa Trp Val Arg Gln Ala
20 25 30

Pro Gly Lys Gly Leu Glu Trp Val Ser Xaa Xaa Arg Phe Thr Ile Ser
35 40 45

Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg
50 55 60

Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Xaa Xaa Xaa Trp Gly
65 70 75 80

Gln Gly Thr Leu Val Thr Val Ser Ser
85

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" framework A
(Xaa = CDR1, Xaa Xaa = CDR2 and Xaa Xaa Xaa = CDR3)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gly Gly Ser Val Gln Gly Gly Gly Ser Leu Arg Leu Ser Cys Ala Ile
1 5 10 15

Ser Gly Xaa Trp Phe Arg Glu Gly Pro Gly Lys Glu Arg Glu Gly Ile
20 25 30

Ala Xaa Xaa Arg Phe Thr Ile Ser Gln Asp Ser Thr Leu Lys Thr Met
35 40 45

Tyr Leu Leu Met Asn Asn Leu Lys Pro Glu Asp Thr Gly Thr Tyr Tyr
50 55 60

Cys Ala Ala Xaa Xaa Xaa Trp Gly Gln Gly Thr Gln Val Thr Val Ser
65 70 75 80

Ser

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" framework B
(Xaa = CDR1, Xaa Xaa = CDR2 and Xaa Xaa Xaa = CDR3)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Ala Ser
1 5 10 15

Ser Ser Xaa Trp Tyr Arg Gln Ala Pro Gly Lys Glu Arg Glu Phe Val
20 25 30

Ser Xaa Xaa Arg Phe Thr Ile Ser Gln Asp Ser Ala Lys Asn Thr Val
35 40 45

Tyr Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr
50 55 60

Cys Lys Ile Xaa Xaa Xaa Trp Gly Gln Gly Thr Gln Val Thr Val Ser
65 70 75 80

Ser

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" framework - short hinge - CH2 fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser Gly Thr Asn Glu Val
1 5 10 15

Cys Lys Cys Pro Lys Cys Pro Ala Pro Glu Leu Pro Gly Gly Pro Ser
20 25 30

Val Phe Val Phe Pro
35

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" framework - long hinge - CH2 fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser Glu Pro Lys Ile Pro
1 5 10 15

Gln Pro Gln Pro Lys Pro Gln Pro Gln Pro Gln Pro Gln Pro Lys Pro
20 25 30

Gln Pro Lys Pro Glu Pro Glu Cys Thr Cys Pro Lys Cys Pro Ala Pro
35 40 45

Glu Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro
50 55 60

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: human gamma-3 CH1 - hinge - CH2 fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Lys Val Asp Lys Arg Val Glu Leu Lys Thr Pro Leu Gly Asp Thr Thr
1 5 10 15

His Thr Cys Pro Arg Cys Pro Glu Pro Lys Cys Ser Asp Thr Pro Pro
20 25 30

Pro Cys Pro Arg Cys Pro Glu Pro Lys Ser Cys Asp Thr Pro Pro Pro
35 40 45

Cys Pro Arg Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
50 55 60

Leu Phe Pro
65

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: human gamma-1 CH1 - hinge - CH2 fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Lys Val Asp Lys Lys Ala Glu Pro Lys Ser Cys Asp Lys Thr His Thr
1 5 10 15

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
20 25 30

Leu Phe Pro
35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: human gamma-2 CH1 - hinge - CH2 fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Lys Val Lys Val Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro
1 5 10 15

Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro
20 25 30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: human gamma-4 CH1 - hinge - CH2 fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Lys Val Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro Pro Cys Pro Ser
1 5 10 15

Cys Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
20 25 30

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: mouse heavy chain V-region

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Phe Thr Phe Ser Asp Phe
20 25 30

Tyr Met Glu Trp Val Arg Gln Pro Pro Gly Lys Arg Leu Glu Trp Ile
35 40 45

Ala Ala Ser Arg Asn Lys Ala Asn Asp Tyr Thr Thr Glu Tyr Ser Ala
50 55 60

Ser Val Lys Gly Arg Phe Ile Val Ser Arg Asp Thr Ser Gln Ser Ile
65 70 75 80

Leu Tyr Leu Gln Met Asn Ala Leu Arg Ala Glu Asp Thr Ala Ile Tyr
85 90 95

Tyr Cys Ala Arg Asp Tyr Tyr Gly Ser Ser Tyr Phe Asp Val Trp Gly
100 105 110

Ala Gly Thr Thr Val Thr Val Ser Ser
115 120

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 131 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: human heavy chain V-region

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ser Xaa Ile Ser Xaa Lys Thr Asp Gly Gly Xaa Thr Tyr Tyr Ala Asp
50 55 60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr
65 70 75 80

Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr
85 90 95

Tyr Cys Ala Arg Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Tyr
100 105 110

Tyr Tyr Tyr His Xaa Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr
115 120 125

Val Ser Ser
130

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 114 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (1)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala
1 5 10 15

Ser Gly Tyr Ser Asn Cys Pro Leu Thr Trp Ser Trp Tyr Arg Gln Phe
20 25 30

Pro Gly Thr Glu Arg Glu Phe Val Ser Ser Met Asp Pro Asp Gly Asn
35 40 45

Thr Lys Tyr Thr Tyr Ser Val Lys Gly Arg Phe Thr Met Ser Arg Gly
50 55 60

Ser Thr Glu Tyr Thr Val Phe Leu Gln Met Asp Asn Leu Lys Pro Glu
65 70 75 80

Asp Thr Ala Met Tyr Tyr Cys Lys Thr Ala Leu Gln Pro Gly Gly Tyr
85 90 95

Cys Gly Tyr Gly Xaa Cys Leu Trp Gly Gln Gly Thr Gln Val Thr Val
100 105 110

Ser Ser

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (2)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Asp Val Gln Leu Val Ala Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Thr Ala Ser Gly Asp Ser Phe Ser Arg Phe
20 25 30

Ala Met Ser Trp Phe Arg Gln Ala Pro Gly Lys Glu Cys Glu Leu Val
35 40 45

Ser Ser Ile Gln Ser Asn Gly Arg Thr Thr Glu Ala Asp Ser Val Gln
50 55 60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Arg Asn Thr Val Tyr Leu
65 70 75 80

Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Gly
85 90 95

Ala Val Ser Leu Met Asp Arg Ile Ser Gln His Gly Cys Arg Gly Gln
100 105 110

Gly Thr Gln Val Thr Val Ser Leu
115 120

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (3)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
Gly Gly Ser Val Gln Thr Gly Gly Ser Leu Arg Leu Ser Cys Ala Val
1               5                  10                  15

Ser Gly Phe Ser Phe Ser Thr Ser Cys Met Ala Trp Phe Arg Gln Ala
            20                  25                  30

Ser Gly Lys Gln Arg Glu Gly Val Ala Ala Ile Asn Ser Gly Gly Gly
        35                  40                  45

Arg Thr Tyr Tyr Asn Thr Tyr Val Ala Glu Ser Val Lys Gly Arg Phe
    50                  55                  60

Ala Ile Ser Gln Asp Asn Ala Lys Thr Thr Val Tyr Leu Asp Met Asn
65                  70                  75                  80

Asn Leu Thr Pro Glu Asp Thr Ala Thr Tyr Tyr Cys Ala Ala Val Pro
                85                  90                  95

Ala His Leu Gly Pro Gly Ala Ile Leu Asp Leu Lys Lys Tyr Lys Tyr
            100                 105                 110

Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
        115                 120
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (7)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
Gly Gly Ser Val Gln Gly Gly Gly Ser Leu Arg Leu Ser Cys Ala Ile
1               5                  10                  15

Ser Gly Tyr Thr Tyr Gly Ser Phe Cys Met Gly Trp Phe Arg Glu Gly
            20                  25                  30

Pro Gly Lys Glu Arg Glu Gly Ile Ala Thr Ile Leu Asn Gly Gly Thr
        35                  40                  45

Asn Thr Tyr Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Gln
    50                  55                  60

Asp Ser Thr Leu Lys Thr Met Tyr Leu Leu Met Asn Asn Leu Lys Pro
65                  70                  75                  80

Glu Asp Thr Gly Thr Tyr Tyr Cys Ala Ala Glu Leu Ser Gly Gly Ser
                85                  90                  95
```

Cys Glu Leu Pro Leu Leu Phe Asp Tyr Trp Gly Gln Gly Thr Gln Val
100 105 110

Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 114 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (9)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Thr Leu Ser Cys Val Tyr
1 5 10 15

Thr Asn Asp Thr Gly Thr Met Gly Trp Phe Arg Gln Ala Pro Gly Lys
20 25 30

Glu Cys Glu Arg Val Ala His Ile Thr Pro Asp Gly Met Thr Phe Ile
35 40 45

Asp Glu Pro Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Gln
50 55 60

Lys Thr Leu Ser Leu Arg Met Asn Ser Leu Arg Pro Glu Asp Thr Ala
65 70 75 80

Val Tyr Tyr Cys Ala Ala Asp Trp Lys Tyr Trp Thr Cys Gly Ala Gln
85 90 95

Thr Gly Gly Tyr Phe Gly Gln Trp Gly Gln Gly Ala Gln Val Thr Val
100 105 110

Ser Ser

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 125 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (11)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Asn Val
1 5 10 15

Ser Gly Ser Pro Ser Ser Thr Tyr Cys Leu Gly Trp Phe Arg Gln Ala
20 25 30

Pro Gly Arg Glu Arg Glu Gly Val Thr Ala Ile Asn Thr Asp Gly Ser
35 40 45

Ile Ile Tyr Ala Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Gln
50 55 60

Asp Thr Ala Lys Glu Thr Val His Leu Gln Met Asn Asn Leu Gln Pro
65 70 75 80

Glu Asp Thr Ala Thr Tyr Tyr Cys Ala Ala Arg Leu Thr Glu Met Gly
85 90 95

Ala Cys Asp Ala Arg Trp Ala Thr Leu Ala Thr Arg Thr Phe Ala Tyr
100 105 110

Asn Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
115 120 125

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 114 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (13)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Gly Gly Ser Val Glu Ala Gly Gly Ser Leu Arg Leu Ser Cys Thr Ala
1 5 10 15

Ser Gly Tyr Val Ser Ser Met Ala Trp Phe Arg Gln Val Pro Gly Gln
20 25 30

Glu Arg Glu Gly Val Ala Phe Val Gln Thr Ala Asp Asn Ser Ala Leu
35 40 45

Tyr Gly Asp Ser Val Lys Gly Arg Phe Thr Ile Ser His Asp Asn Ala
50 55 60

Lys Asn Thr Leu Tyr Leu Gln Met Arg Asn Leu Gln Pro Asp Asp Thr
65 70 75 80

Gly Val Tyr Tyr Cys Ala Ala Gln Lys Lys Asp Arg Thr Arg Trp Ala
85 90 95

Glu Pro Arg Glu Trp Asn Asn Trp Gly Gln Gly Thr Gln Val Thr Ala
100 105 110

Ser Ser

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (16)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Gly Gly Ser Ala Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala
1 5 10 15

His Gly Ile Pro Leu Asn Gly Tyr Tyr Ile Ala Trp Phe Arg Gln Ala
20 25 30

Pro Gly Lys Gly Arg Glu Gly Val Ala Thr Ile Asn Gly Gly Arg Asp
35 40 45

Val Thr Tyr Tyr Ala Asp Ser Val Thr Gly Arg Phe Thr Ile Ser Arg
50 55 60

Asp Ser Pro Lys Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Lys Pro
65 70 75 80

Glu Asp Thr Ala Ile Tyr Phe Cys Ala Ala Gly Ser Arg Phe Ser Ser
85 90 95

Pro Val Gly Ser Thr Ser Arg Leu Glu Ser Ser Asp Tyr Asn Tyr Trp
100 105 110

Gly Gln Gly Ile Gln Val Thr Ala Ser Ser
115 120

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (17)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Gly Gly Ser Val Gln Pro Gly Gly Ser Leu Thr Leu Ser Cys Thr Val
1 5 10 15

Ser Gly Ala Thr Tyr Ser Asp Tyr Ser Ile Gly Trp Ile Arg Gln Ala
20 25 30

Pro Gly Lys Asp Arg Glu Val Val Ala Ala Ala Asn Thr Gly Ala Thr
35 40 45

Ser Lys Phe Tyr Val Asp Phe Val Lys Gly Arg Phe Thr Ile Ser Gln
50 55 60

Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln Met Ser Phe Leu Lys Pro
65 70 75 80

Glu Asp Thr Ala Ile Tyr Tyr Cys Ala Ala Ala Asp Pro Ser Ile Tyr
85 90 95

Tyr Ser Ile Leu Xaa Ile Glu Tyr Lys Tyr Trp Gly Gln Gly Thr Gln
100 105 110

Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (18)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Thr Gly
1 5 10 15

Ser Gly Phe Pro Tyr Ser Thr Phe Cys Leu Gly Trp Phe Arg Gln Ala
20 25 30

Pro Gly Lys Glu Arg Glu Gly Val Ala Gly Ile Asn Ser Ala Gly Gly
35 40 45

Asn Thr Tyr Tyr Ala Asp Ala Val Lys Gly Arg Phe Thr Ile Ser Gln
50 55 60

Gly Asn Ala Lys Asn Thr Val Phe Leu Gln Met Asp Asn Leu Lys Pro
65 70 75 80

Glu Asp Thr Ala Ile Tyr Tyr Cys Ala Ala Asp Ser Pro Cys Tyr Met
85 90 95

Pro Thr Met Pro Ala Pro Pro Ile Arg Asp Ser Phe Gly Trp Asp Asp
100 105 110

Phe Gly Gln Gly Thr Gln Val Thr Val Ser Ser
115 120

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (19)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala
1 5 10 15

Ser Asp Tyr Thr Ile Thr Asp Tyr Cys Met Ala Trp Phe Arg Gln Ala
20 25 30

Pro Gly Lys Glu Arg Glu Leu Val Ala Ala Ile Gln Val Val Arg Ser
35 40 45

Asp Thr Arg Leu Thr Asp Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr
50 55 60

Ile Ser Gln Gly Asn Thr Lys Asn Thr Val Asn Leu Gln Met Asn Ser
65 70 75 80

Leu Thr Pro Glu Asp Thr Ala Ile Tyr Ser Cys Ala Ala Thr Ser Ser
85 90 95

Phe Tyr Trp Tyr Cys Thr Thr Ala Pro Tyr Asn Val Trp Gly Gln Gly
100 105 110

Thr Gln Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (20)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Gly Gly Ser Val Gln Val Gly Gly Ser Leu Arg Leu Ser Cys Val Ala
1 5 10 15

Ser Thr His Thr Asp Ser Ser Thr Cys Ile Gly Trp Phe Arg Gln Ala
20 25 30

Pro Gly Lys Glu Arg Glu Gly Val Ala Ser Ile Tyr Phe Gly Asp Gly
35 40 45

Gly Thr Asn Tyr Arg Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Gln
50 55 60

Leu Asn Ala Gln Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Lys Pro
65 70 75 80

Glu Asp Ser Ala Met Tyr Tyr Cys Ala Ile Thr Glu Ile Glu Trp Tyr
85 90 95

Gly Cys Asn Leu Arg Thr Thr Phe Thr Arg Trp Gly Gln Gly Thr Gln
100 105 110

Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 125 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (21)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Gly Gly Ser Val Gln Val Gly Gly Ser Leu Lys Leu Ser Cys Lys Ile
1 5 10 15

Ser Gly Gly Thr Pro Asp Arg Val Pro Lys Ser Leu Ala Trp Phe Arg
20 25 30

Gln Ala Pro Glu Lys Glu Arg Glu Gly Ile Ala Val Leu Ser Thr Lys
35 40 45

Asp Gly Lys Thr Phe Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile
50 55 60

Phe Leu Asp Asn Asp Lys Thr Thr Phe Ser Leu Gln Leu Asp Arg Leu
65 70 75 80

Asn Pro Glu Asp Thr Ala Asp Tyr Tyr Cys Ala Ala Asn Gln Leu Ala
85 90 95

Gly Gly Trp Tyr Leu Asp Pro Asn Tyr Trp Leu Ser Val Gly Ala Tyr
100 105 110

Ala Ile Trp Gly Gln Gly Thr His Val Thr Val Ser Ser
115 120 125

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 125 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (24)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Asn Val
1 5 10 15

Ser Gly Ser Pro Ser Ser Thr Tyr Cys Leu Gly Trp Phe Arg Gln Ala
20 25 30

Pro Gly Lys Glu Arg Glu Gly Val Thr Ala Ile Asn Thr Asp Gly Ser
35 40 45

Val Ile Tyr Ala Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Gln
50 55 60

Asp Thr Ala Lys Lys Thr Val Tyr Leu Gln Met Asn Asn Leu Gln Pro
65 70 75 80

Glu Asp Thr Ala Thr Tyr Tyr Cys Ala Ala Arg Leu Thr Glu Met Gly
85 90 95

Ala Cys Asp Ala Arg Trp Ala Thr Leu Ala Thr Arg Thr Phe Ala Tyr
100 105 110

Asn Tyr Trp Gly Arg Gly Thr Gln Val Thr Val Ser Ser
115 120 125

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 129 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (25)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Gly Gly Ser Val Gln Thr Gly Gly Ser Leu Arg Leu Ser Cys Glu Ile
1 5 10 15

Ser Gly Leu Thr Phe Asp Asp Ser Asp Val Gly Trp Tyr Arg Gln Ala
20 25 30

Pro Gly Asp Glu Cys Lys Leu Val Ser Gly Ile Leu Ser Asp Gly Thr
35 40 45

Pro Tyr Thr Lys Ser Gly Asp Tyr Ala Glu Ser Val Arg Gly Arg Val
50 55 60

Thr Ile Ser Arg Asp Asn Ala Lys Asn Met Ile Tyr Leu Gln Met Asn
65 70 75 80

Asp Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr Cys Ala Val Asp Gly
85 90 95

Trp Thr Arg Lys Glu Gly Gly Ile Gly Leu Pro Trp Ser Val Gln Cys
100 105 110

Glu Asp Gly Tyr Asn Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser
115 120 125

Ser

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (27)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Ala Ser
1 5 10 15

Ser Ser Lys Tyr Met Pro Cys Thr Tyr Asp Met Thr Trp Tyr Arg Gln
20 25 30

Ala Pro Gly Lys Glu Arg Glu Phe Val Ser Ser Ile Asn Ile Asp Gly
35 40 45

Lys Thr Thr Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Gln
50 55 60

Asp Ser Ala Lys Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Lys Pro
65 70 75 80

Glu Asp Thr Ala Met Tyr Tyr Cys Lys Ile Asp Ser Tyr Pro Cys His
85 90 95

Leu Leu Asp Val Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
100 105 110

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 112 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region (29)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Gly Gly Ser Val Gln Ala Gly Gly Ser Leu Arg Leu Ser Cys Val Ala
1 5 10 15

Ser Gly Phe Asn Phe Glu Thr Ser Arg Met Ala Trp Tyr Arg Gln Thr
20 25 30

Pro Gly Asn Val Cys Glu Leu Val Ser Ser Ile Tyr Ser Asp Gly Lys
35 40 45

Thr Tyr Tyr Val Asp Arg Met Lys Gly Arg Phe Thr Ile Ser Arg Glu
50 55 60

Asn Ala Lys Asn Thr Leu Tyr Leu Gln Leu Ser Gly Leu Lys Pro Glu
65 70 75 80

Asp Thr Ala Met Tyr Tyr Cys Ala Pro Val Glu Tyr Pro Ile Ala Asp
85 90 95

Met Cys Ser Arg Tyr Gly Asp Pro Gly Thr Gln Val Thr Val Ser Ser
100 105 110

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 416 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region followed by the FLAG sequence (pB03)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..408

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
CAG GTG AAA CTG CTC GAG TCT GGG GGA GGC TCG GTG CAG GCT GGG GGG        48
Gln Val Lys Leu Leu Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1               5                  10                  15

TCT CTG ACA CTC TCT TGT GTA TAC ACC AAC GAT ACT GGG ACC ATG GGA        96
Ser Leu Thr Leu Ser Cys Val Tyr Thr Asn Asp Thr Gly Thr Met Gly
            20                  25                  30

TGG TTT CGC CAG GCT CCA GGG AAA GAG TGC GAA AGG GTC GCG CAT ATT       144
Trp Phe Arg Gln Ala Pro Gly Lys Glu Cys Glu Arg Val Ala His Ile
        35                  40                  45

ACG CCT GAT GGT ATG ACC TTC ATT GAT GAA CCC GTG AAG GGG CGA TTC       192
Thr Pro Asp Gly Met Thr Phe Ile Asp Glu Pro Val Lys Gly Arg Phe
    50                  55                  60

ACG ATC TCC CGA GAC AAC GCC CAG AAA ACG TTG TCT TTG CGA ATG AAT       240
Thr Ile Ser Arg Asp Asn Ala Gln Lys Thr Leu Ser Leu Arg Met Asn
65                  70                  75                  80

AGT CTG AGG CCT GAG GAC ACG GCC GTG TAT TAC TGT GCG GCA GAT TGG       288
Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala Ala Asp Trp
                85                  90                  95

AAA TAC TGG ACT TGT GGT GCC CAG ACT GGA GGA TAC TTC GGA CAG TGG       336
Lys Tyr Trp Thr Cys Gly Ala Gln Thr Gly Gly Tyr Phe Gly Gln Trp
            100                 105                 110

GGT CAG GGG GCC CAG GTC ACC GTC TCC TCA CTA GCT AGT TAC CCG TAC       384
Gly Gln Gly Ala Gln Val Thr Val Ser Ser Leu Ala Ser Tyr Pro Tyr
        115                 120                 125

GAC GTT CCG GAC TAC GGT TCT TAATAGAATT C                              416
Asp Val Pro Asp Tyr Gly Ser
    130                 135
```

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 135 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```
Gln Val Lys Leu Leu Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1               5                  10                  15

Ser Leu Thr Leu Ser Cys Val Tyr Thr Asn Asp Thr Gly Thr Met Gly
            20                  25                  30

Trp Phe Arg Gln Ala Pro Gly Lys Glu Cys Glu Arg Val Ala His Ile
        35                  40                  45

Thr Pro Asp Gly Met Thr Phe Ile Asp Glu Pro Val Lys Gly Arg Phe
    50                  55                  60
```

```
Thr Ile Ser Arg Asp Asn Ala Gln Lys Thr Leu Ser Leu Arg Met Asn
 65                  70                  75                  80

Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala Ala Asp Trp
                 85                  90                  95

Lys Tyr Trp Thr Cys Gly Ala Gln Thr Gly Gly Tyr Phe Gly Gln Trp
            100                 105                 110

Gly Gln Gly Ala Gln Val Thr Val Ser Ser Leu Ala Ser Tyr Pro Tyr
        115                 120                 125

Asp Val Pro Asp Tyr Gly Ser
    130                 135
```

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 443 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel "heavy chain immunoglobulin" V-region followed by the FLAG sequence (pB09)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..435

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

```
CAG GTG AAA CTG CTC GAG TCT GGA GGA GGC TCG GTG CAG ACT GGA GGA        48
Gln Val Lys Leu Leu Glu Ser Gly Gly Gly Ser Val Gln Thr Gly Gly
  1               5                  10                  15

TCT CTG AGA CTC TCC TGT GCA GTC TCT GGA TTC TCC TTT AGT ACC AGT        96
Ser Leu Arg Leu Ser Cys Ala Val Ser Gly Phe Ser Phe Ser Thr Ser
            20                  25                  30

TGT ATG GCC TGG TTC CGC CAG GCT TCA GGA AAG CAG CGT GAG GGG GTC       144
Cys Met Ala Trp Phe Arg Gln Ala Ser Gly Lys Gln Arg Glu Gly Val
        35                  40                  45

GCA GCC ATT AAT AGT GGC GGT GGT AGG ACA TAC TAC AAC ACA TAT GTC       192
Ala Ala Ile Asn Ser Gly Gly Gly Arg Thr Tyr Tyr Asn Thr Tyr Val
    50                  55                  60

GCC GAG TCC GTG AAG GGC CGA TTC GCC ATC TCC CAA GAC AAC GCC AAG       240
Ala Glu Ser Val Lys Gly Arg Phe Ala Ile Ser Gln Asp Asn Ala Lys
65                  70                  75                  80

ACC ACG GTA TAT CTT GAT ATG AAC AAC CTA ACC CCT GAA GAC ACG GCT       288
Thr Thr Val Tyr Leu Asp Met Asn Asn Leu Thr Pro Glu Asp Thr Ala
                85                  90                  95

ACG TAT TAC TGT GCG GCG GTC CCA GCC CAC TTG GGA CCT GGC GCC ATT       336
Thr Tyr Tyr Cys Ala Ala Val Pro Ala His Leu Gly Pro Gly Ala Ile
            100                 105                 110

CTT GAT TTG AAA AAG TAT AAG TAC TGG GGC CAG GGG ACC CAG GTC ACC       384
Leu Asp Leu Lys Lys Tyr Lys Tyr Trp Gly Gln Gly Thr Gln Val Thr
        115                 120                 125
```

GTC TCC TCA CTA GCT AGT TAC CCG TAC GAC GTT CCG GAC TAC GGT TCT 432
Val Ser Ser Leu Ala Ser Tyr Pro Tyr Asp Val Pro Asp Tyr Gly Ser
130 135 140

TAATAGAATT C 443

145

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Gln Val Lys Leu Leu Glu Ser Gly Gly Gly Ser Val Gln Thr Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Val Ser Gly Phe Ser Phe Ser Thr Ser
20 25 30

Cys Met Ala Trp Phe Arg Gln Ala Ser Gly Lys Gln Arg Glu Gly Val
35 40 45

Ala Ala Ile Asn Ser Gly Gly Gly Arg Thr Tyr Tyr Asn Thr Tyr Val
50 55 60

Ala Glu Ser Val Lys Gly Arg Phe Ala Ile Ser Gln Asp Asn Ala Lys
65 70 75 80

Thr Thr Val Tyr Leu Asp Met Asn Asn Leu Thr Pro Glu Asp Thr Ala
85 90 95

Thr Tyr Tyr Cys Ala Ala Val Pro Ala His Leu Gly Pro Gly Ala Ile
100 105 110

Leu Asp Leu Lys Lys Tyr Lys Tyr Trp Gly Gln Gly Thr Gln Val Thr
115 120 125

Val Ser Ser Leu Ala Ser Tyr Pro Tyr Asp Val Pro Asp Tyr Gly Ser
130 135 140

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 449 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: camel heavy chain immunoglobulin" V-region followed by the FLAG sequence (pB24)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..441

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

```
CAG GTG AAA CTG CTC GAG TCT GGG GGA GGG TCG GTG CAG GCT GGA GGG          48
Gln Val Lys Leu Leu Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
  1               5                  10                  15

TCT CTG AGA CTC TCC TGT AAT GTC TCT GGC TCT CCC AGT AGT ACT TAT          96
Ser Leu Arg Leu Ser Cys Asn Val Ser Gly Ser Pro Ser Ser Thr Tyr
            20                  25                  30

TGC CTG GGC TGG TTC CGC CAG GCT CCA GGG AAG GAG CGT GAG GGG GTC         144
Cys Leu Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
        35                  40                  45

ACA GCG ATT AAC ACT GAT GGC AGT GTC ATA TAC GCA GCC GAC TCC GTG         192
Thr Ala Ile Asn Thr Asp Gly Ser Val Ile Tyr Ala Ala Asp Ser Val
    50                  55                  60

AAG GGC CGA TTC ACC ATC TCC CAA GAC ACC GCC AAG AAA ACG GTA TAT         240
Lys Gly Arg Phe Thr Ile Ser Gln Asp Thr Ala Lys Lys Thr Val Tyr
65                  70                  75                  80

CTC CAG ATG AAC AAC CTG CAA CCT GAG GAT ACG GCC ACC TAT TAC TGC         288
Leu Gln Met Asn Asn Leu Gln Pro Glu Asp Thr Ala Thr Tyr Tyr Cys
                85                  90                  95

GCG GCA AGA CTG ACG GAG ATG GGG GCT TGT GAT GCG AGA TGG GCG ACC         336
Ala Ala Arg Leu Thr Glu Met Gly Ala Cys Asp Ala Arg Trp Ala Thr
            100                 105                 110

TTA GCG ACA AGG ACG TTT GCG TAT AAC TAC TGG GGC CGG GGG ACC CAG         384
Leu Ala Thr Arg Thr Phe Ala Tyr Asn Tyr Trp Gly Arg Gly Thr Gln
        115                 120                 125

GTC ACC GTC TCC TCA CTA GCT AGT TAC CCG TAC GAC GTT CCG GAC TAC         432
Val Thr Val Ser Ser Leu Ala Ser Tyr Pro Tyr Asp Val Pro Asp Tyr
    130                 135                 140

GGT TCT TAATAGAATT C                                                   449
Gly Ser
145
```

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 146 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

```
Gln Val Lys Leu Leu Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
  1               5                  10                  15

Ser Leu Arg Leu Ser Cys Asn Val Ser Gly Ser Pro Ser Ser Thr Tyr
            20                  25                  30

Cys Leu Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
        35                  40                  45

Thr Ala Ile Asn Thr Asp Gly Ser Val Ile Tyr Ala Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Gln Asp Thr Ala Lys Lys Thr Val Tyr
65                  70                  75                  80
```

Leu Gln Met Asn Asn Leu Gln Pro Glu Asp Thr Ala Thr Tyr Tyr Cys
85 90 95

Ala Ala Arg Leu Thr Glu Met Gly Ala Cys Asp Ala Arg Trp Ala Thr
100 105 110

Leu Ala Thr Arg Thr Phe Ala Tyr Asn Tyr Trp Gly Arg Gly Thr Gln
115 120 125

Val Thr Val Ser Ser Leu Ala Ser Tyr Pro Tyr Asp Val Pro Asp Tyr
130 135 140

Gly Ser
145

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

AATTTAGCGG CCGCCCAGGT GAAACTGCTC GAGTAAGTGA CTAAGGTCAC CGTCTCCTCA 60

GAACAAAAAC TCATCTCAGA AGAGGATCTG AATTAATGAG AATTCATCAA ACGGTGATA 119

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

AGCTTATCAC CGTTTGATGA ATTCTCATTA ATTCAGATCC TCTTCTGAGA TGAGTTTTTG 60

TTCTGAGGAG ACGGTGACCT TAGTCACTTA CTCGAGCAGT TTCACCTGGG CGGCCGCTAA 120

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Ala Gln Val Lys Leu Leu Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Val Thr Val Ser Ser Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 19

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

AATTTAGTCG CGACAGGTGA AACTGCTCGA GTAAGTGACT AAGGTCACCG TCTCCTCAGA 60

ACAAAAACTC ATCTCAGAAG AGGATCTGAA TTAATGAGAA TTCATCTTAA GGTGATA 117

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 19

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

AGCTTATCAC CTTAAGATGA ATTCTCATTA ATTCAGATCC TCTTCTGAGA TGAGTTTTTG 60

TTCTGAGGAG ACGGTGACCT TAGTCACTTA CTCGAGCAGT TTCACCTGTC GCGACTA 117

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 19

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

Arg Gln Val Lys Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(B) CLONE: See figure 19

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

Val Thr Val Ser Ser Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Gln Val Lys Leu
1

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Val Thr Val Ser Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

GTCACCGTCT CCTCATAATG A 21

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

AGCTTCATTA TGAGGAGACG 20

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

GTCACCGTCT CCTCATAATG ATCTTAAGGT GATA 34

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

AGCTTATCAC CTTAAGATCA TTATGAGGAG ACG 33

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

AATTGCGGCC GC 12

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

CATGCAGTCT TCGGGC 16

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

TTAAGCCCGA AGACTG 16

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

TCACTGAATT CGGGATCATG AGGACTCTCC TTGTGAGCTC GCTT 44

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

ATGTCACAAA GCTTAAGCAC GAAGACAGTC GACCGTGCGG CCGGAGAC 48

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

CGCGTCCATG CAGTCCTCAG GTGGATCATC CCAGGTGAAA CTGC 44

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

TCGAGCAGTT TCACCTGGGA TGATCCACCT GAGGACTGCA TGGA 44

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

Ser Met Gln Ser Ser Gly Gly Ser Ser Gln Val Lys Leu Leu Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

CATGGCCAGG TGAAACTGCT CGAGTAAGTG ACTAAGGTCA CCGTCTCCTC AGC 53

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

GGCCGCTGAG GAGACGGTGA CCTTAGTCAC TTACTCGAGC AGTTTCACCT GGC 53

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

Ser Ser Gly Gly Ser Ser
1 5

CLAIMS

1. A process for the production of an antibody or a fragment or functionalized fragment thereof using a transformed lower eukaryotic host containing an expressible DNA sequence encoding the antibody or (functionalized) fragment thereof, wherein the antibody or (functionalized) fragment thereof is derived from a heavy chain immunoglobulin of *Camelidae* and is devoid of light chains, and wherein the lower eukaryotic host is a mould or a yeast.

2. A process according to claim 1, in which the mould belongs to the genera *Aspergillus* or *Trichoderma*.

3. A process according to claim 1, in which the yeast belongs to the genera *Saccharomyces*, *Kluyveromcyes*, *Hansenula*, or *Pichia*.

4. A process according to claim 1, in which the heavy chain fragment at least contains the whole variable domain.

5. A process according to claim 1, in which the antibody or (functionalized) fragment thereof derived from a heavy chain immunoglobulin of *Camelidae* comprises a complementary determining region (CDR) different from the CDR belonging to the natural antibody ex *Camelidae* grafted on the framework of the variable domain of the heavy chain immunoglobulin ex *Camelidae*.

6. A process according to claim 1, in which the immunoglobulin to be produced is a catalytic antibody raised in *Camelidae*.

7. A process according to claim 1, in which the functionalized antibody or fragment thereof comprises a fusion protein of both a heavy chain immunoglobulin from *Camelidae* or a fragment thereof and another polypeptide.

8. A process according to claim 1, in which the DNA sequence encodes a modified heavy chain immunoglobulin or (functionalized) fragment thereof derived from *Camelidae* and being devoid of light chains, and is made by random or directed mutagenesis or both.

9. A process according to claim 8, in which the resulting immunoglobulin or (functionalized) fragment thereof is modified such that
   - it is better adapted for production by the host cell, or
   - it is optimized for secretion by the lower eukaryotic host into the fermentation medium, or
   - its binding properties (k_{on} and k_{off}) are optimized, or
   - its catalytic activity is improved, or
   - it has acquired a metal chelating activity, or
   - its physical stability is improved.

10. A composition containing a product produced by a process as claimed in any one of claims 1-9.

11. New product obtainable by a process as claimed in any one of claims 1-9.

12. A composition containing a new product as claimed in claim 11.

* * * * *

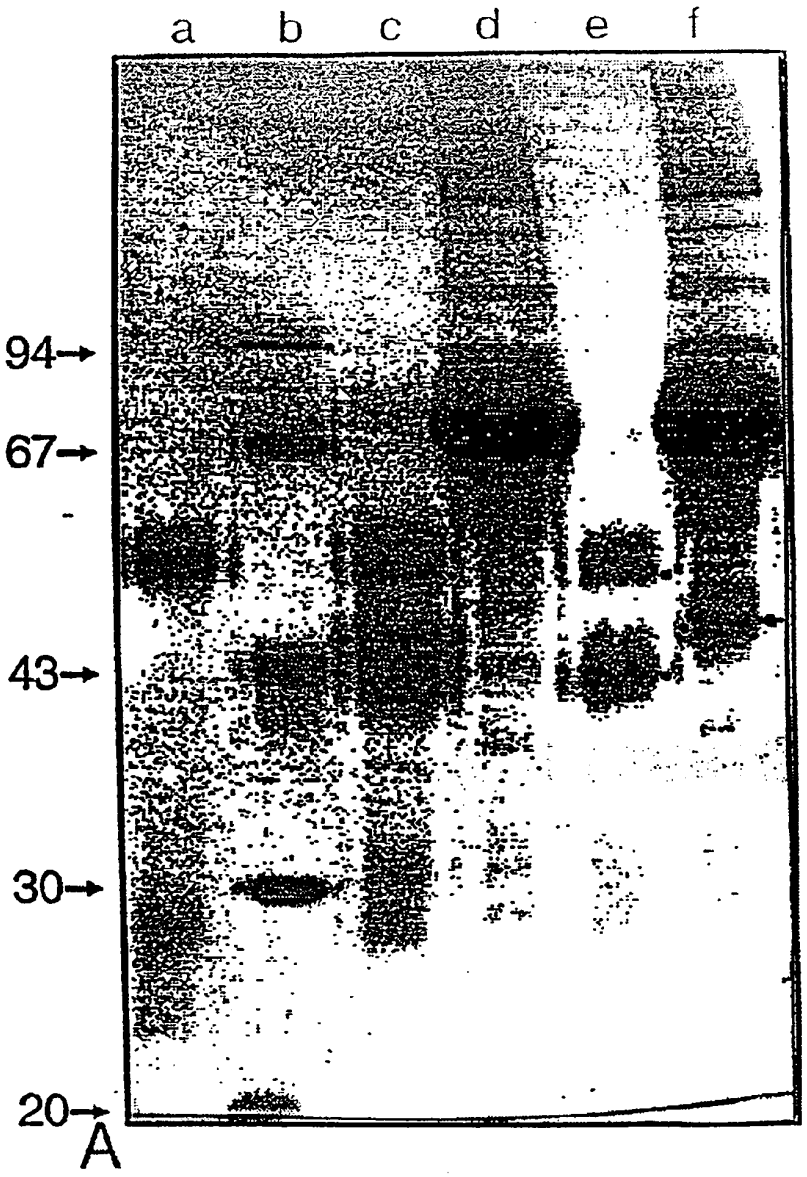


FIGURE 1A



FIGURE 1B

FIGURE 1C

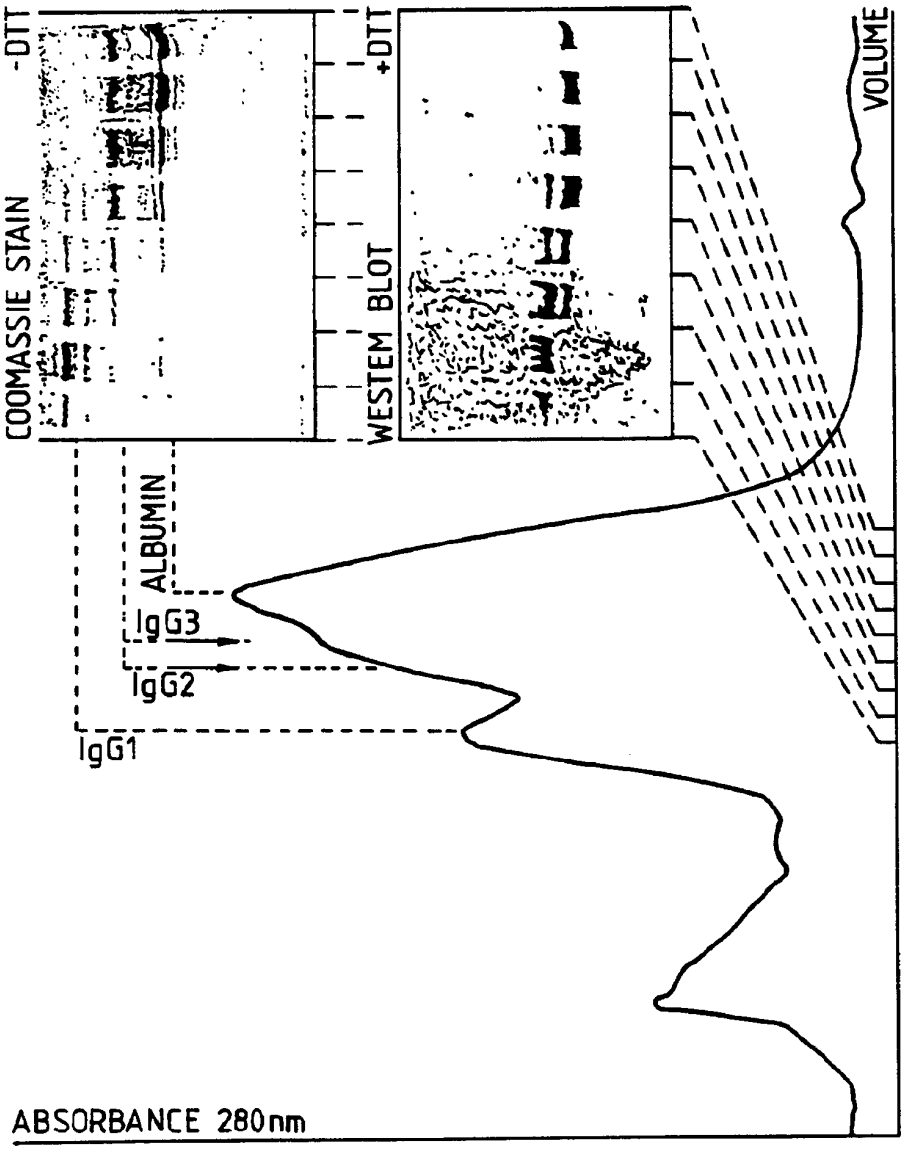
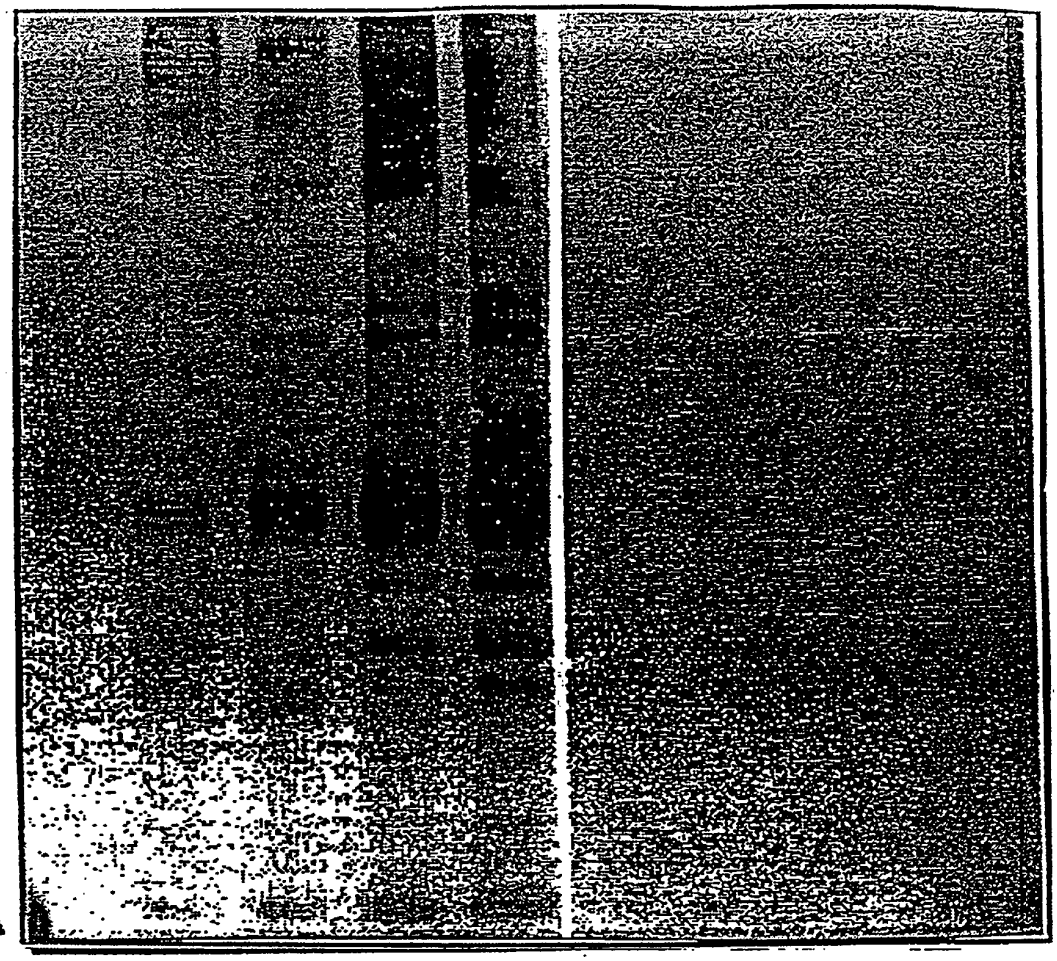

-DTT
+DTT
COOMASSIE STAIN
WESTEM BLOT
VOLUME
ALBUMIN
IgG3
IgG2
IgG1
ABSORBANCE 280nm

Fig.1D



A

	Prot.A	Ig1	Ig2	Ig3	Tot.Ser	Ig1	Ig2	Ig3	Tot.Ser
	Control		T. evansi infected				Healthy		
counts/5µl	65	1258	1214	2700	2978	147	157	160	107

FIGURE 2A

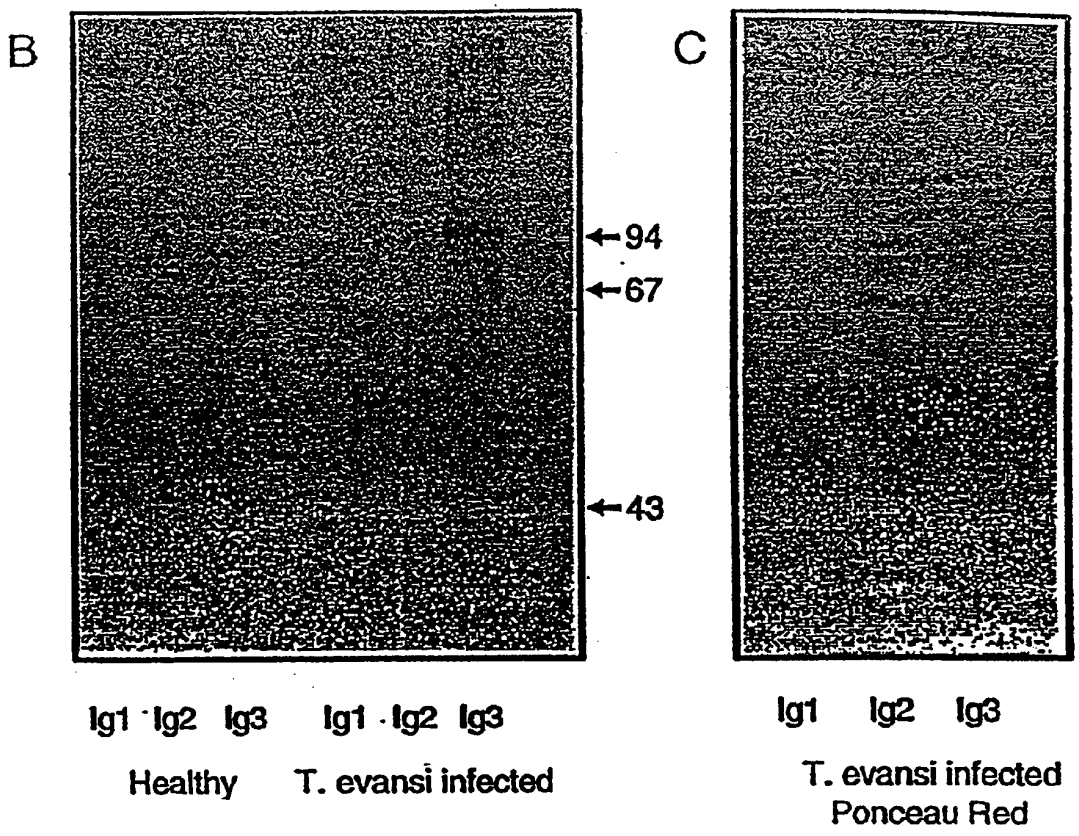


FIGURE 2B

FIGURE 2C

Fig.3.

10	20		40		
EVQLVESGGG	*LVQPGGSLRL*	*SCAASG*	*CDR1*	*WVRQA PGKGLEWVS*	*CDR2*
GG	SVQGGGSLRL	SCAISG	CDR1	WFREG PGKEREGIA	CDR2
GG	SVQAGGSLRL	SCASSS	CDR1	WYRQA PGKEREFVS	CDR2

70	80	90		110	
RFTIS	*RDNSKNTLYL*	*QMNSLRAEDTAVY YCAR*	*CDR3*	*WGQGTLVT VSS*	
RFTIS	QDSTLKTMYL	LMNNLKPEDTGTY YCAA	CDR3	WGQGTQVT VSS	
RFTIS	QDSAKNTVYL	QMNSLKPEDTAMY YCKI	CDR3	WGQGTQVT VSS	

	camel V_H	hinge	C_H2
camel	WGQGTQVT VSS	GTNEVCKCPKCP	APELPGG PSVFVFP
	WGQGTQVT VSS	EPKIPQPQPKPQPQP	
		QPQPKPQP	
		KPEPECTCPKCP	APELLGG PSVFIFP

	human C_H1	hinge	C_H2
human gamma 3	*KVDKRV*	*ELKTPLGDTTHTCPRCP*	
		EPKCSDTPPPCPRCP	
		EPKSCDTPPPCPRCP	*APELLGG PSVFLFP*
human gamma 1	*KVDKK*	*AEPKSCDKTHTCPPCP*	*APELLGG PSVFLFP*
human gamma 2	*KVKVTV*	*ERKCCVECPPCP*	*APPVAG- PSVFLFP*
human gamma 4	*KVDKRV*	*ESKYGPPCPSCP*	*APEFLGG PSVFLFP*

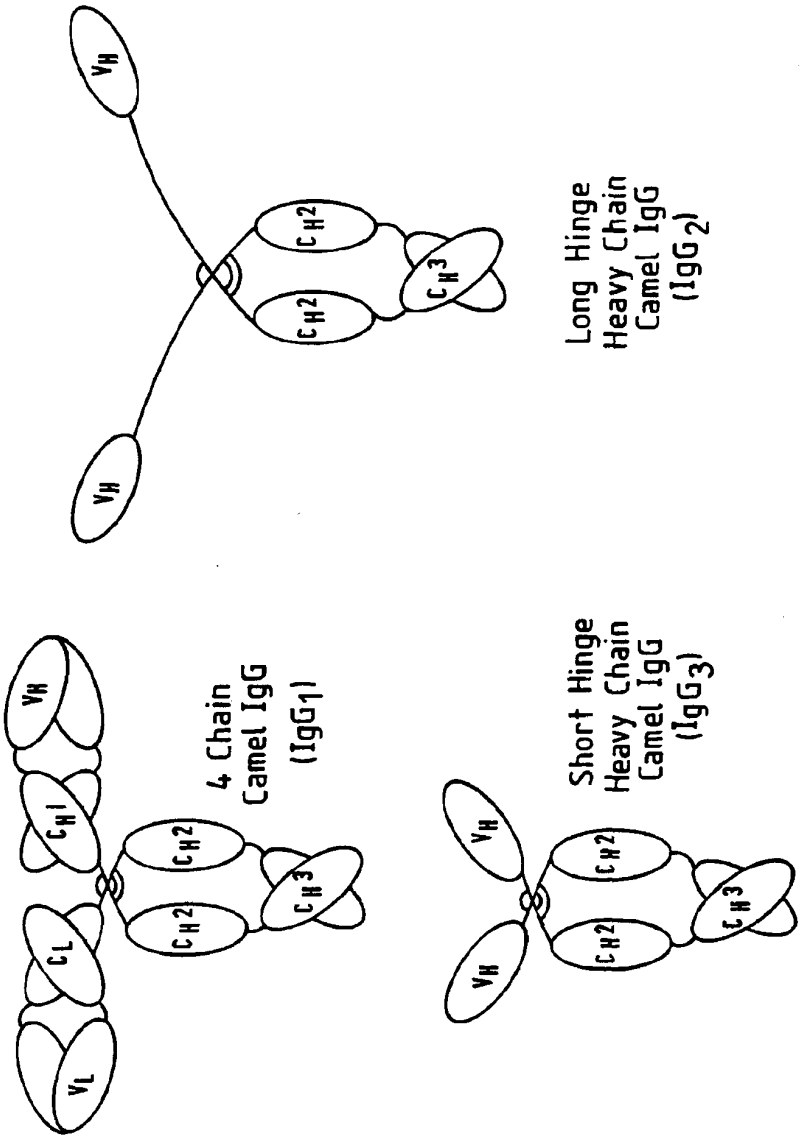

Long Hinge
Heavy Chain
Camel IgG
(IgG2)
4 Chain
Camel IgG
(IgG1)
Short Hinge
Heavy Chain
Camel IgG
(IgG3)

Fig.4.

Fig.5A.

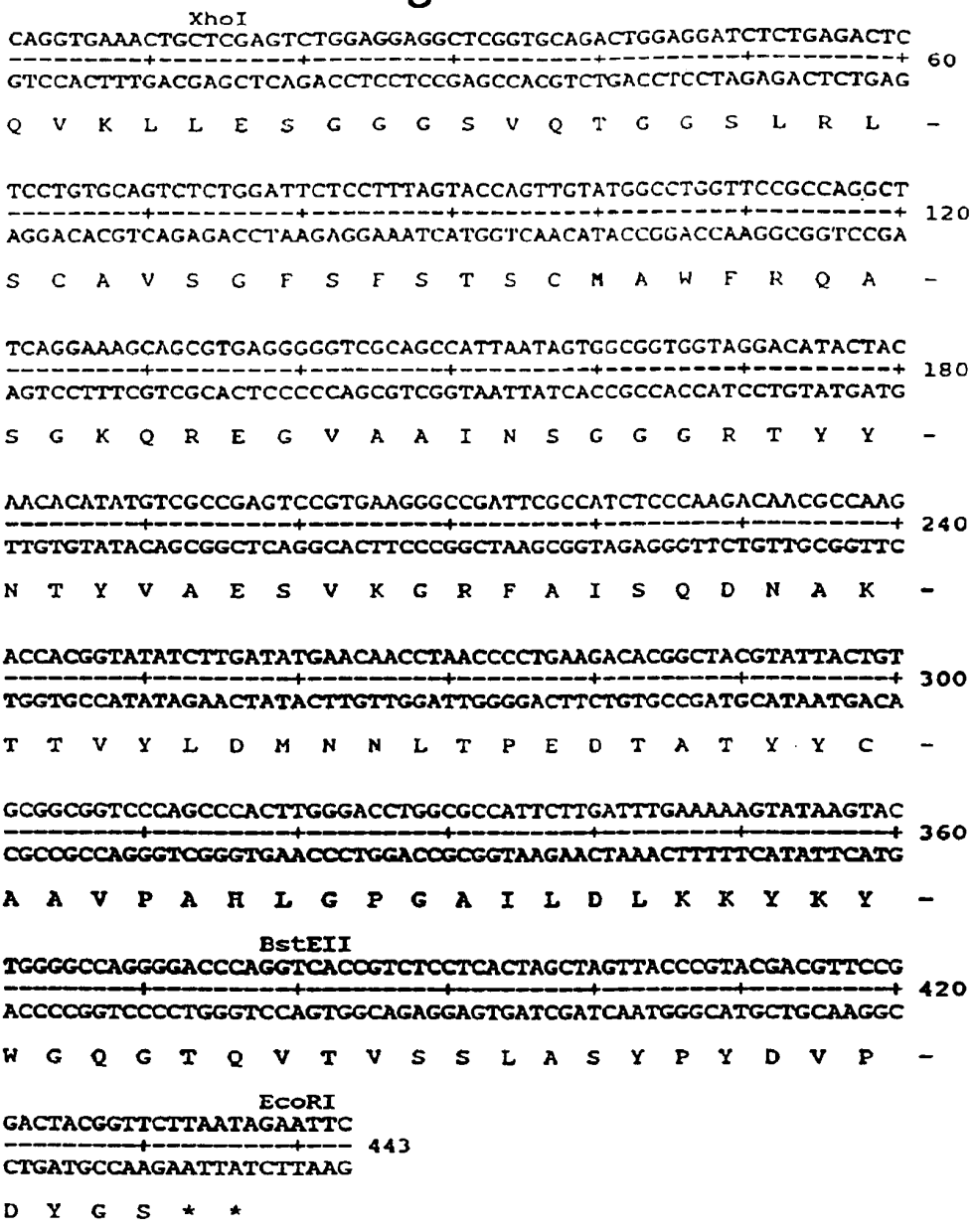

XhoI
CAGGTGAAACTGCTCGAGTCTGGAGGAGGCTCGGTGCAGACTGGAGGATCTCTGAGACTC
GTCCACTTTGACGAGCTCAGACCTCCTCCGAGCCACGTCTGACCTCCTAGAGACTCTGAG
Q V K L L E S G G G S V Q T G G S L R L -
TCCTGTGCAGTCTCTGGATTCTCCTTTAGTACCAGTTGTATGGCCTGGTTCCGCCAGGCT
AGGACACGTCAGAGACCTAAGAGGAAATCATGGTCAACATACCGGACCAAGGCGGTCCGA
S C A V S G F S F S T S C M A W F R Q A -
TCAGGAAAGCAGCGTGAGGGGGTCGCAGCCATTAATAGTGGCGGTGGTAGGACATACTAC
AGTCCTTTCGTCGCACTCCCCCAGCGTCGGTAATTATCACCGCCACCATCCTGTATGATG
S G K Q R E G V A A I N S G G G R T Y Y -
AACACATATGTCGCCGAGTCCGTGAAGGGCCGATTCGCCATCTCCCAAGACAACGCCAAG
TTGTGTATACAGCGGCTCAGGCACTTCCCGGCTAAGCGGTAGAGGGTTCTGTTGCGGTTC
N T Y V A E S V K G R F A I S Q D N A K -
ACCACGGTATATCTTGATATGAACAACCTAACCCCTGAAGACACGGCTACGTATTACTGT
TGGTGCCATATAGAACTATACTTGTTGGATTGGGGACTTCTGTGCCGATGCATAATGACA
T T V Y L D M N N L T P E D T A T Y Y C -
GCGGCGGTCCCAGCCCACTTGGGACCTGGCGCCATTCTTGATTTGAAAAAGTATAAGTAC
CGCCGCCAGGGTCGGGTGAACCCTGGACCGCGGTAAGAACTAAACTTTTTCATATTCATG
A A V P A H L G P G A I L D L K K Y K Y -
BstEII
TGGGGCCAGGGGACCCAGGTCACCGTCTCCTCACTAGCTAGTTACCCGTACGACGTTCCG
ACCCCGGTCCCCTGGGTCCAGTGGCAGAGGAGTGATCGATCAATGGGCATGCTGCAAGGC
W G Q G T Q V T V S S L A S Y P Y D V P -
EcoRI
GACTACGGTTCTTAATAGAATTC
CTGATGCCAAGAATTATCTTAAG
D Y G S * *
421
443

Fig.5B.

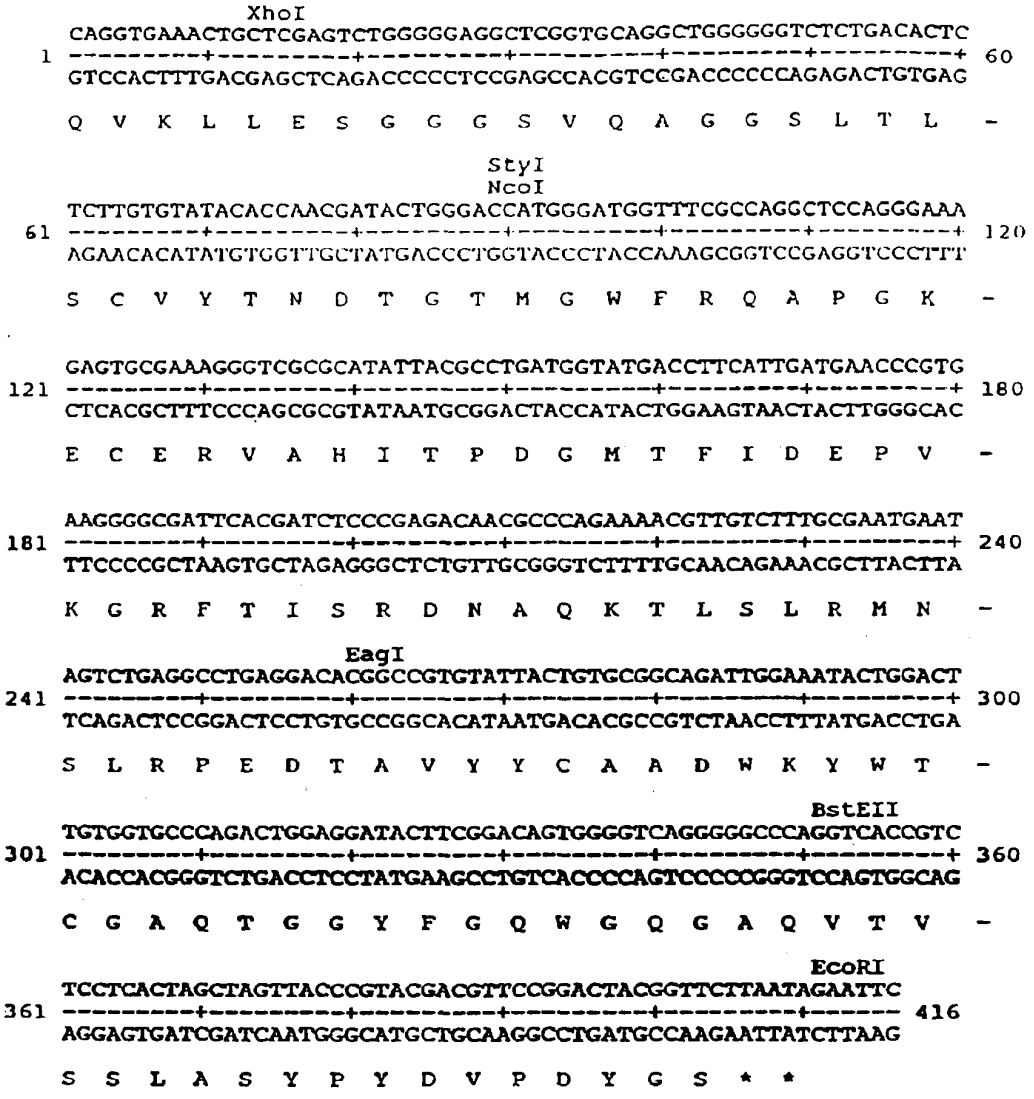

XhoI
CAGGTGAAACTGCTCGAGTCTGGGGGAGGCTCGGTGCAGGCTGGGGGGTCTCTGACACTC
1
GTCCACTTTGACGAGCTCAGACCCCCTCCGAGCCACGTCCGACCCCCCAGAGACTGTGAG 60
Q V K L L E S G G G S V Q A G G S L T L -
StyI
NcoI
TCTTGTGTATACACCAACGATACTGGGACCATGGGATGGTTTCGCCAGGCTCCAGGGAAA
61
AGAACACATATGTGGTTGCTATGACCCTGGTACCCTACCAAAGCGGTCCGAGGTCCCTTT 120
S C V Y T N D T G T M G W F R Q A P G K -
GAGTGCGAAAGGGTCGCGCATATTACGCCTGATGGTATGACCTTCATTGATGAACCCGTG
121
CTCACGCTTTCCCAGCGCGTATAATGCGGACTACCATACTGGAAGTAACTACTTGGGCAC 180
E C E R V A H I T P D G M T F I D E P V -
AAGGGGCGATTCACGATCTCCCGAGACAACGCCCAGAAAACGTTGTCTTTGCGAATGAAT
181
TTCCCCGCTAAGTGCTAGAGGGCTCTGTTGCGGGTCTTTTGCAACAGAAACGCTTACTTA 240
K G R F T I S R D N A Q K T L S L R M N -
EagI
AGTCTGAGGCCTGAGGACACGGCCGTGTATTACTGTGCGGCAGATTGGAAATACTGGACT
241
TCAGACTCCGGACTCCTGTGCCGGCACATAATGACACGCCGTCTAACCTTTATGACCTGA 300
S L R P E D T A V Y Y C A A D W K Y W T -
BstEII
TGTGGTGCCCAGACTGGAGGATACTTCGGACAGTGGGGTCAGGGGGCCCAGGTCACCGTC
301
ACACCACGGGTCTGACCTCCTATGAAGCCTGTCACCCCAGTCCCCCGGGTCCAGTGGCAG 360
C G A Q T G G Y F G Q W G Q G A Q V T V -
EcoRI
TCCTCACTAGCTAGTTACCCGTACGACGTTCCGGACTACGGTTCTTAATAGAATTC
361
AGGAGTGATCGATCAATGGGCATGCTGCAAGGCCTGATGCCAAGAATTATCTTAAG 416
S S L A S Y P Y D V P D Y G S * *

Fig.5C.

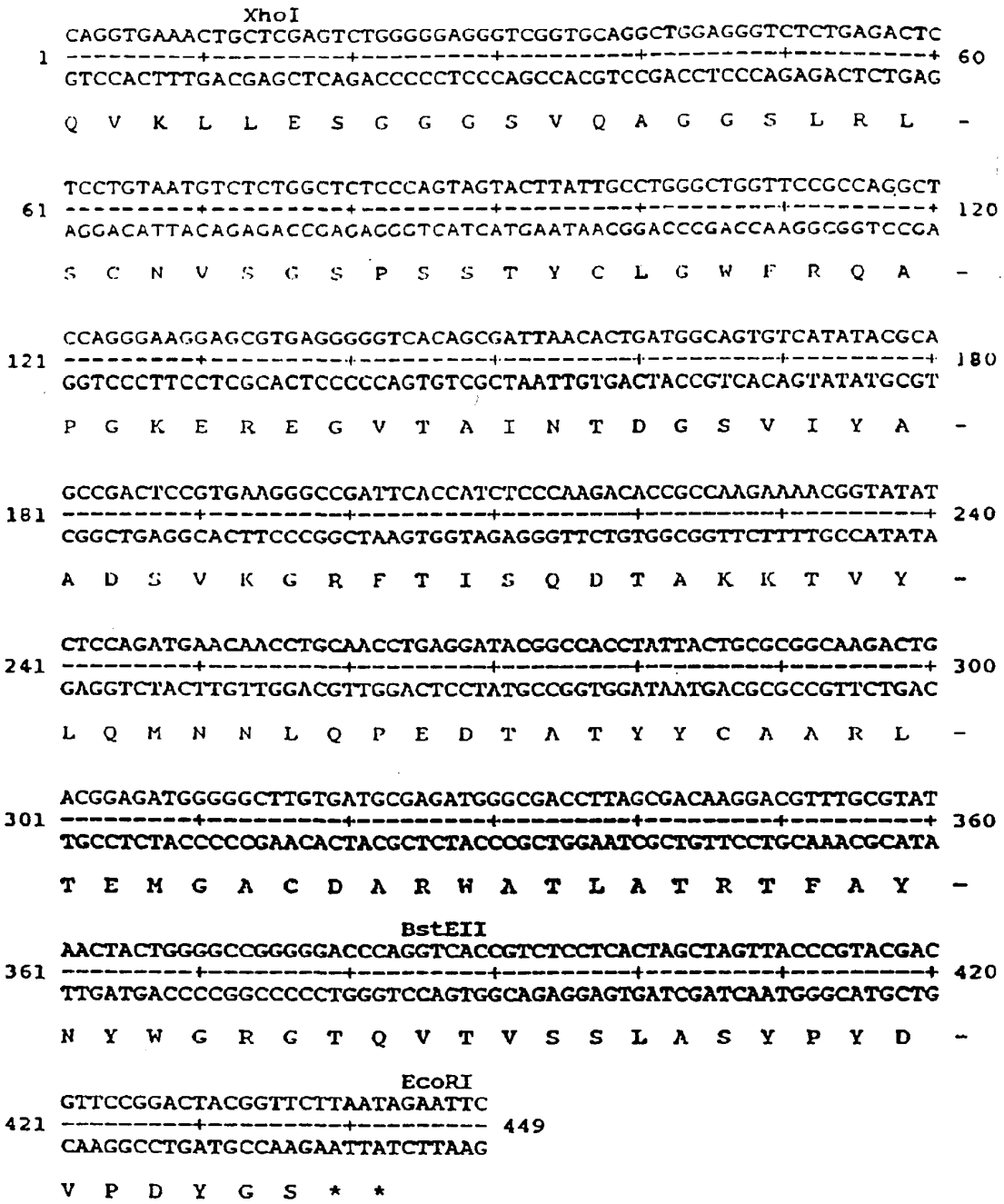

XhoI
CAGGTGAAACTGCTCGAGTCTGGGGGAGGGTCGGTGCAGGCTGGAGGGTCTCTGAGACTC
GTCCACTTTGACGAGCTCAGACCCCCTCCCAGCCACGTCCGACCTCCCAGAGACTCTGAG
Q V K L L E S G G G S V Q A G G S L R L -
TCCTGTAATGTCTCTGGCTCTCCCAGTAGTACTTATTGCCTGGGCTGGTTCCGCCAGGCT
AGGACATTACAGAGACCGAGAGGGTCATCATGAATAACGGACCCGACCAAGGCGGTCCGA
S C N V S G S P S S T Y C L G W F R Q A -
CCAGGGAAGGAGCGTGAGGGGGTCACAGCGATTAACACTGATGGCAGTGTCATATACGCA
GGTCCCTTCCTCGCACTCCCCCAGTGTCGCTAATTGTGACTACCGTCACAGTATATGCGT
P G K E R E G V T A I N T D G S V I Y A -
GCCGACTCCGTGAAGGGCCGATTCACCATCTCCCAAGACACCGCCAAGAAAACGGTATAT
CGGCTGAGGCACTTCCCGGCTAAGTGGTAGAGGGTTCTGTGGCGGTTCTTTTGCCATATA
A D S V K G R F T I S Q D T A K K T V Y -
CTCCAGATGAACAACCTGCAACCTGAGGATACGGCCACCTATTACTGCGCGGCAAGACTG
GAGGTCTACTTGTTGGACGTTGGACTCCTATGCCGGTGGATAATGACGCGCCGTTCTGAC
L Q M N N L Q P E D T A T Y Y C A A R L -
ACGGAGATGGGGGCTTGTGATGCGAGATGGGCGACCTTAGCGACAAGGACGTTTGCGTAT
TGCCTCTACCCCCGAACACTACGCTCTACCCGCTGGAATCGCTGTTCCTGCAAACGCATA
T E M G A C D A R W A T L A T R T F A Y -
BstEII
AACTACTGGGGCCGGGGGACCCAGGTCACCGTCTCCTCACTAGCTAGTTACCCGTACGAC
TTGATGACCCCGGCCCCCTGGGTCCAGTGGCAGAGGAGTGATCGATCAATGGGCATGCTG
N Y W G R G T Q V T V S S L A S Y P Y D -
EcoRI
GTTCCGGACTACGGTTCTTAATAGAATTC
CAAGGCCTGATGCCAAGAATTATCTTAAG
V P D Y G S * *

Fig.6.

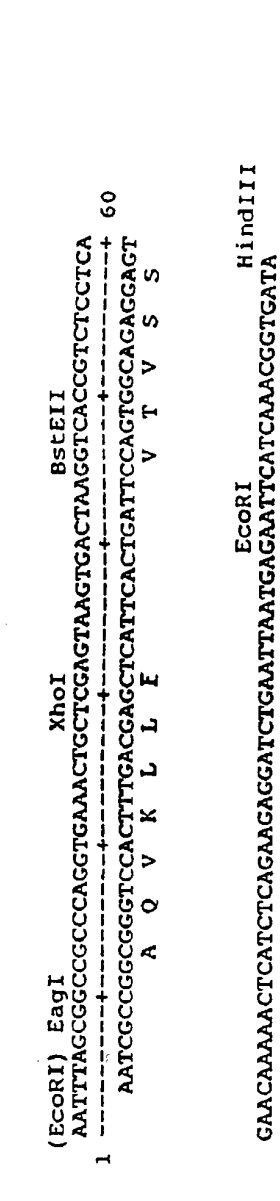

(EcoRI) EagI
XhoI
BstEII
AATTTAGCGGCCGCCCCAGGTGAAACTGCTCGAGTAAGTGACTAAGGTCACCGTCTCCTCA
1
60
AATCGCCGGCGGGGTCCACTTTGACGAGCTCATTCACTGATTCCAGTGGCAGAGGAGT
A Q V V K L L L E
V T V S S S
EcoRI
HindIII
GAACAAAAACTCATCTCAGAAGAGGATCTGAATTAATGAGAATTCATCAAACGGTGATA
61
120
CTTGTTTTTGAGTAGAGTCTTCTCCTAGACTTAATTACTCTTAAGTAGTTTGCCACTATT
E Q K L I S E E D L N * *
121 --- 123
CGA

Fig.19.

(EcoRI) NruI
XhoI
BstEII
AATTTAGTCGCGACAGGTGAAACTGCTCGAGTAAGTGACTAAGGTCACCGTCTCCTCAGA
1
60
ATCAGCGCTGTCCACTTTGACGAGCTCATTCACTGATTCCAGTGGCAGAGGAGTCT
R Q V V K L L L
V T V S S S E
EcoRI
AflII
HindIII
ACAAAAACTCATCTCAGAAGAGGATCTGAATTAATGAGAATTCATCTTAAGGTGATA
61
120
TGTTTTTGAGTAGAGTCTTCTCCTAGACTTAATTACTCTTAAGTAGAATTCCACTATTCG
Q K L I S E E D L N * *
121 - 121
A

Fig.7.

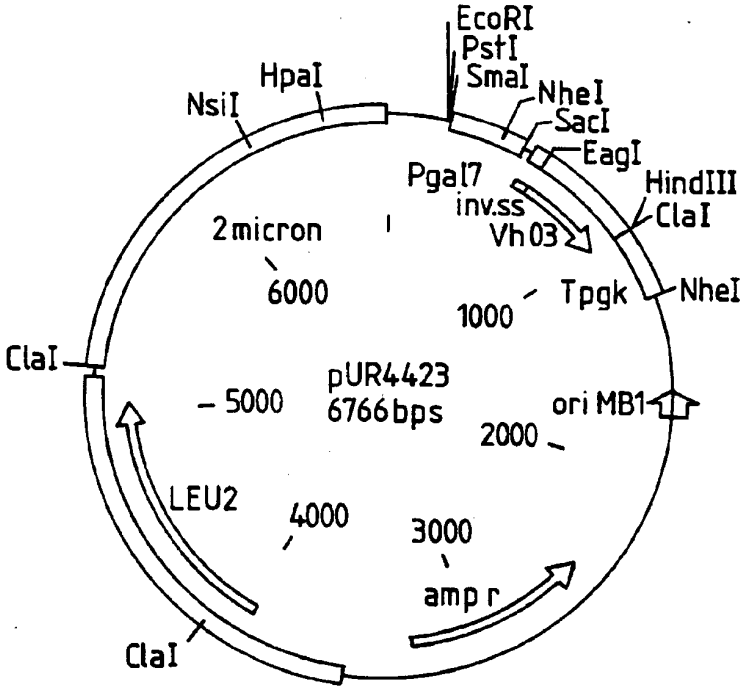

EcoRI
PstI
SmaI
NheI
SacI
EagI
HindIII
ClaI
NheI
HpaI
NsiI
Pgal7
inv.ss
Vh03
2micron
6000
1000
Tpgk
ClaI
pUR4423
6766bps
5000
ori MB1
2000
LEU2
4000
3000
amp r
ClaI

Fig.8.

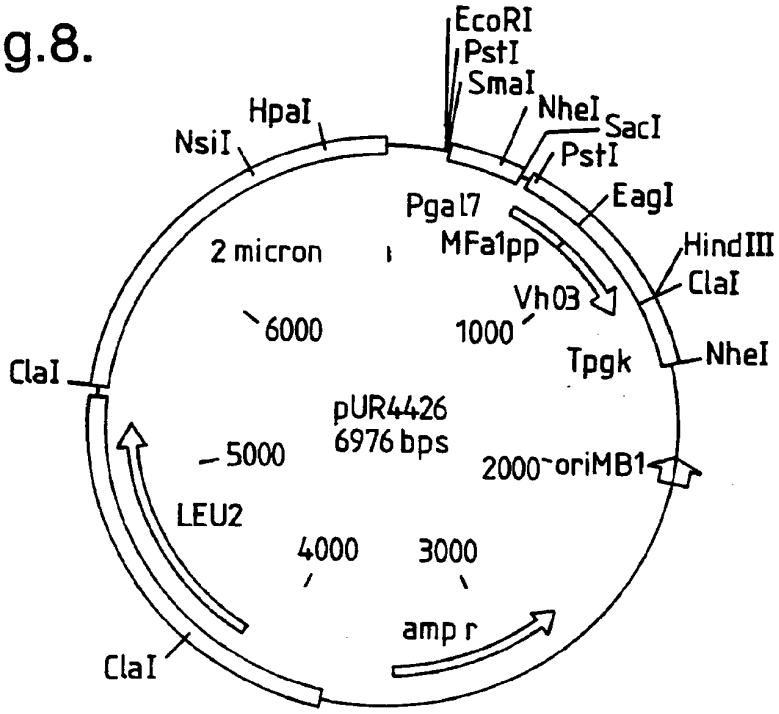

EcoRI
PstI
SmaI
NheI
SacI
PstI
HpaI
NsiI
EagI
Pgal7
MFa1pp
2 micron
HindIII
ClaI
Vh03
6000
1000
Tpgk
NheI
ClaI
pUR4426
6976 bps
5000
2000 oriMB1
LEU2
4000
3000
amp r
ClaI

Fig.9.

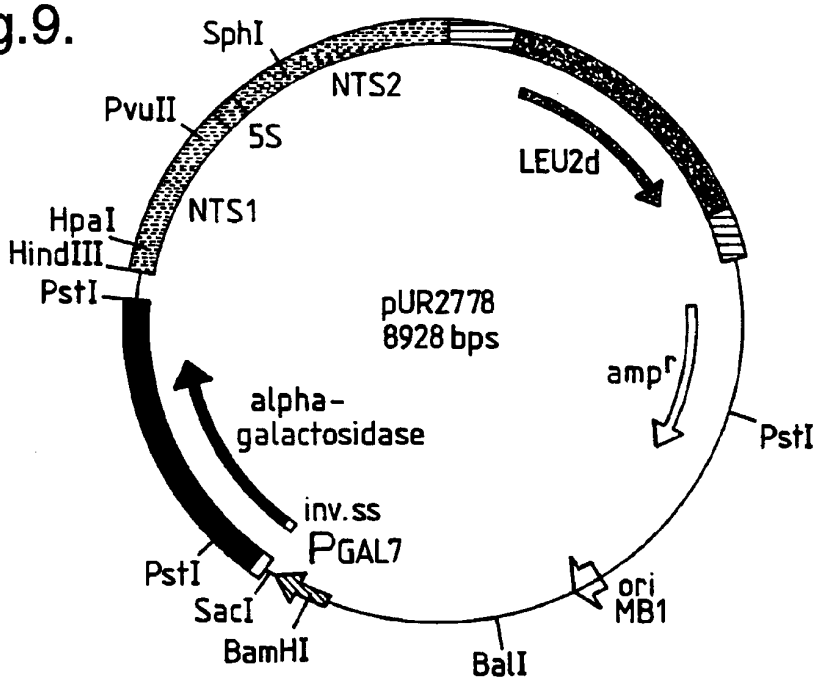

SphI
NTS2
PvuII
5S
LEU2d
HpaI
NTS1
HindIII
PstI
pUR2778
8928 bps
amp^r
alpha-
galactosidase
PstI
inv.ss
PGAL7
PstI
SacI
BamHI
ori
MB1
BalI

Fig.10.

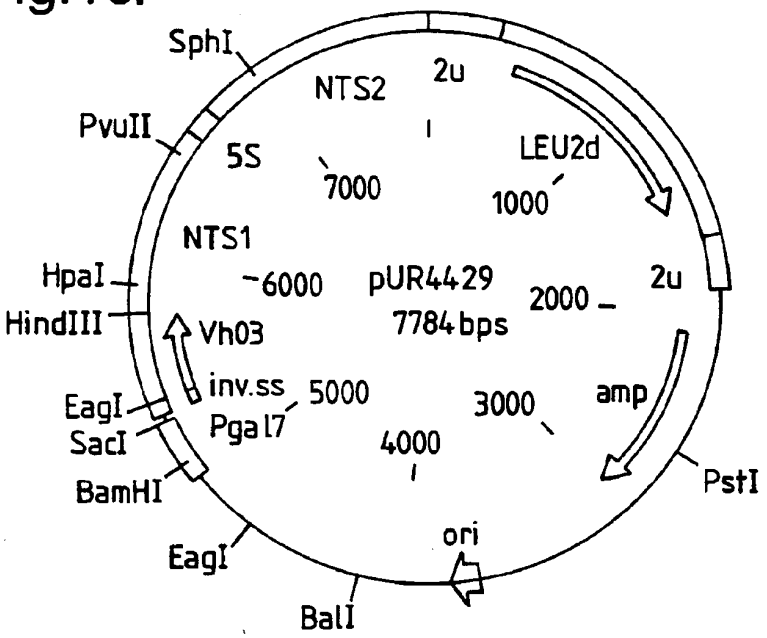

SphI
NTS2
2u
PvuII
5S
LEU2d
7000
1000
NTS1
HpaI
6000
pUR4429
2000
2u
HindIII
Vh03
7784 bps
inv.ss
5000
3000
amp
EagI
Pgal7
SacI
4000
BamHI
PstI
ori
EagI
BalI

Fig.11.

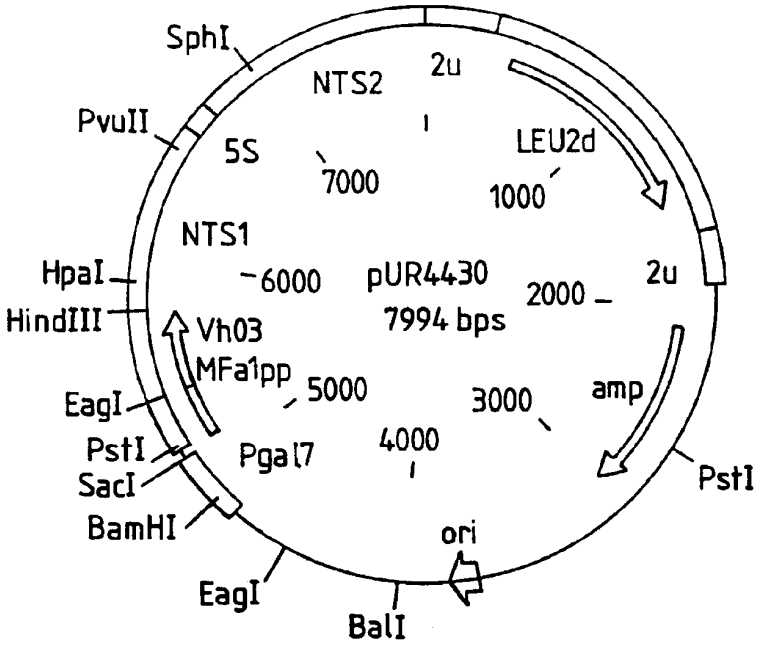

SphI
NTS2
2u
PvuII
5S
7000
LEU2d
1000
NTS1
HpaI
6000
pUR4430
2000
2u
HindIII
Vh03
7994 bps
MFa1pp
5000
3000
amp
EagI
PstI
Pgal7
4000
SacI
BamHI
ori
EagI
PstI
BalI

Fig.12.

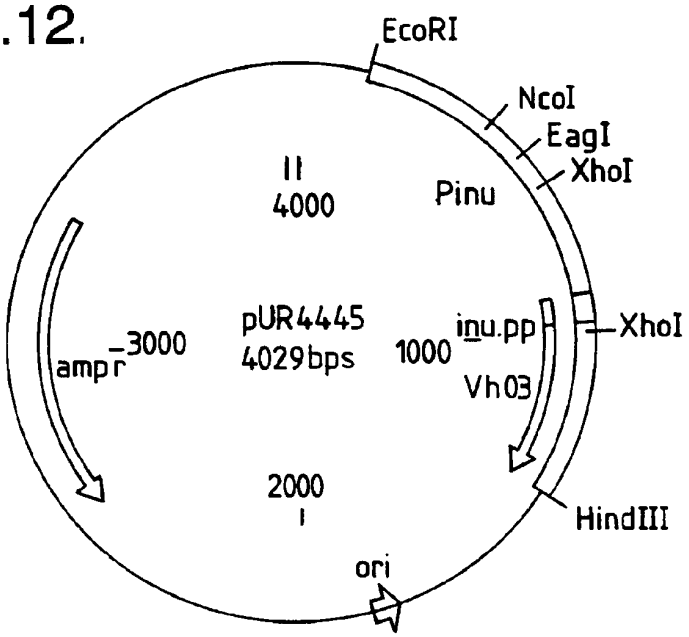

EcoRI
NcoI
EagI
XhoI
II
4000
Pinu
pUR4445
4029bps
inu.pp
XhoI
ampr
3000
1000
Vh03
2000
HindIII
ori

Fig.13.

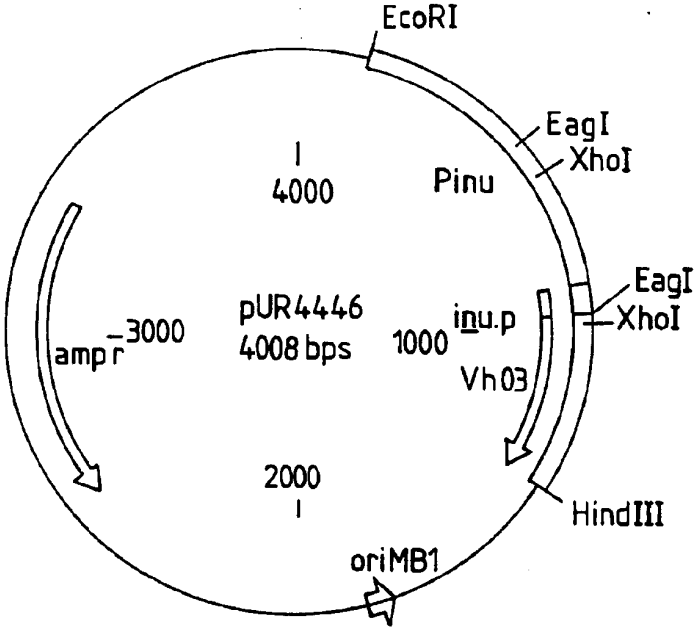

EcoRI
EagI
XhoI
4000
Pinu
EagI
XhoI
pUR4446
4008 bps
amp r
3000
1000
inu.p
Vh03
2000
HindIII
oriMB1

Fig.14.

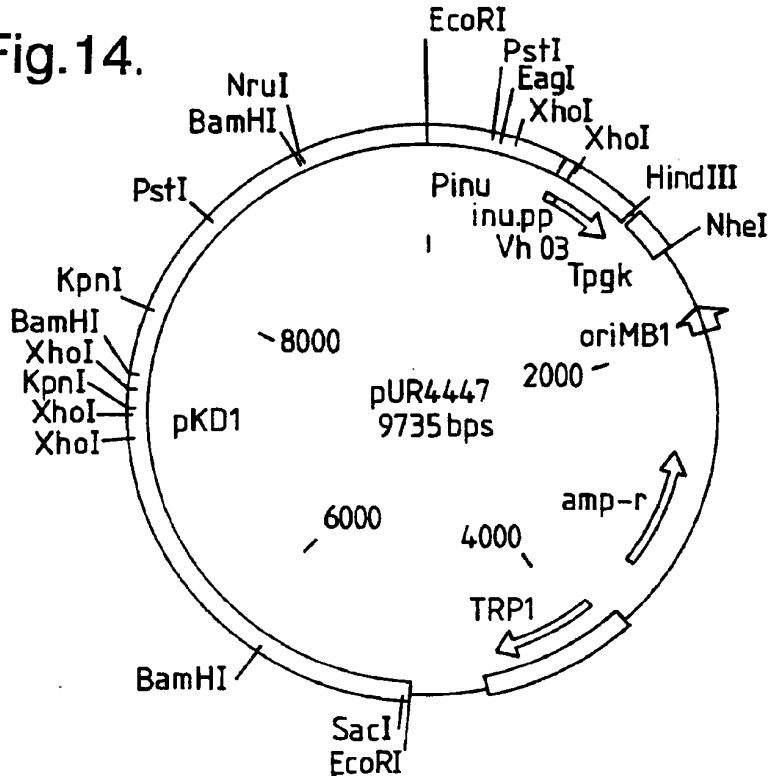

EcoRI
PstI
EagI
XhoI
NruI
BamHI
XhoI
HindIII
PstI
Pinu
inu.pp
Vh 03
NheI
KpnI
Tpgk
BamHI
XhoI
KpnI
XhoI
XhoI
8000
oriMB1
2000
pUR4447
9735 bps
pKD1
amp-r
6000
4000
TRP1
BamHI
SacI
EcoRI

Fig.15.

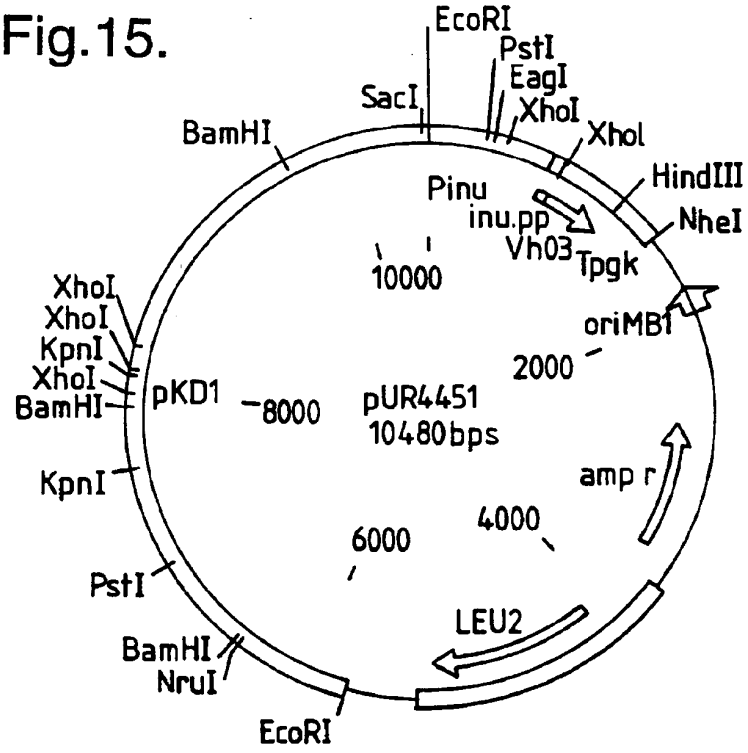

EcoRI
PstI
EagI
XhoI
XhoI
SacI
BamHI
HindIII
NheI
Pinu
inu.pp
Vh03
Tpgk
10000
oriMB1
2000
XhoI
XhoI
KpnI
XhoI
BamHI
pKD1
8000
pUR4451
10480bps
KpnI
amp r
4000
6000
PstI
LEU2
BamHI
NruI
EcoRI

Fig.16.

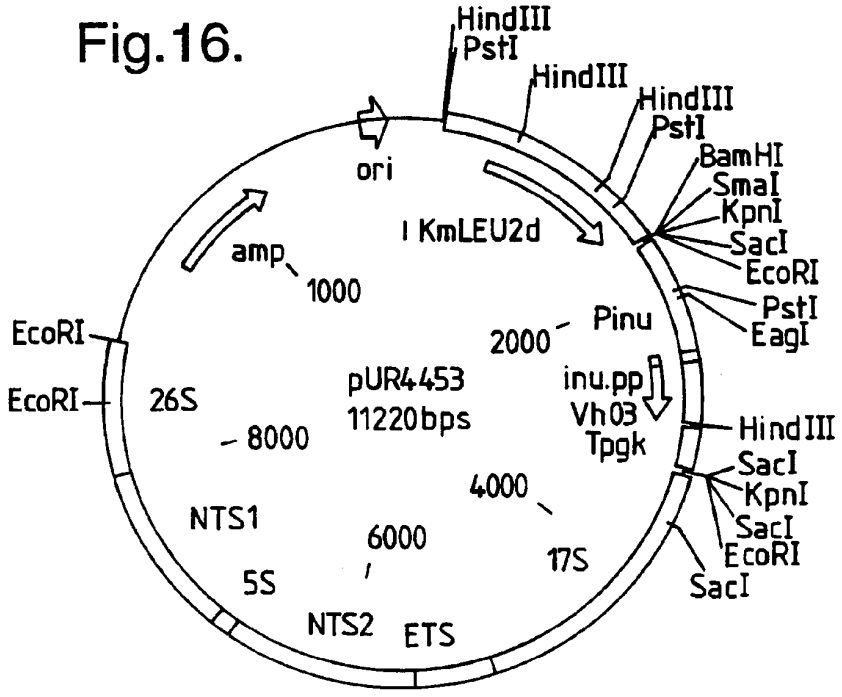

HindIII
PstI
HindIII
HindIII
PstI
BamHI
SmaI
KpnI
SacI
EcoRI
PstI
EagI
ori
I KmLEU2d
amp
1000
EcoRI
2000
Pinu
EcoRI
26S
pUR4453
11220bps
inu.pp
Vh03
Tpgk
8000
HindIII
SacI
4000
KpnI
SacI
NTS1
6000
EcoRI
17S
5S
SacI
NTS2
ETS

Fig.17.

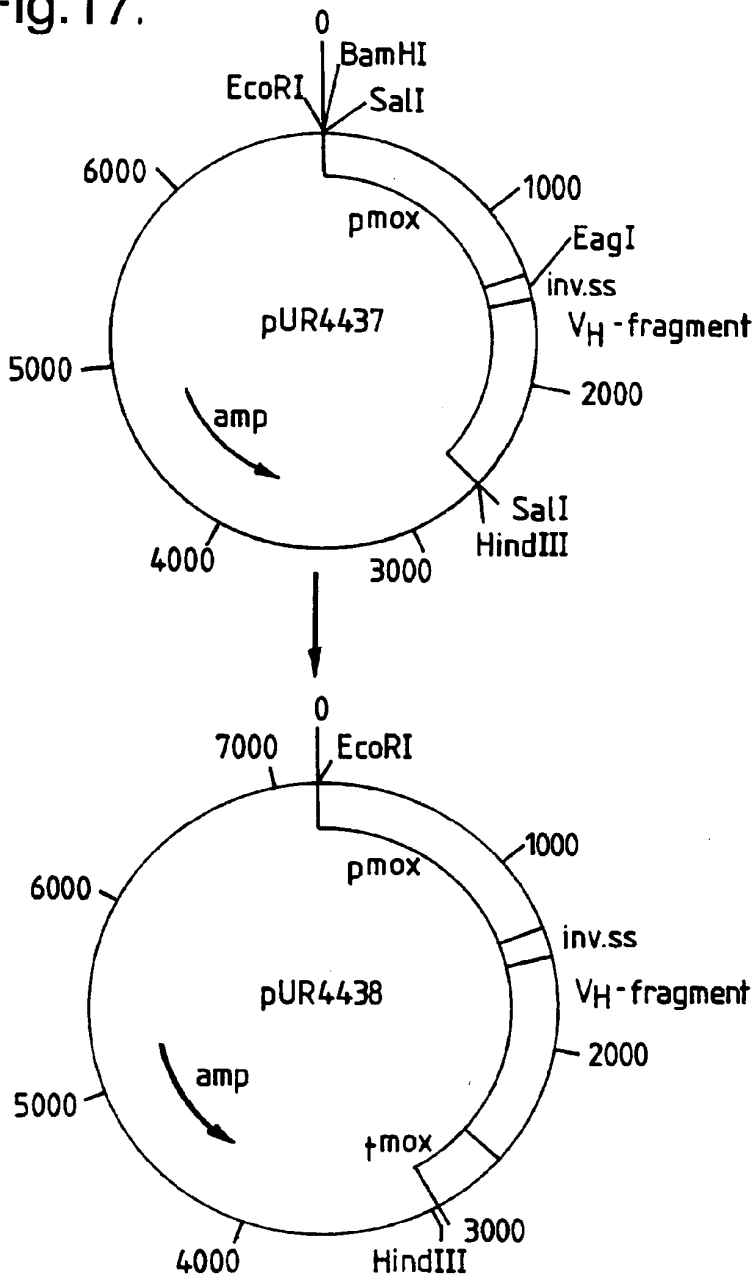
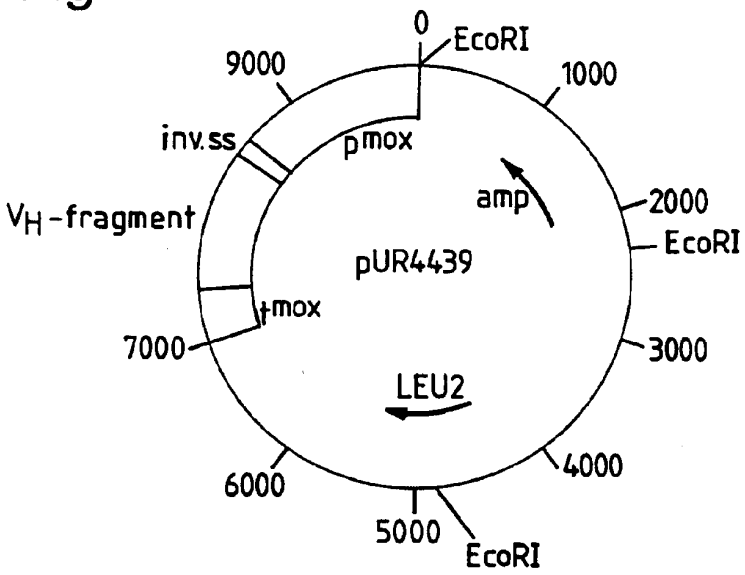

0
BamHI
EcoRI
SalI
6000
1000
pmox
EagI
inv.ss
pUR4437
VH-fragment
5000
2000
amp
SalI
HindIII
4000
3000
0
7000
EcoRI
1000
pmox
6000
inv.ss
pUR4438
VH-fragment
2000
amp
5000
tmox
3000
4000
HindIII

Fig.18.


0
EcoRI
1000
2000
EcoRI
3000
4000
EcoRI
5000
6000
7000
9000
inv.ss
pmox
amp
pUR4439
tmox
LEU2
VH-fragment

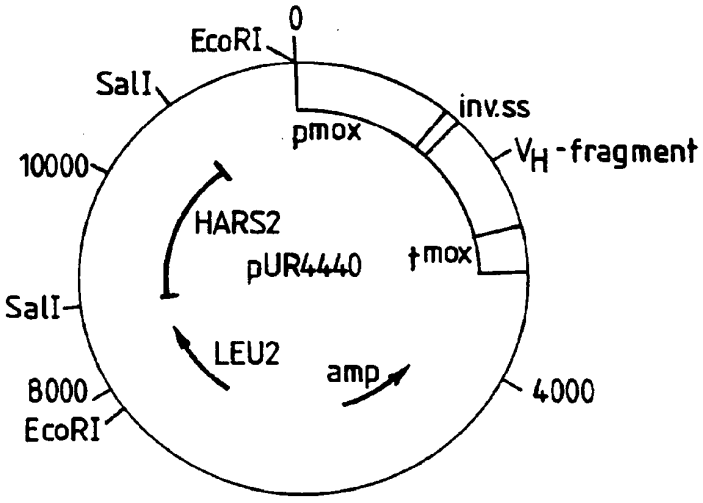
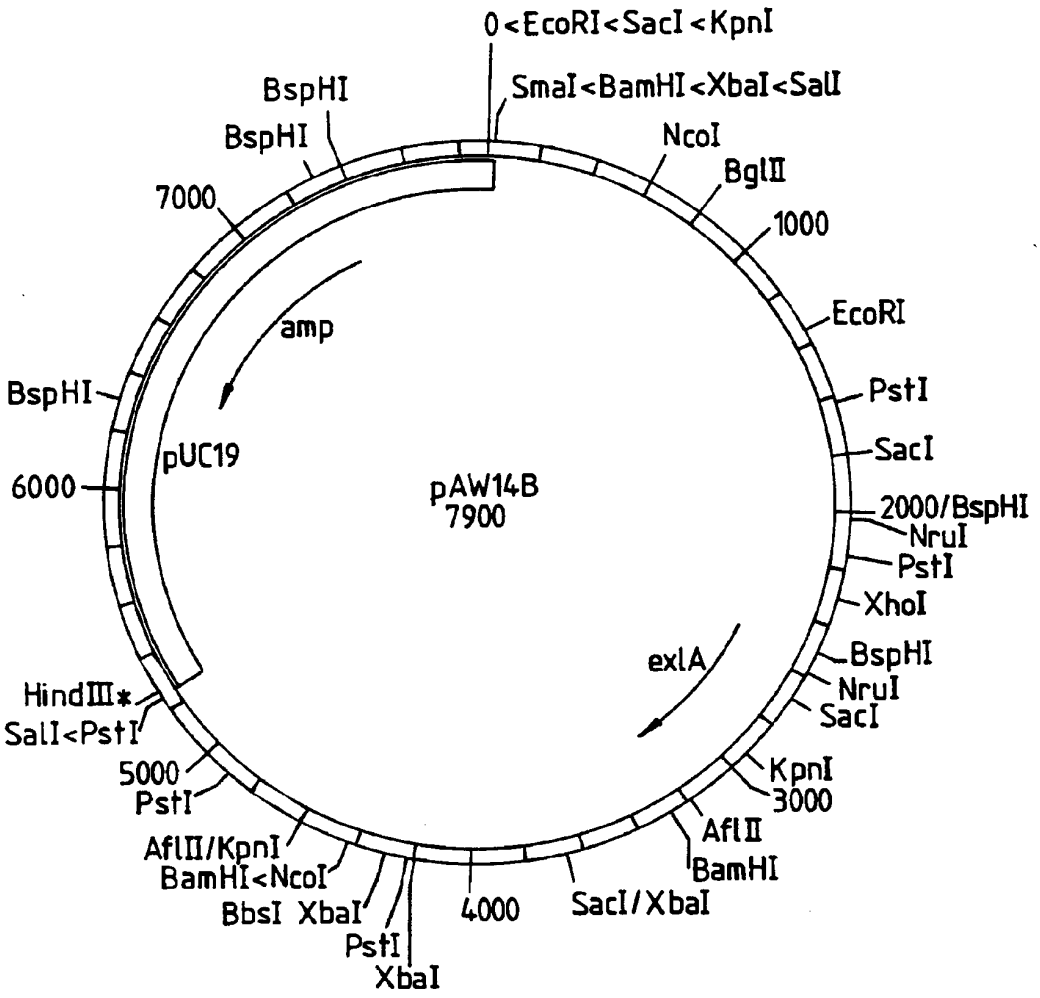

0
EcoRI
SalI
inv.ss
pmox
VH-fragment
10000
HARS2
pUR4440
tmox
SalI
LEU2
amp
8000
EcoRI
4000

Fig.20.


0<EcoRI<SacI<KpnI
SmaI<BamHI<XbaI<SalI
NcoI
BglII
1000
EcoRI
PstI
SacI
2000/BspHI
NruI
PstI
XhoI
BspHI
NruI
SacI
KpnI
3000
AflII
BamHI
SacI/XbaI
4000
PstI
XbaI
BbsI XbaI
BamHI<NcoI
AflII/KpnI
5000
PstI
SalI<PstI
HindIII*
BspHI
6000
pUC19
amp
BspHI
BspHI
7000
pAW14B
7900
exlA

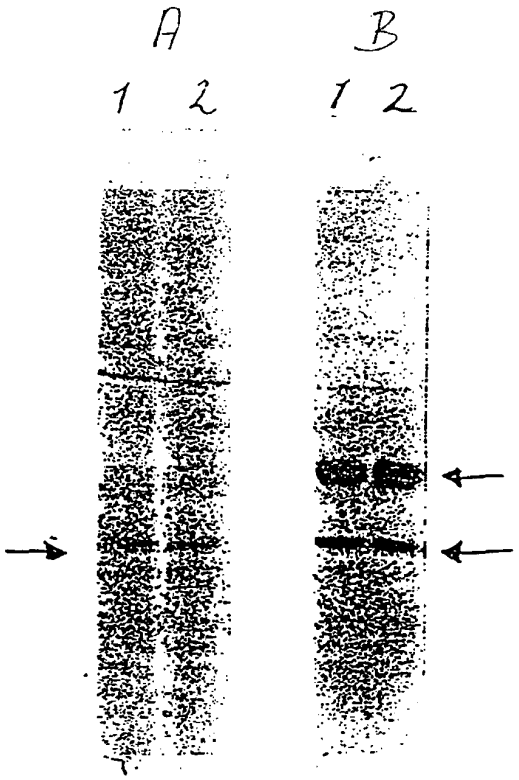


FIGURE 21

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 94/01442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/13 C07K15/28 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 256 421 (PHILLIPS PETROLEUM COMPANY) 24 February 1988 cited in the application see the whole document ---	1,3
P,X	NATURE vol. 363, no. 6428 , 3 June 1993 , LONDON, GB pages 446 - 448 C. HAMERS-CASTERMAN ET AL. 'Naturally occurring antibodies devoid of light chains.' cited in the application see the whole document --- -/--	1,4, 10-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 1994	26 -08- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 94/01442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	FEBS LETTERS vol. 339, no. 3 , 21 February 1994 , AMSTERDAM, THE NETHERLANDS pages 285 - 290 J. DAVIES ET AL. ''Camelising' human antibody fragments: NMR studies on VH domains.' see the whole document	1,5, 10-12
P,X	WO,A,94 04678 (C. CASTERMAN ET AL.) 3 March 1994 see the whole document	1,3,4,6, 10-12

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter nal Application No
PCT/EP 94/01442

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0256421	24-02-88	AU-B-	620667	20-02-92
		AU-A-	4590789	22-03-90
		AU-B-	594476	08-03-90
		AU-A-	7474787	18-02-88
		JP-A-	63044899	25-02-88
WO-A-9404678	03-03-94	EP-A-	0584421	02-03-94
		AU-B-	4949793	15-03-94

Form PCT/ISA/210 (patent family annex) (July 1992)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 May 2004 (21.05.2004)

PCT

(10) International Publication Number
WO 2004/041862 A2

(51) International Patent Classification[7]: C07K 16/00

(21) International Application Number:
PCT/BE2003/000192

(22) International Filing Date:
7 November 2003 (07.11.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/425,073	8 November 2002 (08.11.2002)	US
60/425,063	8 November 2002 (08.11.2002)	US
03447005.4	10 January 2003 (10.01.2003)	EP
PCT/EP03/06581	23 June 2003 (23.06.2003)	EP
PCT/EP03/07313	8 July 2003 (08.07.2003)	EP

(71) Applicant *(for all designated States except US)*: **ABLYNX N.V.** [BE/BE]; Technologiepark 4, B-9052 Zwijnaarde (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SILENCE, Karen** [BE/BE]; Jean Tombeurstraat 21, B-3090 Overijse (BE). **LAUWEREYS, Marc** [BE/BE]; Wilgenstraat 2, B-9450 Haaltert (BE). **DE HAARD, Hans** [NL/NL]; 't Zwint 1, NL-4436 NA Oudelande (NL).

(74) Agent: **BRANTS, Johan, Philippe, Emi**; De Clercq, Brants & Partners, E. Gevaertdreef 10a, B-9830 Sint-Martens-Latem (BE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SINGLE DOMAIN ANTIBODIES DIRECTED AGAINST TUMOUR NECROSIS FACTOR-ALPHA AND USES THEREFOR

(57) Abstract: The present invention relates to polypeptides derived from single domain heavy chain antibodies directed to Tumor Necrosis Factor-alpha. It further relates to single domain antibodies that are Camelidae VHHs. It further relates to methods of administering said polypeptides. It further relates to protocols for screening for agents that modulate the TNF-alpha receptor, and the agents resulting from said screening.

WO 2004/041862 A2

SINGLE DOMAIN ANTIBODIES DIRECTED AGAINST TUMOUR NECROSIS FACTOR-ALPHA AND USES THEREFOR

FIELD OF THE INVENTION

The present invention provides polypeptides comprising one or more single domain antibodies directed towards tumor necrosis factor alpha (TNF-alpha). The present invention further relates to their use in diagnosis and therapy. Such antibodies may have a framework sequence with high homology to the human framework sequences. Compositions comprising antibodies to tumor necrosis factor alpha (TNF-alpha) alone or in combination with other drugs are described.

BACKGROUND TO THE INVENTION

Tumor necrosis factor alpha (TNF-alpha) is believed to play an important role in various disorders, for example in inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis. Both TNF-alpha and the receptors (CD120a, CD120b) have been studied in great detail. TNF-alpha in its bioactive form is a trimer and the groove formed by neighboring subunits is important for the cytokine-receptor interaction. Several strategies to antagonize the action of the cytokine have been developed and are currently used to treat various disease states.

A TNF-alpha inhibitor which has sufficient specificity and selectivity to TNF-alpha may be an efficient prophylactic or therapeutic pharmaceutical compound for preventing or treating disorders where TNF-alpha has been implicated as causative agent. Methods of treating toxic shock (EP 486526), tumor regression, inhibition of cytotoxicity (US 6448380, US 6451983, US 6498237), autoimmune disease such as RA and Crohn's disease (EP 663836, US 5672347, US 5656272), graft versus host reaction (US 5672347), bacterial meningitis (EP 585705) by means of an antibody to TNF-alpha have been described.

Yet none of the presently available drugs are completely effective for the treatment of autoimmune disease, and most are limited by severe toxicity. In addition, it is extremely difficult and a lengthy process to develop a new chemical entitiy (NCE) with sufficient potency and selectivity to such target sequence. Antibody-based therapeutics on the other hand have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. In addition, the development time can be reduced considerably when compared to the development of new chemical entities (NCE's). However, conventional antibodies are difficult to raise against multimeric proteins where the receptor-binding domain of the ligand is embedded in a groove, as is the case with

TNF-alpha. Heavy chain antibodies described in the invention which are derived from *Camelidae*, are known to have cavity-binding propensity (WO97/49805; Lauwereys *et al*, EMBO J. 17, 5312, 1998)). Therefore, such heavy chain antibodies are inherently suited to bind to receptor binding domains of such ligands as TNF. In addition, such antibodies are known to be stable over long periods of time, therefore increasing their shelf-life (Perez *et al*, Biochemistry, 40, 74, 2001). Furthermore, such heavy chain antibody fragments can be produced 'en-masse' in fermentors using cheap expression systems compared to mammalian cell culture fermentation, such as yeast or other microorganisms (EP 0 698 097).

The use of antibodies derived from sources such as mouse, sheep, goat, rabbit etc., and humanised derivatives thereof as a treatment for conditions which require a modulation of inflammation is problematic for several reasons. Traditional antibodies are not stable at room temperature, and have to be refrigerated for preparation and storage, requiring necessary refrigerated laboratory equipment, storage and transport, which contribute towards time and expense. Refrigeration is sometimes not feasible in developing countries. Furthermore, the manufacture or small-scale production of said antibodies is expensive because the mammalian cellular systems necessary for the expression of intact and active antibodies require high levels of support in terms of time and equipment, and yields are very low. Furthermore the large size of conventional antibodies, would restrict tissue penetration, for example, at the site of inflamed tissue. Furthermore, traditional antibodies have a binding activity which depends upon pH, and hence are unsuitable for use in environments outside the usual physiological pH range such as, for example, in treating gastric bleeding, gastric surgery. Furthermore, traditional antibodies are unstable at low or high pH and hence are not suitable for oral administration. However, it has been demonstrated that camelidae antibodies resist harsh conditions, such as extreme pH, denaturing reagents and high temperatures (Dumoulin *et al*, Protein Science 11, 500, 2002), so making them suitable for delivery by oral administration. Furthermore, traditional antibodies have a binding activity, which depends upon temperature, and hence are unsuitable for use in assays or kits performed at temperatures outside biologically active-temperature ranges (*e.g.* 37 ± 20°C).

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, it is known by the skilled addressee that an antibody which has been obtained for a therapeutically useful target requires additional modification in order

to prepare it for human therapy, so as to avoid an unwanted immunological reaction in a human individual upon administration thereto. The modification process is commonly termed "humanisation". It is known by the skilled artisan that antibodies raised in species, other than in humans, require humanisation to render the antibody therapeutically useful in humans ((1) CDR grafting : Protein Design Labs: US 6180370, US 5693761; Genentech US 6054297; Celltech: 460167, EP 626390, US 5859205; (2) Veneering: Xoma: US 5869619, US 5766886, US 5821123). There is a need for a method for producing antibodies which avoids the requirement for substantial humanisation, or which completely obviates the need for humanisation. There is a need for a new class of antibodies which have defined framework regions or amino acid residues and which can be administered to a human subject without the requirement for substantial humanisation, or the need for humanisation at all.

Another important drawback of conventional antibodies is that they are complex, large molecules and therefore relatively unstable, and they are sensitive to breakdown by proteases. This means that conventional antibody drugs cannot be administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation because they are not resistant to the low pH at these sites, the action of proteases at these sites and in the blood and/or because of their large size. They have to be administered by injection (intravenously, subcutaneously, etc.) to overcome some of these problems. Administration by injection requires specialist training in order to use a hypodermic syringe or needle correctly and safely. It further requires sterile equipment, a liquid formulation of the therapeutic polypeptide, vial packing of said polypeptide in a sterile and stable form and, of the subject, a suitable site for entry of the needle. Furthermore, subjects commonly experience physical and psychological stress prior to and upon receiving an injection. Therefore, there is need for a method for the delivery of therapeutic polypeptides which avoids the need for injection which is not only cost/time saving, but which would also be more convenient and more comfortable for the subject.

Single domain antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, improving further their intrinsic and functional affinity can lead to many benefits for a patient such as reduced dose of therapeutic, faster therapy, and reduced side effects.

THE AIMS OF THE PRESENT INVENTION

It is an aim of the present invention is to provide polypeptides comprising one or more single domain antibodies which bind to TNF-alpha, homologues of said polypeptides, functional portions of homologues of said polypeptides. Said polypeptides modify the biological activity of TNF-alpha upon binding. Such polypeptides might bind into the receptor-binding groove of TNF-alpha, or might not bind in the receptor binding groove. Such polypeptides are single domain antibodies.

It is a further aim of the present invention to provide single domain antibodies which may be any of the art, or any future single domain antibodies. Examples include, but are not limited to, heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional 4-chain antibodies, engineered antibodies and single domain scaffolds other than those derived from antibodies. According to one aspect of the invention, a single domain antibody as used herein is a naturally occurring single domain antibody known as heavy chain antibody devoid of light chains (WO 9404678). For clarity reasons, this variable domain derived from a heavy chain antibody devoid of light chain will be called VHH or nanobody to distinguish it from the conventional VH of four chain immunoglobulins. Such a VHH molecule can be derived from antibodies raised in *Camelidae* species, for example in camel, llama, dromedary, alpaca and guanaco.

It is a further aim of the invention to provide a method of administering anti-TNF-alpha polypeptides intravenously, subcutaneously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

It is a further aim of the invention to enhance the binding affinity of monovalent single domain antibodies.

SUMMARY OF THE INVENTION

One embodiment of the present invention is an anti-TNF-alpha polypeptide comprising at least one anti-TNF-alpha single domain antibody.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 16 and 79 to 84.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above further comprising at least one single domain antibody directed against a serum protein.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above wherein said serum protein is any of serum albumin, serum immunoglobulins, thyroxine-binding protein, transferring, or fibrinogen.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above wherein a single domain anti-serum protein single domain antibody correspond to a sequence represented by any of SEQ ID NOs: 26 to 29 and 85 to 97.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above corresponding to a sequence represented by any of SEQ ID NOs: 30 to 43.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above further comprising at least one single domain antibody selected from the group consisting of anti-IFN-gamma single domain antibody, anti-TNF-alpha receptor single domain antibody and anti-IFN-gamma receptor single domain antibody.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, wherein the number of single domain antibodies directed against TNF-alpha is at least two.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above corresponding to a sequence represented by any of SEQ ID NOs: 73 to 76.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, wherein at least one single domain antibody is a humanized Camelidae VHHs.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above wherein a humanized Camelidae VHH corresponds to a sequence represented by any of SEQ ID NOs: 17 to 19 and 21 to 24.

Another embodiment of the present invention is a composition comprising an anti-TNF-alpha polypeptide as described above and at least one single domain antibody from the group consisting of anti-IFN-gamma single domain antibody, anti-TNF-alpha receptor single domain antibody and anti-IFN-gamma receptor single domain antibody, for simultaneous, separate or sequential administration to a subject.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a composition as described above wherein at least one anti-IFN-gamma single domain antibody correspond to a sequence represented by any of SEQ ID NOs: 44 to 72.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a composition as described above, wherein said single domain antibody is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length single domain antibody.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a composition as described above, wherein the anti-TNF-alpha polypeptide is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length anti-TNF-alpha polypeptide.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a composition as described above wherein at least one single domain antibody is a Camelidae VHH.

Another embodiment of the present invention is a nucleic acid encoding an anti-TNF-alpha polypeptide as described above.

Another embodiment of the present invention is a method of identifying an agent that modulates the binding of an anti-TNF-alpha polypeptide as described above, to Tumor Necrosis Factor-alpha comprising the steps of:
(a) contacting an anti-TNF-alpha polypeptide as described above with a target that is Tumor Necrosis Factor alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and
(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in

the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of an anti-TNF-alpha polypeptide as described above and Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a method of identifying an agent that modulates Tumor Necrosis Factor-alpha-mediated disorders through the binding of an anti-TNF-alpha polypeptide as described above to Tumor Necrosis Factor-alpha comprising:

(a) contacting an anti-TNF-alpha polypeptide as described above with a target that is Tumor Necrosis Factor alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified, said candidate modulator as an agent that modulates Tumor Necrosis Factor alpha-mediated disorders.

Another embodiment of the present invention is a method of identifying an agent that modulates the binding of Tumor Necrosis Factor alpha to its receptor through the binding of an anti-TNF-alpha polypeptide as described above to Tumor Necrosis Factor-alpha comprising:

(a) contacting an anti-TNF-alpha polypeptide as described above with a target that is Tumor Necrosis Factor-alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of Tumor Necrosis Factor-alpha to its receptor.

Another embodiment of the present invention is a kit for screening for agents that modulate Tumor Necrosis Factor-alpha-mediated disorders comprising an anti-TNF-alpha polypeptide as described above and Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is an unknown agent that modulates the binding of an anti-TNF-alpha polypeptide as described above to Tumor Necrosis Factor-alpha, identified according to the method as described above.

Another embodiment of the present invention is an unknown agent that modulates Tumor Necrosis Factor-alpha-mediated disorders, identified according to the methods as described above.

Another embodiment of the present invention is an unknown agent as described above wherein said disorders are one or more of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above, or a nucleic acid as described above, or a composition as described above, or an agent as described above for treating and/or preventing and/or alleviating disorders relating to inflammatory processes.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a nucleic acid as described above, or a composition as described above, or an agent as described above for the preparation of a medicament for treating and/or preventing and/or alleviating disorders relating to inflammatory reactions.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the gastric environment without the substance being inactivated.

Another embodiment of the present invention is an use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the gastric environment without the substance being inactivated.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the vaginal and/or rectal tract.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the vaginal and/or rectal tract.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the nose, upper respiratory tract and/or lung.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the nose, upper respiratory tract and/or lung.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the tissues beneath the tongue effectively.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a

medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the tissues beneath the tongue effectively.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as described above or a composition as described above, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the skin effectively.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above or a composition as described above, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the skin effectively.

Another embodiment of the present invention is a method as described above, a kit as described above, a nucleic acid or agent as described above, use of a nucleic acid or agent as described above, a composition as described above, use of a composition as described above, an anti-TNF-alpha polypeptide as described above, use of an anti-TNF-alpha polypeptide as described above wherein said disorders are any of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, multiple sclerosis, Addison's disease, Autoimmune hepatitis, Autoimmune parotitis, Diabetes Type I, Epididymitis, Glomerulonephritis, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, Hemolytic anemia, Systemic lupus erythematosus, Male infertility, Multiple sclerosis, Myasthenia Gravis, Pemphigus, Psoriasis, Rheumatic fever, Rheumatoid arthritis, Sarcoidosis, Scleroderma, Sjogren's syndrome, Spondyloarthropathies, Thyroiditis, and Vasculitis.

Another embodiment of the present invention is a composition comprising a nucleic acid or agent as described above, an anti-TNF-alpha polypeptide as described above, or a composition as described above, and a suitable pharmaceutical vehicle.

Another embodiment of the present invention is a method of diagnosing a disorder characterised by the dysfunction of Tumor Necrosis Factor-alpha comprising:
(a) contacting a sample with an anti-TNF-alpha polypeptide as described above,
(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a kit for screening for a disorder as cited above, using a method as described above.

Another embodiment of the present invention is a kit for screening for a disorder as cited above comprising an isolated anti-TNF-alpha polypeptide as described above.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above for the purification of said Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as described above for inhibiting the interaction between Tumor Necrosis Factor-alpha and one or more Tumor Necrosis Factor-alpha receptors.

Another embodiment of the present invention is a method for producing an anti-TNF-alpha polypeptide as described above comprising the steps of:
(a) obtaining double stranded DNA encoding a Camelidae VHH directed to Tumor Necrosis Factor alpha,
(b) cloning and expressing the DNA selected in step (b).

Another embodiment of the present invention is a method of producing an anti-TNF-alpha polypeptide as described above comprising:
(a) culturing host cells comprising nucleic acid capable of encoding an anti-TNF-alpha polypeptide as described above, under conditions allowing the expression of the polypeptide, and,
(b) recovering the produced polypeptide from the culture.

Another embodiment of the present invention is a method as described above, wherein said host cells are bacterial or yeast.

Another embodiment of the present invention is a kit for screening for any of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome or multiple sclerosis comprising an anti-TNF-alpha polypeptide as described above.

BRIEF DESCRIPTION OF FIGURES AND TABLES

Figure 1 Alignment of anti-human TNF VHH's as described in Example 1

Figure 2 Dilution series of anti-human TNF-alpha VHHs as tested in ELISA according to Example 1.

Figure 3 Antagonistic effect of VHH as determined in cytotoxicity assay using human cell line KYM according to Example 1.

Figure 4 In vitro receptor binding assay of wild type VHH#12B and mutant A74S+Y76N+K83R+P84A

Figure 5 In vitro receptor binding assay of wild type VHH#12B and mutant 1E + Q5LA74S + Y76N + K83R + P84A

Figure 6 Binding in ELISA of wild type VHH#3E and mutant VHH's

Figure 7 In vitro receptor binding assay of wild type VHH#3E and mutant VHH's

Figure 8 Alignment of antagonistic anti-mouse TNF's as described in Example 3.

Figure 9 Antagonistic effect of anti-mouse TNF VHH as determined in cytotoxicity assay using murine cell line L929 according to Example 3.

Figure 10 EcoRI – HindIII insert of vector pAX11 (pUC119 backbone) for production of bi-valent or bispecific VHH.

Figure 11 Coomassie-stained PAGE (15%) of IMAC-purified mono-(lane 8), bi- (lane 1), tri- (lanes 2, 3 and 5) and tetravalent (lanes 4, 6 and 7) anti- TNFa VHH.

Figure 12 Chromatogram of the analysis by gel filtration on Superdex 75HR of the mono-, bi-, tri and tetravalent VHH.

Figure 13 Comparison of the antagonistic characteristics of the mono-, bi-, tri- and tetravalent form of the anti-human TNF VHH with the clinically used products Remicade and Enbrel.

Figure 14 Antagonistic behaviour of the mono- and bivalent VHH's directed against mouse TNFalpha .

Figure 15 Coomassie stained PAGE of VHH-Fc-fusion derived from human IgG1 described in Example 4.

Figure 16 Antagonistic efficacy of VHH-Fc fusion derived from VHH#3E compared with bivalent format of VHH#3E as determined in bioassay.

Figure 17 ELISA of reference and pepsin-treated TNF3E at pH2.2, pH3.2 and pH4.2 (100% is the signal measured at a 1/100 dilution)

Figure 18 Experimental setting

Table 1 Amino acid sequence listing of the peptides of aspects of present invention directed against TNF-alpha.

Table 2 List of mutagenesis reactions, mutagenic primers and templates used for mutagenesis of VHH#12B.

Table 3 List of mutagenesis reactions, mutagenic primers and templates used for mutagenesis of VHH#3E.

Table 4 Overview of humanised and wild type VHH

Table 5 Anti-mouse serum albumin/anti TNF-alpha

Table 6 Amino acid sequence listing of VHH's directed against human IFN-gamma.

Table 7 Sequences of bivalent (BIV 3E, BIV#m3F), trivalent (TRI3E) or tetravalent (TETRA 3E) VHH directed against TNF-alpha.

Table 8 Fractional homologies between the amino acid sequences of anti-mouse serum albumin VHHs of the invention.

Table 9 Fractional homologies between anti-TNF-alpha VHHs of the invention.

Table 10 Percentage homologies between anti-IFN-gamma VHHs of the invention.

Table 11 Treatment schedule

DETAILED DESCRIPTION

The present invention relates to an anti-tumour necrosis factor-alpha (TNF-alpha) polypeptide, comprising one or more single domain antibodies which are directed against TNF-alpha. The invention also relates to nucleic acids capable of encoding said polypeptides.

Single domain antibodies are antibodies whose complementary determining regions are part of a single domain polypeptide. Examples include, but are not limited to, heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional 4-chain antibodies, engineered antibodies and single domain scaffolds other than those derived from antibodies. Single domain antibodies may be any of the art, or any future single domain antibodies. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, goat, rabbit, bovine. According to one aspect of the invention, a single domain antibodies as used herein is a naturally occurring single domain antibody known as heavy chain antibody devoid of light chains. Such single domain antibodies are disclosed in WO 94/04678 for example. For clarity reasons, this variable domain derived from a heavy chain antibody naturally devoid of light chain is known herein as a *VHH* or *nanobody* to distinguish it from the conventional VH of four chain immunoglobulins. Such a VHH molecule can be derived from antibodies raised in *Camelidae* species, for example in camel, dromedary, llama, alpaca and guanaco. Other species besides *Camelidae* may produce heavy chain antibodies naturally devoid of light chain; such VHHs are within the scope of the invention.

VHHs, according to the present invention, and as known to the skilled addressee are heavy chain variable domains derived from immunoglobulins naturally devoid of light chains such as those derived from *Camelidae* as described in WO 94/04678 (and referred to hereinafter as VHH domains or nanobodies). VHH molecules are about 10x smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies. Furthermore, *in vitro* expression of VHHs produces high yield, properly folded functional VHHs. In addition, antibodies generated in

Camelids will recognize epitopes other than those recognised by antibodies generated *in vitro* through the use of antibody libraries or via immunisation of mammals other than *Camelids* (WO 9749805). As such, anti-TNF-alpha VHH's may interact more efficiently with TNF-alpha than conventional antibodies, thereby blocking its interaction with the TNF-alpha receptor more efficiently.

According to the invention, TNF-alpha is derived from any species. Examples of species relevant to the invention include as rabbits, goats, mice, rats, cows, calves, camels, llamas, monkeys, donkeys, guinea pigs, chickens, sheep, dogs, cats, horses, and preferably humans.

TNF-alpha is also a fragment of TNF-alpha, capable of eliciting an immune response. TNF-alpha is also a fragment of TNF-alpha, capable of binding to a single domain antibody raised against the full length TNF-alpha.

A single domain antibody directed against TNF-alpha means single domain antibody that it is capable of binding to TNF-alpha with an affinity of better than 10^{-6} M.

One embodiment of the present invention is an anti-TNF polypeptide, wherein the single domain antibodies comprise *Camelidae* VHH directed against TNF-alpha.

The one or more single domain antibodies of the anti-TNF polypeptide which are directed against a TNF-alpha may be of the same sequence. Alternatively they may not all have the same sequence. It is within the scope of the invention that an anti-TNF polypeptide comprises anti-TNF-alpha single domain antibodies which do not all share the same sequence, but which are directed against the same target, one or more antigens thereof.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 16 and 79 to 84 as shown in Table 1. Said sequences are derived from *Camelidae* heavy chain antibodies (VHHs) which are directed against TNF-alpha.

The present invention further relates to an anti-TNF-alpha polypeptide, wherein said single domain antibody is a VHH directed against TNF-alpha, wherein the VHH belongs to a class having human-like sequences. The class is characterised in that the VHHs carry an amino acid from the group consisting of glycine, alanine, valine, leucine, isoleucine,

proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, or glutamine at position 45, such as, for example, L45 and a tryptophan at position 103, according to the Kabat numbering. The new class of *Camelidae* single-domain antibodies described in this invention (Table 1, Example 1) is represented by VHH#2B (SEQ ID NO: 3) and VHH#12B (SEQ ID No. 14) containing the hydrophobic residues in FR2 in combination with the hydrophobic residue tryptophan at position 103.

Another human-like class of *Camelidae* single domain antibodies represented by sequences VHH#1A (SEQ ID NO. 1), VHH#4B (SEQ ID NO. 12), VHH#8-29 (SEQ ID NO. 81), VHH#8-41 (SEQ ID NO. 82), VHH#8-42 (SEQ ID NO. 83) and VHH#8-44 (SEQ ID NO. 84) (Table 1, Example 1) have been described in WO03035694 and contain the hydrophobic FR2 residues typically found in conventional antibodies of human origin or from other species, but compensating this loss in hydrophilicity by the charged arginine residue on position 103 that substitutes the conserved tryptophan residue present in VH from double-chain antibodies. As such, peptides belonging to these two classes show a high amino acid sequence homology to human VH framework regions and said peptides might be administered to a human directly without expectation of an unwanted immune response therefrom, and without the burden of further humanisation. The invention also relates to nucleic acids capable of encoding said polypeptides.

Therefore, one aspect of the present invention allows for the direct administration of an anti-TNF-alpha polypeptide, wherein the single domain antibodies belong to the humanized class of VHH, and comprise a sequence represented by any of SEQ ID NO:1, 3, 12, 14, 81, 82, 83, and 84 to a patient in need of the same.

Any of the VHHs as used by the invention may be of the traditional class or of the classes of human-like *Camelidae* antibodies. Said antibodies may be directed against whole TNF-alpha or a fragment thereof, or a fragment of a homologous sequence thereof. These polypeptides include the full length *Camelidae* antibodies, namely Fc and VHH domains, chimeric versions of heavy chain *Camelidae* antibodies with a human Fc domain or VHH's by themselves or derived fragments.

Anti-serum albumin VHH's may interact in a more efficient way with serum albumin than conventional antibodies which is known to be a carrier protein. As a carrier protein some of the epitopes of serum albumin may be inaccessible by bound proteins, peptides and small chemical compounds. Since VHH's are known to bind into 'unusual' or non-

conventional epitopes such as cavities (WO 97/49805), the affinity of such VHH's to circulating albumin may be increased.

The present invention also relates to the finding that an anti-TNF polypeptide as described herein further comprising one or more single domain antibodies directed against one or more serum proteins of a subject, surprisingly has significantly prolonged half-life in the circulation of said subject compared with the half-life of the anti-TNF-alpha single domain antibody when not part of said construct. Examples of such polypeptides are represented in Table 5 by SEQ ID NOs: 30 to 43. Furthermore, the said polypeptides were found to exhibit the same favourable properties of single domain antibodies such as high stability remaining intact in mice, extreme pH resistance, high temperature stability and high target affinity.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide further comprising one or more single domain antibodies directed against one or more serum proteins, said anti-TNF alpha polypeptide comprising a sequence corresponding to any represented by SEQ ID NOs: 30 to 43 (Table 5).

Another embodiment of the present invention is an anti-TNF-alpha polypeptide, wherein an anti-serum protein single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 26 to 29 and 85 to 97 as shown in Table 5.

The serum protein may be any suitable protein found in the serum of subject. In one aspect of the invention, the serum protein is serum albumin, serum immunoglobulins, thyroxine-binding protein, transferrin, or fibrinogen. Depending on the intended use such as the required half-life for effective treatment and/or compartimentalisation of the target antigen, the VHH-partner can be directed to one of the above serum proteins.

Another aspect of the invention is an anti-TNF-alpha polypeptide as disclosed herein further comprising at least one polypeptide selected from the group consisting of an anti-IFN-gamma polypeptide, an anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide.

It is an embodiment of the invention that a single domain antibody directed against IFN-gamma corresponds to a sequence represented by any of SEQ ID NOs: 44 to 72 as shown in Table 6.

According to one aspect of the invention, a single domain antibody is directed against TNF-alpha receptor. Said single domain antibody may be a *Camelidae* VHH.

According to one aspect of the invention, a single domain antibody is directed against IFN-gamma receptor. Said single domain antibody may be a *Camelidae* VHH.

Another aspect of the invention is a method of treating an autoimmune disease or condition as cited herein, comprising administering to a patient an effective amount of an anti-TNF-alpha polypeptide further comprising a least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, such polypeptides joined to each other as described below.

Such multi-specific constructs may have improved potency as inflammatory therapeutic compound over mono-specific constructs.

One aspect of the invention is a composition comprising an anti-TNF-alpha polypeptide as disclosed herein and at least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, for simultaneous, separate or sequential administration to a subject.

One aspect of the invention is a method for treating autoimmune disease comprising administering to an individual an effective amount of an anti-TNF-alpha polypeptide and a least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, simultaneously, separately or sequentially.

Another aspect of the invention is a kit containing an anti-TNF-alpha polypeptide and a least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide for simultaneous, separate or sequential administration to a subject. It is an aspect of the invention that the kit may be used according to the invention. It is an aspect of the invention that the kit may be used to treat the diseases as cited herein.

By simultaneous administration means the polypeptides are administered to a subject at the same time. For example, as a mixture of the polypeptides or a composition comprising

said polypeptides. Examples include, but are not limited to a solution administered intraveneously, a tablet, liquid, topical cream, etc., wherein each preparation comprises the polypeptides of interest.

By separate administration means the polypeptides are administered to a subject at the same time or substantially the same time. The polypeptides are present in the kit as separate, unmixed preparations. For example, the different polypeptides may be present in the kit as individual tablets. The tablets may be administered to the subject by swallowing both tablets at the same time, or one tablet directly following the other.

By sequential administration means the polypeptides are administered to a subject sequentially. The polypeptides are present in the kit as separate, unmixed preparations. There is a time interval between doses. For example, one polypeptide might be administered up to 336, 312, 288, 264, 240, 216, 192, 168, 144, 120, 96, 72, 48, 24, 20, 16, 12, 8, 4, 2, 1, or 0.5 hours after the other component.

In sequential administration, one polypeptide may be administered once, or any number of times and in various doses before and/or after administration of another polypeptide. Sequential administration may be combined with simultaneous or sequential administration.

The medical uses of the anti-TNF-alpha polypeptide described below, also apply to the composition comprising an anti-TNF-alpha polypeptide as disclosed herein and at least one polypeptide selected from the group consisting of anti-IFN-gamma polypeptide, anti-TNF-alpha receptor polypeptide and anti-IFN-gamma receptor polypeptide, for simultaneous, separate or sequential administration to a subject as disclosed here above.

According to one aspect of the invention, an anti-IFN-gamma polypeptide anti-TNF-alpha a single domain antibody directed against IFN-gamma. Said single domain antibody may be a *Camelidae* VHH.

It is an embodiment of the invention that a single domain antibody directed against IFN-gamma corresponds to a sequence represented by any of SEQ ID NOs: 44 to 72 as shown in Table 6.

According to one aspect of the invention, anti-TNF-alpha a single domain antibody directed against TNF-alpha receptor. Said single domain antibody may be a *Camelidae* VHH.

According to one aspect of the invention, an anti-IFN-gamma receptor polypeptide anti-TNF-alpha a single domain antibody directed against IFN-gamma receptor. Said single domain antibody may be a *Camelidae* VHH.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein, wherein the number of single domain antibodies directed against TNF-alpha is two or more. Such multivalent anti-TNF-alpha polypeptides have the advantage of unusually high functional affinity for the target, displaying much higher than expected inhibitory properties compared to their monovalent counterparts.

The multivalent anti-TNF-alpha polypeptides have functional affinities that are several orders of magnitude higher than the monovalent parent anti-TNF-alpha polypeptides. The inventors have found that the functional affinities of these multivalent polypeptides are much higher than those reported in the prior art for bivalent and multivalent antibodies. Surprisingly, anti-TNF-alpha polypeptides of the present invention linked to each other directly (SEQ ID No. 77 and 78) or via a short linker sequence show the high functional affinities expected theoretically with multivalent conventional four-chain antibodies.

The inventors have found that such large increased functional activities can be detected preferably with antigens composed of multidomain and multimeric proteins, either in straight binding assays or in functional assays, *e.g.* cytotoxicity assays.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein, wherein the number of single domain antibodies directed against TNF-alpha is two or more, said anti-TNF-alpha polypeptide comprising a sequence corresponding to any represented by SEQ ID NOs: 73 to 76.

The single domain antibodies may be joined to form any of the polypeptides disclosed herein comprising more than one single domain antibody using methods known in the art or any future method. For example, they may be fused by chemical cross-linking by reacting amino acid residues with an organic derivatising agent such as described by Blattler *et al*, Biochemistry 24,1517-1524; EP294703. Alternatively, the single domain

antibody may be fused genetically at the DNA level *i.e.* a polynucleotide construct formed which encodes the complete polypeptide construct comprising one or more anti-target single domain antibodies and one or more anti-serum protein single domain antibodies. A method for producing bivalent or multivalent VHH polypeptide constructs is disclosed in PCT patent application WO 96/34103. One way of joining multiple single domain antibodies is via the genetic route by linking single domain antibody coding sequences either directly or *via* a peptide linker. For example, the C-terminal end of the first single domain antibody may be linked to the N-terminal end of the next single domain antibody. This linking mode can be extended in order to link additional single domain antibodies for the construction and production of tri-, tetra-, etc. functional constructs.

According to one aspect of the present invention, the single domain antibodies are linked to each other directly, without use of a linker. Contrary to joining bulky conventional antibodies where a linker sequence is needed to retain binding activity in the two subunits, polypeptides of the invention can be linked directly (SEQ ID No. 77 and 78) thereby avoiding potential problems of the linker sequence, such as antigenicity when administered to a human subject, instability of the linker sequence leading to dissociation of the subunits.

According to another aspect of the present invention, the single domain antibodies are linked to each other via a peptide linker sequence. Such linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. The linker sequence is expected to be non-immunogenic in the subject to which the anti-TNF-alpha polypeptide is administered. The linker sequence may provide sufficient flexibility to the multivalent anti-TNF-alpha polypeptide, at the same time being resistant to proteolytic degradation. A non-limiting example of a linker sequences is one that can be derived from the hinge region of VHHs described in WO 96/34103.

According to another aspect of the invention, multivalent single domain antibodies comprising more than two single domain antibodies can be linked to each other either directly or via a linker sequence. Such constructs are difficult to produce with conventional antibodies and due to steric hindrance of the bulky subunits, functionality will be lost or greatly diminished rather than increased considerably as seen with VHH's of the invention compared to the monovalent construct (see Figure 12 for gel filtration analyses of such multivalent VHH constructs).

The polypeptide constructs disclosed herein may be made by the skilled artisan according to methods known in the art or any future method. For example, VHHs may be obtained using methods known in the art such as by immunising a camel and obtaining hybridomas therefrom, or by cloning a library of single domain antibodies using molecular biology techniques known in the art and subsequent selection by using phage display.

According to an aspect of the invention an anti-TNF-alpha polypeptide may be a homologous sequence of a full-length anti-TNF-alpha polypeptide. According to another aspect of the invention, an anti-TNF-alpha polypeptide may be a functional portion of a full-length anti-TNF-alpha polypeptide. According to another aspect of the invention, an anti-TNF-alpha polypeptide may be a homologous sequence of a full-length anti-TNF-alpha polypeptide. According to another aspect of the invention, an anti-TNF-alpha polypeptide may be a functional portion of a homologous sequence of a full-length anti-TNF-alpha polypeptide. According to an aspect of the invention an anti-TNF-alpha polypeptide may comprise a sequence of an anti-TNF-alpha polypeptide.

According to an aspect of the invention a single domain antibody used to form an anti-TNF-alpha polypeptide may be a complete single domain antibody (*e.g.* a VHH) or a homologous sequence thereof. According to another aspect of the invention, a single domain antibody used to form the polypeptide construct may be a functional portion of a complete single domain antibody. According to another aspect of the invention, a single domain antibody used to form the polypeptide construct may be a homologous sequence of a complete single domain antibody. According to another aspect of the invention, a single domain antibody used to form the polypeptide construct may be a functional portion of a homologous sequence of a complete single domain antibody.

As used herein, an homologous sequence of the present invention may comprise additions, deletions or substitutions of one or more amino acids, which do not substantially alter the functional characteristics of the polypeptides of the invention. The number of amino acid deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

A homologous sequence according to the present invention may a polypeptide modified by the addition, deletion or substitution of amino acids, said modification not substantially altering the functional characteristics compared with the unmodified polypeptide.

A homologous sequence according to the present invention may be a polypeptide modified by the addition, deletion or substitution of amino acids, said modification not substantially altering the functional characteristics compared with the unmodified polypeptide.

A homologous sequence according to the present invention may be a sequence which exists in other *Camelidae* species such as, for example, camel, dromedary, llama, alpaca, guanaco etc.

Where homologous sequence indicates sequence identity, it means a sequence which presents a high sequence identity (more than 70%, 75%, 80%, 85%, 90%, 95% or 98% sequence identity) with the parent sequence and is preferably characterised by similar properties of the parent sequence, namely affinity, said identity calculated using known methods.

Alternatively, an homologous sequence may also be any amino acid sequence resulting from allowed substitutions at any number of positions of the parent sequence according to the formula below:
Ser substituted by Ser, Thr, Gly, and Asn;
Arg substituted by one of Arg, His, Gln, Lys, and Glu;
Leu substituted by one of Leu, Ile, Phe, Tyr, Met, and Val;
Pro substituted by one of Pro, Gly, Ala, and Thr;
Thr substituted by one of Thr, Pro, Ser, Ala, Gly, His, and Gln;
Ala substituted by one of Ala, Gly, Thr, and Pro;
Val substituted by one of Val, Met, Tyr, Phe, Ile, and Leu;
Gly substituted by one of Gly, Ala, Thr, Pro, and Ser;
Ile substituted by one of Ile, Met, Tyr, Phe, Val, and Leu;
Phe substituted by one of Phe, Trp, Met, Tyr, Ile, Val, and Leu;
Tyr substituted by one of Tyr, Trp, Met, Phe, Ile, Val, and Leu;
His substituted by one of His, Glu, Lys, Gln, Thr, and Arg;
Gln substituted by one of Gln, Glu, Lys, Asn, His, Thr, and Arg;
Asn substituted by one of Asn, Glu, Asp, Gln, and Ser;

Lys substituted by one of Lys, Glu, Gln, His, and Arg;
Asp substituted by one of Asp, Glu, and Asn;
Glu substituted by one of Glu, Asp, Lys, Asn, Gln, His, and Arg;
Met substituted by one of Met, Phe, Ile, Val, Leu, and Tyr.

A homologous nucleotide sequence according to the present invention may refer to nucleotide sequences of more than 50, 100, 200, 300, 400, 500, 600, 800 or 1000 nucleotides able to hybridize to the reverse-complement of the nucleotide sequence capable of encoding the patent sequence, under stringent hybridisation conditions (such as the ones described by Sambrook *et al.*, Molecular Cloning, Laboratory Manuel, Cold Spring, Harbor Laboratory press, New York).

As used herein, a functional portion refers to a sequence of a single domain antibody that is of sufficient size such that the interaction of interest is maintained with affinity of 1×10^{-6} M or better.

Alternatively, a functional portion comprises a partial deletion of the complete amino acid sequence and still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with the target.

As used herein, a functional portion refers to less than 100% of the complete sequence (*e.g.,* 99%, 90%, 80%, 70%, 60% 50%, 40%, 30%, 20%, 10%, 5%, 1% etc.), but comprises 5 or more amino acids or 15 or more nucleotides.

Targets as mentioned herein such as TNF-alpha, TNF-alpha receptor, serum proteins (*e.g.* serum albumin, serum immunoglobulins, thyroxine-binding protein, transferrin, fibrinogen) and IFN-gamma, IFN-gamma receptor may be fragments of said targets. Thus a target is also a fragment of said target, capable of eliciting an immune response. A target is also a fragment of said target, capable of binding to a single domain antibody raised against the full length target.

A fragment as used herein refers to less than 100% of the sequence (e.g., 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% etc.), but comprising 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acids. A fragment is of sufficient length such that the interaction of interest is maintained with affinity of 1 x 10-6 M or better.

A fragment as used herein also refers to optional insertions, deletions and substitutions of one or more amino acids which do not substantially alter the ability of the target to bind to a single domain antibody raised against the wild-type target. The number of amino acid insertions deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

A homologous sequence of the present invention may include an anti-TNF-alpha polypeptide which has been humanised. The humanisation of antibodies of the new class of VHHs would further reduce the possibility of unwanted immunological reaction in a human individual upon administration.

One embodiment of the present invention relates to a method for preparing modified polypeptides based upon llama antibodies by determining the amino acid residues of the antibody variable domain (VHH) which may be modified without diminishing the native affinity of the domain for antigen and while reducing its immunogenicity with respect to a heterologous species; the use of VHHs having modifications at the identified residues which are useful for administration to heterologous species; and to the VHH so modified.

More specifically, the invention relates to the preparation of modified VHHs, which are modified for administration to humans, the resulting VHH themselves, and the use of such "humanized" VHHs in the treatment of diseases in humans. By humanised is meant mutated so that immunogenicity upon administration in human patients is minor or nonexistent. Humanising a polypeptide, according to the present invention, comprises a step of replacing one or more of the *Camelidae* amino acids by their human counterpart as found in the human consensus sequence, without that polypeptide losing its typical character, *i.e.* the humanisation does not significantly affect the antigen binding capacity of the resulting polypeptide. Such methods are known by the skilled addressee.

Humanization of *Camelidae* single domain antibodies requires the introduction and mutagenesis of a limited amount of amino acids in a single polypeptide chain. This is in contrast to humanization of scFv, Fab, (Fab)2 and IgG, which requires the introduction of amino acid changes in two chains, the light and the heavy chain and the preservation of the assembly of both chains.

As a non-limited example, the polypeptide of VHH#12B containing human-like residues in FR2 was humanized. Humanization required mutagenesis of residues in FR1 at position 1 and 5 which were introduced by the primer used for repertoire cloning and do not occur naturally in the llama sequence. Mutagenesis of those residues did not result in loss of binding and/or inhibition activity. Humanization also required mutagenesis of residues in FR3 at position 74, 76, 83, 84, 93. Mutagenesis of those residues did not result in a dramatic loss of binding and/or inhibition activity (see Figure 4). Combining the mutations of FR1 and FR3 therefore did not affect the binding and/or inhibition activity (Figure 5). Humanization also required mutagenesis of residues in FR4 at position 108. Mutagenesis of Q108L resulted in lower production level in *Escherichia coli*. Position 108 is solvent exposed in camelid VHH, while in human antibodies this position is buried at the VH-VL interface (Spinelli, 1996; Nieba, 1997). In isolated VHs position 108 is solvent exposed. The introduction of a non-polar hydrophobic Leu instead of polar uncharged Gln can have a drastic effect on the intrinsic folding/stability of the molecule.

As a non-limited example, the polypeptide represented in the VHH#3E containing camelid hallmark residues at position 37, 44, 45 and 47 with hydrophilic characteristics, was humanized. Replacement of the hydrophilic residues by human hydrophobic residues at positions 44 and 45 (E44G and R45L), did not have an effect on binding and/or inhibition. However, loss of binding and/or inhibition activity was observed when F37V and F47W were introduced. Modeling data confirmed the critical residue 37 to preserve the integrity of the CDR3 loop conformation and hence on activity (see Figure 6)(all numbering according to the Kabat).

SEQ ID NO: 3 and 14 display more than 90% amino acid sequence homology to human VH framework regions and therefore said VHH might be administered to patients directly without expectation of an immune response therefrom, and without the additional burden of humanisation. Therefore, one aspect of the present invention allows for the direct administration of the polypeptide comprising SEQ ID NO: 3 and 14, homologous sequence thereof, or a functional portion of an homologous sequence thereof to a patient in need of the same.

One embodiment of the present invention is a method for humanizing a VHH comprising the steps of replacing of any of the following residues either alone or in combination:

FR1 position 1, 5, 28 and 30,

the hallmark amino acid at position 44 and 45 in FR2,

FR3 residues 74, 75, 76, 83, 84, 93 and 94 ,
and positions 103, 104, 108 and 111 in FR4 ;
numbering according to the Kabat numbering.

One embodiment of the present invention is an anti-TNF-alpha polypeptide, or a nucleic acid capable of encoding said polypeptide for use in treating, preventing and/or alleviating the symptoms of disorders relating to inflammatory processes. TNF-alpha is involved in inflammatory processes, and the blocking of TNF-alpha action can have an anti-inflammatory effect, which is highly desirable in certain disease states such as, for example, Crohn's disease. Our Examples demonstrate VHHs according to the invention which bind TNF-alpha and moreover, block its binding to the TNF-alpha receptor.

The anti-TNF-alpha polypeptides of the present invention are applicable to autoimmune diseases, such as Addison's disease (adrenal), Autoimmune diseases of the ear (ear), Autoimmune diseases of the eye (eye), Autoimmune hepatitis (liver), Autoimmune parotitis (parotid glands), Crohn's disease (intestine), Diabetes Type I (pancreas), Epididymitis (epididymis), Glomerulonephritis (kidneys), Graves' disease (thyroid), Guillain-Barre syndrome (nerve cells), Hashimoto's disease (thyroid), Hemolytic anemia (red blood cells), Systemic lupus erythematosus (multiple tissues), Male infertility (sperm), Multiple sclerosis (nerve cells), Myasthenia Gravis (neuromuscular junction), Pemphigus (primarily skin), Psoriasis (skin), Rheumatic fever (heart and joints), Rheumatoid arthritis (joint lining), Sarcoidosis (multiple tissues and organs), Scleroderma (skin and connective tissues), Sjogren's syndrome (exocrine glands, and other tissues), Spondyloarthropathies (axial skeleton, and other tissues), Thyroiditis (thyroid), Vasculitis (blood vessels). Within parenthesis is the tissue affected by the disease. This listing of autoimmune diseases is intended to be exemplary rather than inclusive.

Autoimmune conditions for which the anti-TNF-alpha polypeptides of the present invention is applicable include, for example, AIDS, atopic allergy, bronchial asthma, eczema, leprosy, schizophrenia, inherited depression, transplantation of tissues and organs, chronic fatigue syndrome, Alzheimer's disease, Parkinson's disease, myocardial infarction, stroke, autism, epilepsy, Arthus's phenomenon, anaphylaxis, and alcohol and drug addiction. In the above-identified autoimmune conditions, the tissue affected is the primary target, in other cases it is the secondary target. These conditions are partly or mostly autoimmune syndromes. Therefore, in treating them, it is possible to use the same

methods, or aspects of the same methods that are herein disclosed, sometimes in combination with other methods.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide according to the invention, or a nucleic acid capable of encoding said polypeptide for the preparation of a medicament for treating a disorder relating to inflammatory processes. Examples of disorders include rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis

Polypeptides and nucleic acids according to the present invention may be administered to a subject by conventional routes, such as intravenously. However, a special property of the anti-TNF-alpha polypeptides of the invention is that they penetrate barriers such as tissue membranes and/or tumours and act locally and act locally thereon, and they are sufficiently stable to withstand extreme environments such as in the stomach. Therefore, another aspect of the present invention relates to the delivery of anti-TNF-alpha polypeptides.

A subject according to the invention can be any mammal susceptible to treatment by therapeutic polypeptides.

Oral delivery of anti-TNF-alpha polypeptides of the invention results in the provision of such molecules in an active form in the colon at local sites that are affected by the disorder. These sites may be highly inflamed and contain TNF-alpha-producing cells. The anti-TNF-alpha polypeptides of the invention which bind to TNF-alpha can neutralise the TNF-alpha locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody or functional portions thereof. Such modified microorganisms can be used as vehicles for local production and delivery of antibodies or functional portions thereof in the intestine. By using a strain which produces an anti-TNF-alpha polypeptide, inflammatory bowel syndrome could be treated.

Another aspect of the invention involves delivering anti-TNF polypeptides by using surface expression on or secretion from non-invasive bacteria, such as Gram-positive host organisms like *Lactococcus spec.* using a vector such as described in WO00/23471.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the gastric environment without the substance being inactivated.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowl syndrome, and multiple sclerosis. As known by persons skilled in the art, once in possession of said polypeptide construct, formulation technology may be applied to release a maximum amount of polypeptide in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating, prevent and/or alleviate the symptoms of disorders whose targets are located in the gut system.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder susceptible to modulation by a TNF-alpha modulating substance which is able pass through the gastric environment without being inactivated, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the gastric environment without being inactivated.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the gut system without said substance being inactivated, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the bloodstream of a subject without the substance being inactivated, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms or disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the vaginal and/or rectal tract.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowl syndrome, and multiple sclerosis. In a non-limiting example, a formulation according to the invention comprises an anti-TNF-alpha polypeptide as disclosed herein, in the form of a gel, cream, suppository, film, or in the form of a sponge or as a vaginal ring that slowly releases the active ingredient over time (such formulations are described in EP 707473, EP 684814, US 5629001).

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the vaginal and/or rectal tract, by vaginally and/or rectally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the vaginal and/or rectal tract.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the vaginal and/or rectal tract without being said substance being inactivated, by administering to the vaginal and/or rectal tract of a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the bloodstream of a subject without said substance being inactivated, by administering to the vaginal and/or rectal tract of a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein, for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the nose, upper respiratory tract and/or lung.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowl syndrome, and multiple sclerosis. In a non-limiting example, a formulation according to the invention,

comprises an anti-TNF-alpha polypeptide as disclosed herein in the form of a nasal spray (e.g. an aerosol) or inhaler. Since the polypeptide construct is small, it can reach its target much more effectively than therapeutic IgG molecules.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the upper respiratory tract and lung, by administering to a subject an anti-TNF-alpha polypeptide as disclosed herein, by inhalation through the mouth or nose.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the nose, upper respiratory tract and/or lung, without said polypeptide being inactivated.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the nose, upper respiratory tract and lung without inactivation, by administering to the nose, upper respiratory tract and/or lung of a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the bloodstream of a subject without inactivation by administering to the nose, upper respiratory tract and/or lung of a subject an anti-TNF-alpha polypeptide as disclosed herein.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa. Because of their small size, an anti-TNF-alpha polypeptide as disclosed herein can pass through the intestinal mucosa and reach the bloodstream more efficiently in subjects suffering from disorders which cause an increase in the permeability of the intestinal mucosa, for example Crohn's disease.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance

delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa, by orally administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

This process can be even further enhanced by an additional aspect of the present invention - the use of active transport carriers. In this aspect of the invention, VHH is fused to a carrier that enhances the transfer through the intestinal wall into the bloodstream. In a non-limiting example, this "carrier" is a second VHH which is fused to the therapeutic VHH. Such fusion constructs are made using methods known in the art. The "carrier" VHH binds specifically to a receptor on the intestinal wall which induces an active transfer through the wall.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the intestinal mucosa without being inactivated, by administering orally to a subject an anti-TNF-alpha polypeptide of the invention.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the bloodstream of a subject without being inactivated, by administering orally to a subject an anti-TNF-alpha polypeptide of the invention.

This process can be even further enhanced by an additional aspect of the present invention - the use of active transport carriers. In this aspect of the invention, an anti-TNF-alpha polypeptide as described herein is fused to a carrier that enhances the transfer through the intestinal wall into the bloodstream. In a non-limiting example, this "carrier" is a VHH which is fused to said polypeptide. Such fusion constructs made using methods known in the art. The "carrier" VHH binds specifically to a receptor on the intestinal wall which induces an active transfer through the wall.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the tissues beneath the tongue effectively.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowl syndrome, and multiple sclerosis. A formulation of said polypeptide construct as disclosed herein, for example, a tablet, spray, drop is placed under the tongue and adsorbed through the mucus membranes into the capillary network under the tongue.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the tissues beneath the tongue effectively, by sublingually administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able to pass through the tissues beneath the tongue.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the tissues beneath the tongue without being inactivated, by administering sublingually to a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the bloodstream of a subject without being inactivated, by administering orally to a subject an anti-TNF-alpha polypeptide as disclosed herein.

One embodiment of the present invention is an anti-TNF-alpha polypeptide as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the skin effectively.

Examples of disorders are any that cause inflammation, including, but not limited to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowl syndrome, and

multiple sclerosis. A formulation of said polypeptide construct, for example, a cream, film, spray, drop, patch, is placed on the skin and passes through.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the skin effectively, by topically administering to a subject an anti-TNF-alpha polypeptide as disclosed herein.

Another embodiment of the present invention is a use of an anti-TNF-alpha polypeptide as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the skin effectively.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the skin without being inactivated, by administering topically to a subject an anti-TNF-alpha polypeptide as disclosed herein.

An aspect of the invention is a method for delivering a TNF-alpha modulating substance to the bloodstream of a subject, by administering topically to a subject an anti-TNF-alpha polypeptide as disclosed herein.

In another embodiment of the present invention, an anti-TNF-alpha polypeptide further comprises a carrier single domain antibody (e.g. VHH) which acts as an active transport carrier for transport said anti-TNF-alpha polypeptide, from the lung lumen to the blood.

An anti-TNF-alpha polypeptide further comprising a carrier binds specifically to a receptor present on the mucosal surface (bronchial epithelial cells) resulting in the active transport of the polypeptide from the lung lumen to the blood. The carrier single domain antibody may be fused to the polypeptide construct. Such fusion constructs may be made using methods known in the art and are describe herein. The "carrier" single domain antibody binds specifically to a receptor on the mucosal surface which induces an active transfer through the surface.

Another aspect of the present invention is a method to determine which single domain antibodies (e.g. VHHs) are actively transported into the bloodstream upon nasal administration. Similarly, a naïve or immune VHH phage library can be administered

nasally, and after different time points after administration, blood or organs can be isolated to rescue phages that have been actively transported to the bloodstream. A non-limiting example of a receptor for active transport from the lung lumen to the bloodstream is the Fc receptor N (FcRn). One aspect of the invention includes the VHH molecules identified by the method. Such VHH can then be used as a carrier VHH for the delivery of a therapeutic VHH to the corresponding target in the bloodstream upon nasal administration.

In one aspect of the invention, one can use an anti-TNF-alpha polypeptide as disclosed herein, in order to screen for agents that modulate the binding of the polypeptide to TNF-alpha. When identified in an assay that measures binding or said polypeptide displacement alone, agents will have to be subjected to functional testing to determine whether they would modulate the action of the antigen *in vivo*. Examples of screening assays are given below primarily in respect of SEQ ID NO: 3, though any anti-TNF-alpha polypeptide as disclosed herein as disclosed herein may be appropriate.

In an example of a displacement experiment, phage or cells expressing TNF-alpha or a fragment thereof are incubated in binding buffer with, for example, a polypeptide represented by SEQ ID NO: 3 which has been labeled, in the presence or absence of increasing concentrations of a candidate modulator. To validate and calibrate the assay, control competition reactions using increasing concentrations of said polypeptide and which is unlabeled, can be performed. After incubation, cells are washed extensively, and bound, labeled polypeptide is measured as appropriate for the given label (*e.g.*, scintillation counting, fluorescence, etc.). A decrease of at least 10% in the amount of labeled polypeptide bound in the presence of candidate modulator indicates displacement of binding by the candidate modulator. Candidate modulators are considered to bind specifically in this or other assays described herein if they displace 50% of labeled polypeptide (sub-saturating polypeptide dose) at a concentration of 1 μM or less.

Alternatively, binding or displacement of binding can be monitored by surface plasmon resonance (SPR). Surface plasmon resonance assays can be used as a quantitative method to measure binding between two molecules by the change in mass near an immobilized sensor caused by the binding or loss of binding of, for example, the polypeptide represented by SEQ ID NO: 3 from the aqueous phase to TNF-alpha immobilized in a membrane on the sensor. This change in mass is measured as resonance units versus time after injection or removal of the said polypeptide or candidate

modulator and is measured using a Biacore Biosensor (Biacore AB). TNF-alpha can be for example immobilized on a sensor chip (for example, research grade CM5 chip; Biacore AB) in a thin film lipid membrane according to methods described by Salamon et al. (Salamon *et al.*, 1996, Biophys J. 71: 283-294; Salamon *et al.*, 2001, Biophys. J. 80: 1557-1567; Salamon *et al.*, 1999, Trends Biochem. Sci. 24: 213-219, each of which is incorporated herein by reference.). Sarrio *et al.* demonstrated that SPR can be used to detect ligand binding to the GPCR A(1) adenosine receptor immobilized in a lipid layer on the chip (Sarrio *et al.*, 2000, Mol. Cell. Biol. 20: 5164-5174, incorporated herein by reference). Conditions for the binding of SEQ ID NO:3 to TNF-alpha in an SPR assay can be fine-tuned by one of skill in the art using the conditions reported by Sarrio *et al.* as a starting point.

SPR can assay for modulators of binding in at least two ways. First, a polypeptide represented by SEQ ID NO: 3, for example, can be pre-bound to immobilized TNF-alpha followed by injection of candidate modulator at a concentration ranging from 0.1 nM to 1 µM. Displacement of the bound polypeptide can be quantitated, permitting detection of modulator binding. Alternatively, the membrane-bound TNF-alpha can be pre-incubated with a candidate modulator and challenged with, for example, a polypeptide represented by SEQ ID NO: 3. A difference in binding affinity between said polypeptide and TNF-alpha pre-incubated with the modulator, compared with that between said polypeptide and TNF-alpha in absence of the modulator will demonstrate binding or displacement of said polypeptide in the presence of modulator. In either assay, a decrease of 10% or more in the amount of said polypeptide bound in the presence of candidate modulator, relative to the amount of said polypeptide bound in the absence of candidate modulator indicates that the candidate modulator inhibits the interaction of TNF-alpha and said polypeptide.

Another method of detecting inhibition of binding of, for example, a polypeptide represented by SEQ ID NO: 3, to TNF-alpha uses fluorescence resonance energy transfer (FRET). FRET is a quantum mechanical phenomenon that occurs between a fluorescence donor (D) and a fluorescence acceptor (A) in close proximity to each other (usually < 100 Å of separation) if the emission spectrum of D overlaps with the excitation spectrum of A. The molecules to be tested, *e.g.* a polypeptide represented by SEQ ID NO: 3 and a TNF-alpha are labelled with a complementary pair of donor and acceptor fluorophores. While bound closely together by the TNF-alpha: polypeptide interaction, the fluorescence emitted upon excitation of the donor fluorophore will have a different wavelength from that emitted in response to that excitation wavelength when the said

polypeptide and TNF-alpha are not bound, providing for quantitation of bound versus unbound molecules by measurement of emission intensity at each wavelength. Donor fluorophores with which to label the TNF-alpha are well known in the art. Of particular interest are variants of the A. Victoria GFP known as Cyan FP (CFP, Donor (D)) and Yellow FP (YFP, Acceptor (A)). As an example, the YFP variant can be made as a fusion protein with TNF-alpha. Vectors for the expression of GFP variants as fusions (Clontech) as well as fluorophore-labeled reagents (Molecular Probes) are known in the art. The addition of a candidate modulator to the mixture of fluorescently-labelled polypeptide and YFP-TNF-alpha will result in an inhibition of energy transfer evidenced by, for example, a decrease in YFP fluorescence relative to a sample without the candidate modulator. In an assay using FRET for the detection of TNF-alpha: polypeptide interaction, a 10% or greater decrease in the intensity of fluorescent emission at the acceptor wavelength in samples containing a candidate modulator, relative to samples without the candidate modulator, indicates that the candidate modulator inhibits the TNF-alpha:polypeptide interaction.

A sample as used herein may be any biological sample containing TNF-alpha such as clinical (*e.g.* cell fractions, whole blood, plasma, serum, tissue, cells, etc.), derived from clinical, agricultural, forensic, research, or other possible samples. The clinical samples may be from human or animal origin. The sample analysed can be both solid or liquid in nature. It is evident when solid materials are used, these are first dissolved in a suitable solution.

A variation on FRET uses fluorescence quenching to monitor molecular interactions. One molecule in the interacting pair can be labelled with a fluorophore, and the other with a molecule that quenches the fluorescence of the fluorophore when brought into close apposition with it. A change in fluorescence upon excitation is indicative of a change in the association of the molecules tagged with the fluorophore:quencher pair. Generally, an increase in fluorescence of the labelled TNF-alpha is indicative that anti-TNF-alpha polypeptide bearing the quencher has been displaced. For quenching assays, a 10% or greater increase in the intensity of fluorescent emission in samples containing a candidate modulator, relative to samples without the candidate modulator, indicates that the candidate modulator inhibits TNF-alpha: anti-TNF-alpha polypeptide interaction.

In addition to the surface plasmon resonance and FRET methods, fluorescence polarization measurement is useful to quantitate binding. The fluorescence polarization

value for a fluorescently-tagged molecule depends on the rotational correlation time or tumbling rate. Complexes, such as those formed by TNF-alpha associating with a fluorescently labelled anti-TNF-alpha polypeptide, have higher polarization values than uncomplexed, labelled polypeptide. The inclusion of a candidate inhibitor of the TNF-alpha:anti-TNF-alpha polypeptide interaction results in a decrease in fluorescence polarization, relative to a mixture without the candidate inhibitor, if the candidate inhibitor disrupts or inhibits the interaction of TNF-alpha with said polypeptide. Fluorescence polarization is well suited for the identification of small molecules that disrupt the formation of TNF-alpha:anti-TNF-alpha polypeptide complexes. A decrease of 10% or more in fluorescence polarization in samples containing a candidate modulator, relative to fluorescence polarization in a sample lacking the candidate modulator, indicates that the candidate modulator inhibits the TNF-alpha : anti-TNF-alpha polypeptide interaction.

Another alternative for monitoring TNF-alpha : anti-TNF-alpha polypeptide interactions uses a biosensor assay. ICS biosensors have been described in the art (Australian Membrane Biotechnology Research Institute; Cornell B, Braach-Maksvytis V, King L, Osman P, Raguse B, Wieczorek L, and Pace R. "A biosensor that uses ion-channel switches" Nature 1997, 387, 580). In this technology, the association of TNF-alpha and a anti-TNF-alpha polypeptide is coupled to the closing of gramacidin-facilitated ion channels in suspended membrane bilayers and thus to a measurable change in the admittance (similar to impedence) of the biosensor. This approach is linear over six orders of magnitude of admittance change and is ideally suited for large scale, high throughput screening of small molecule combinatorial libraries. A 10% or greater change (increase or decrease) in admittance in a sample containing a candidate modulator, relative to the admittance of a sample lacking the candidate modulator, indicates that the candidate modulator inhibits the interaction of TNF-alpha and said polypeptide. It is important to note that in assays testing the interaction of TNF-alpha with an anti-TNF-alpha polypeptide, it is possible that a modulator of the interaction need not necessarily interact directly with the domain(s) of the proteins that physically interact with said polypeptide. It is also possible that a modulator will interact at a location removed from the site of interaction and cause, for example, a conformational change in the TNF-alpha. Modulators (inhibitors or agonists) that act in this manner are nonetheless of interest as agents to modulate the binding of TNF-alpha to its receptor.

Any of the binding assays described can be used to determine the presence of an agent in a sample, e.g., a tissue sample, that binds to TNF-alpha , or that affects the binding of,

for example, a polypeptide represented by SEQ ID NO: 3 to the TNF-alpha. To do so a TNF-alpha is reacted with said polypeptide in the presence or absence of the sample, and polypeptide binding is measured as appropriate for the binding assay being used. A decrease of 10% or more in the binding of said polypeptide indicates that the sample contains an agent that modulates the binding of said polypeptide to the TNF-alpha. Of course, the above-generalized method might easily be applied to screening for candidate modulators which alter the binding between any anti-TNF-alpha polypeptide of the invention, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof, and TNF-alpha or a fragment thereof.

One embodiment of the present invention is an unknown agent identified by the method disclosed herein.

One embodiment of the present invention is an unknown agent identified by the method disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders relating to inflammatory processes.

Another embodiment of the present invention is a use of an unknown agent identified by the method disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders relating to inflammatory processes.

Examples of disorders include rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis

A cell that is useful according to the invention is preferably selected from the group consisting of bacterial cells such as, for example, *E. coli*, yeast cells such as, for example, *S. cerevisiae*, *P. pastoris*, insect cells or mammal cells.

A cell that is useful according to the invention can be any cell into which a nucleic acid sequence encoding a polypeptide comprising an anti-TNF-alpha of the invention, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof according to the invention can be introduced such that the polypeptide is expressed at natural levels or above natural levels, as defined herein. Preferably a polypeptide of the invention that is expressed in a cell exhibits normal or near normal pharmacology, as defined herein. Most preferably a polypeptide of the invention that is expressed in a cell comprises the nucleotide sequence capable of encoding any

one of the amino acid sequences presented in Table 1 or capable of encoding an amino acid sequence that is at least 70% identical to the amino acid sequence presented in Table 1.

According to a preferred embodiment of the present invention, a cell is selected from the group consisting of COS7-cells, a CHO cell, a LM (TK-) cell, a NIH-3T3 cell, HEK-293 cell, K-562 cell or a 1321N1 astrocytoma cell but also other transfectable cell lines.

In general, "therapeutically effective amount", "therapeutically effective dose" and "effective amount" means the amount needed to achieve the desired result or results (modulating TNF-alpha binding; treating or preventing inflammation). One of ordinary skill in the art will recognize that the potency and, therefore, an "effective amount" can vary for the various compounds that modulate TNF-alpha binding used in the invention. One skilled in the art can readily assess the potency of the compound.

As used herein, the term "compound" refers to an anti-TNF-alpha polypeptide of the present invention, a composition, or a nucleic acid capable of encoding said polypeptide or an agent identified according to the screening method described herein or said polypeptide comprising one or more derivatised amino acids.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual along with the compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Anti-TNF-alpha polypeptides as disclosed herein is useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound or composition.

Anti-TNF polypeptides of the present invention are useful for treating or preventing conditions relating to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis in a subject and comprises administering a pharmaceutically effective amount of a compound or composition that binds TNF-alpha.

Anti-TNF-alpha polypeptides as disclosed here in are useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, aspirin.

The anti-TNF-alpha polypeptides as disclosed here in are useful for treating or preventing conditions relating to rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, aspirin.

The present invention is not limited to the administration of formulations comprising a single compound of the invention. It is within the scope of the invention to provide combination treatments wherein a formulation is administered to a patient in need thereof that comprises more than one compound of the invention.

Conditions mediated by TNF-alpha include, but are not limited rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis.

A compound useful in the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient or a domestic animal in a variety of forms adapted to the chosen route of administration, *i.e.*, orally or parenterally, by intranassally by inhalation, intravenous, intramuscular, topical or subcutaneous routes.

A compound of the present invention can also be administered using gene therapy methods of delivery. See, *e.g.*, U.S. Patent No. 5,399,346, which is incorporated by reference in its entirety. Using a gene therapy method of delivery, primary cells transfected with the gene for the compound of the present invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells.

Thus, the present compound may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined

with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage.

The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compound may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, hydroxyalkyls or glycols or water-alcohol/glycol blends, in which the present compound can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the compound to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compound can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the compound(s) in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician. Also the dosage of the compound varies depending on the target cell, tumor, tissue, graft, or organ.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen could include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill

in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

The invention provides for an agent that is a modulator of TNF-alpha / TNF-alpha-receptor interactions.

The candidate agent may be a synthetic agent, or a mixture of agents, or may be a natural product (*e.g.* a plant extract or culture supernatant). A candidate agent according to the invention includes a small molecule that can be synthesized, a natural extract, peptides, proteins, carbohydrates, lipids etc.

Candidate modulator agents from large libraries of synthetic or natural agents can be screened. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based agents. Synthetic agent libraries are commercially available from a number of companies including Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Combinatorial libraries are available and can be prepared. Alternatively, libraries of natural agents in the form of bacterial, fungal, plant and animal extracts are available from e.g., Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible by methods well known in the art. Additionally, natural and synthetically produced libraries and agents are readily modified through conventional chemical, physical, and biochemical means.

Useful agents may be found within numerous chemical classes. Useful agents may be organic agents, or small organic agents. Small organic agents have a molecular weight of more than 50 yet less than about 2,500 daltons, preferably less than about 750, more preferably less than about 350 daltons. Exemplary classes include heterocycles, peptides, saccharides, steroids, and the like. The agents may be modified to enhance efficacy, stability, pharmaceutical compatibility, and the like. Structural identification of an agent may be used to identify, generate, or screen additional agents. For example, where peptide agents are identified, they may be modified in a variety of ways to enhance their stability, such as using an unnatural amino acid, such as a D-amino acid, particularly D-alanine, by functionalizing the amino or carboxylic terminus, e.g. for the amino group,

acylation or alkylation, and for the carboxyl group, esterification or amidification, or the like.

For primary screening, a useful concentration of a candidate agent according to the invention is from about 10 mM to about 100 µM or more (*i.e.* 1 mM, 10 mM, 100 mM, 1 M etc.). The primary screening concentration will be used as an upper limit, along with nine additional concentrations, wherein the additional concentrations are determined by reducing the primary screening concentration at half-log intervals (*e.g.* for 9 more concentrations) for secondary screens or for generating concentration curves.

High throughput screening kit

A high throughput screening kit according to the invention comprises all the necessary means and media for performing the detection of an agent that modulates TNF-alpha/TNF-alpha receptor interactions by interacting with TNF-alpha in the presence of a polypeptide, preferably at a concentration in the range of 1µM to 1 mM.

The kit comprises the following. Recombinant cells of the invention, comprising and expressing the nucleotide sequence encoding TNF-alpha , which are grown according to the kit on a solid support, such as a microtiter plate, more preferably a 96 well microtiter plate, according to methods well known to the person skilled in the art especially as described in WO 00/02045. Alternatively TNF-alpha is supplied in a purified form to be immobilized on, for example, a 96 well microtiter plate by the person skilled in the art. Alternatively TNF-alpha is supplied in the kit pre-immobilized on, for example, a 96 well microtiter plate. The TNF-alpha may be whole TNF-alpha or a fragment thereof.

Modulator agents according to the invention, at concentrations from about 1 µM to 1 mM or more, are added to defined wells in the presence of an appropriate concentration of anti-TNF-alpha polypeptide, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof, said concentration of said polypeptide preferably in the range of 1 µM to 1 mM. Kits may contain one or more anti-TNF-alpha polypeptide (*e.g.* one or more of a polypeptide represented by any of the SEQ ID NOs: 1 to 15 or other anti-TNF-alpha polypeptides, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof).

Binding assays are performed as according to the methods already disclosed herein and the results are compared to the baseline level of, for example TNF-alpha binding to an

anti-TNF-alpha polypeptide, an homologous sequence thereof, a functional portion thereof or a functional portion of an homologous sequence thereof , but in the absence of added modulator agent. Wells showing at least 2 fold, preferably 5 fold, more preferably 10 fold and most preferably a 100 fold or more increase or decrease in TNF-alpha-polypeptide binding (for example) as compared to the level of activity in the absence of modulator, are selected for further analysis.

Other Kits Useful According to the Invention

The invention provides for kits useful for screening for modulators of TNF-alpha/TNF-alpha receptor binding, as well as kits useful for diagnosis of disorders characterised by dysfunction of TNF-alpha. The invention also provides for kits useful for screening for modulators of disorders as well as kits for their diagnosis, said disorders characterised by one or more process involving TNF-alpha. Kits useful according to the invention can include an isolated TNF-alpha . Alternatively, or in addition, a kit can comprise cells transformed to express TNF-alpha. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding TNF-alpha. In a still further embodiment, a kit according to the invention may comprise the specific primers useful for amplification of TNF-alpha. Kits useful according to the invention can comprise an isolated TNF-alpha polypeptide, a homologue thereof, or a functional portion thereof. A kit according to the invention can comprise cells transformed to express said polypeptide. Kits may contain more than one polypeptide. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding TNF-alpha . In a still further embodiment, a kit according to the invention may comprise the specific primers useful for amplification of a macromolecule such as, for example, TNF-alpha. All kits according to the invention will comprise the stated items or combinations of items and packaging materials therefore. Kits will also include instructions for use.

EXAMPLES

The invention is illustrated by the following non-limiting example.

Example 1: Example of *Camelidae* antibodies against human tumor necrosis factor alpha

1) Immunization and library constructions

A llama (*Llama glama*) was immunized with human TNF-alpha. For immunization, the cytokine was formulated as an emulsion with an appropriate, animal-friendly adjuvant (Specoll, CEDI Diagnostics B.V.). The antigen cocktail was administered by double-spot injections intramuscularly in the neck. The animal received 6 injections of the emulsion, containing 100 μg of TNF-alpha at weekly intervals. At different time points during immunization, 10-ml blood samples were collected from the animal and sera were prepared. The induction of an antigen specific humoral immune response was verified using the serum samples in an ELISA experiment with TNF (data not shown). Five days after the last immunization, a blood sample of 150 ml was collected. From this sample, conventional and heavy-chain antibodies (HcAbs) were fractionated (Lauwereys et al. 1998) and used in an ELISA, which revealed that the HcAbs were responsible for the antigen specific humoral immune response (data not shown). Peripheral blood lymphocytes (PBLs), as the genetic source of the llama heavy chain immunoglobulins (HcAbs), were isolated from the 150-ml blood sample using a Ficoll-Paque gradient (Amersham Biosciences) yielding $5x10^8$ PBLs. The maximal diversity of antibodies is expected to be equal to the number of sampled B-lymphocytes, which is about 10 % of the number of PBLs ($5x10^7$). The fraction of heavy-chain antibodies in llama is up to 20 % of the number of B-lymphocytes. Therefore, the maximal diversity of HcAbs in the 150 ml blood sample is calculated as 10^7 different molecules. Total RNA (around 400 µg) was isolated from these cells using an acid guanidinium thiocyanate extraction (Chomczynski and Sacchi, 1987).

cDNA was prepared on 100 µg total RNA with M-MLV Reverse Transcriptase (Gibco BRL) and oligo-dT-primer or hexanucleotide random primers (Amersham Biosciences) as described before (de Haard *et al.*, 1999). The cDNA was purified with a phenol/chloroform extraction combined with an ethanol precipitation and subsequently used as template to specifically amplify the VHH repertoire.

The VHH repertoire was amplified using oligo-dT primed cDNA as template with a single degenerated framework1 (FR1) primer ABL013 (5'-GAGGTBCAR**CTGCAG**GASTCYGG-3'), introducing a *Pst*I restriction site (in bold), in combination with the oligo-dT primer as is described in EP01205100.9. This amplification yields two fragments of 1650 bp and 1300 bp, the latter being the product derived from the CH1-deleted HcAb genes. The smaller PCR-product was gel purified and subsequently digested with *Pst*I and *Bst*EII. The *Bst*EII-site frequently occurs within the FR4 of heavy-chain derived VHH encoding DNA-fragments.

Alternatively, the VHH-repertoire was amplified in a hinge-dependent approach using two IgG specific oligonucleotide primers. In a single PCR reaction a short (5'-AACAGTTAAGCTTCCGCTT**GCGGCCGC**GGAGCTGGGGTCTTCGCTGTGGTGCG-3') or long (5'-AACAGTTAAGCTTCCGCTT**GCGGCCGC**TGGTTGTGGTTTTGGTGTCTTGGGTT-3') hinge primer known to be specific for HcAbs was combined with the FR1-primer ABL013 (see above). A *Pst*I and *Not*I (bold underlined) restriction site was introduced within the FR1 and hinge primers respectively, to allow cloning. Subsequently, the DNA fragments were ligated into *Pst*I-*Bst*EII or *Pst*I-*Not*I digested phagemid vector pAX004, which is identical to pHEN1 (Hoogenboom *et al.*, 1991), but encodes a carboxyterminal $(His)_6$- and c-myc-tag for purification and detection, respectively. The ligation mixture was desalted on a Microcon filter (YM-50, Millipore) and electroporated into *E. coli* TG1 cells to obtain a library containing $1.8x10^7$ clones. The transformed cells were grown overnight at 37°C on a single 20x20 cm plate with LB containing 100 µg/ml ampicillin and 2 % glucose. The colonies were scraped from plates using 2xTY medium and stored at -80°C in 20 % glycerol.

As quality control the percentage of insert containing clones was verified on 24 clones for each library by PCR using a combination of vector based primers. This analysis revealed that 95 % of the clones contained a VHH encoding insert. The variability was examined by *Hin*fI fingerprint analysis of the amplified VHH fragment of these 24 clones, thereby showing that all clones were indeed different (data not shown).

2) Selection of antagonistic anti-TNF VHH's

From both libraries phage was prepared. To rescue the polyclonal phage repertoire, libraries were grown to logarithmic phase (OD600 = 0.5) at 37°C in 2xTY containing 100 µg/ml ampicillin and 2 % glucose and subsequently superinfected with M13K07 helper phage for 30 minutes at 37°C. Infected cells were pelleted for 5 minutes at 4000 rpm and

resuspended in 2xTY containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Bacteriophage was propagated by overnight growth at 37°C and 250 rpm. Overnight cultures were centrifuged for 15 minutes at 4500 rpm and phage was precipitated with one fifth volume of a [20% polyethyleneglycol 6000, 1.5 M NaCl]-solution by incubation for 30 minutes on ice. Phage was pelleted by centrifugation for 15 minutes at 4000xg and 4°C. After resuspension of the phages in PBS, cell debris was pelleted by centrifugation for 1 minute at maximal speed (15000xg) in microcentrifuge tubes. The supernatant containing the phage particles was transferred to a new tube and again phage was precipitated as described above. Phage was dissolved in PBS and separated from remaining cell debris as mentioned above. The titer of phage was determined by infection of logarithmic TG1 cells followed by plating on selective medium.

The library was selected using *in vitro* biotinylated TNF-alpha. The biotinylation was carried out as described by Magni et al (Anal Biochem 2001, 298, 181-188). The incorporation of biotin in TNF was evaluated by SDS-PAGE analysis and detection with Extravidin-alkaline phosphatase conjugate (Sigma). The functionality of the modified protein was evaluated for its ability to bind to the solid phase coated recombinant a p75 receptor.

VHH were selected by capturing biotinylated TNF-alpha (10 to 400 ng per well during 2 hours at room temperature) on streptavidin coated microtiter plates (coated with 100 μl of 10 μg/ml streptavidin during 16 hours at +4°C). Antagonistic VHH were obtained by elution with an excess of receptor, either the extracellular ligand binding domain or with cells expressing the receptor. After 2 hours incubation of phage with captured cytokine, the non-specific phage was washed away, while specific phage displaying antagonistic VHH was eluted for 30 minutes with receptor (extracellular domain of CD120b or p75; 10 μM) or with receptor displaying cells (>10^5 KYM cells per well). High enrichments, i.e. the ratio of the number of phage eluted with receptor and those eluted by serum albumin (50 μg per well), of more than a factor of 20 suggested the successful selection of TNF-alpha specific clones. Alternatively, instead of elution with receptor a standard procedure was applied, in which a low pH causes the denaturation of VHH and / or antigen (0.1 M glycine buffer pH 2.5). Log phase growing *E. coli* cells were infected with the eluted and neutralized phage and plated on selective medium.
Individual clones were picked and grown in microtiter plate for the production of VHH in culture supernatant. ELISA screening with TNF-alpha captured on Extravidin coated plates revealed about 50% positive clones. *Hin*fI-fingerprint analysis showed that 13

different clones were selected, which were grown and induced on 50 ml scale. The sequences of said clones are shown in Table 1.

Five clones, coded VHH#1A, #2B, #3E, #3G, #7B and #12B, with different sequences (Figure 1) were characterized in more detail. VHH#3E, #3G and #7B are single-domain antibody fragments carrying the typical hydrophilic residue at position 45 (arginine) and the phenylalanine to tryptophan substitution in position 47 in FR2 thereby conferring the advantageous characteristics in terms of solubility. VHH#1A contains the hydrophobic FR2 residues typically found in double-chain antibodies of human origin or from other species, but compensating this loss in hydrophilicity by the charged arginine residue on position 103 that substitutes the conserved tryptophan residue present in VH from double-chain antibodies (PCT/EP02/07804). A new class of humanised *Camelidae* single-domain antibodies described in this invention is represented by VHH#2B and VHH#12B, which contains the hydrophobic residues in FR2 in combination with the hydrophobic residue tryptophan at position 103. Larger amounts of antibody fragments were expressed by cultivation on 50 ml scale and purified by IMAC using TALON resin (Clontech). After dialysis against PBS to remove the eluent imidazol the amount of VHH was determined by OD280; approximately 300 µg of VHH was obtained from each clone.

This material was used for determining the sensitivity of detection of (biotinylated) TNF in ELISA. For this purpose a streptavidin (10 µg/ml) coated microtiterplate was employed for capture of biotinylated TNF (1 µg/ml), VHH was diluted in 0.2 % casein / PBS and incubated for 2 hours at room temperature. Bound VHH was detected with anti-MYC mAB 9E10 (0.5 µg/ml) and anti-mouse AP conjugate (1000-fold diluted, Sigma). The results are shown in Figure 2.

3) Determination of antagonistic effect in cytotoxicity assay with KYM cell line

TNF-alpha-induced cytostasis/cytotoxicity was determined by the colorimetric MTT assay as described by Vandenabeele and colleagues (Vandenabeele, P., Declercq, W., Vercammen, D., Van de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., Fiers, W. (1992) Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T cell hybridoma. J. Exp. Med. 176, 1015-1024.). MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. KYM cells (Sekiguchi M, Shiroko Y, Suzuki T, Imada M, Miyahara M, Fujii G. (1985) Characterization of a human rhabdomyosarcoma cell strain in tissue

culture. Biomed. Pharmacother. 39, 372-380.) were seeded in 96 well microtiterplates and cultured in the presence or absence of TNF-alpha (0.216 ng/ml or approx. 5 pM of trimer). In addition to TNF variable amounts of antibody (VHH or Remicade) were included during cultivation. For the assay MTT was added to the culture medium at a final concentration of 500 µg/ml and the plates were incubated at 37°C to achieve cleavage of MTT by mitochondrial enzymes. The formed formazon product, which appear as black, fuzzy crystals on the bottom of the well were dissolved by addition of acid isopropanol (40 nM HCl in isopropanol) or DMSO. The absorbance is measured at 570 nm.

The MTT assay (Figure 3) shows that VHH#1A, which has arginine on position 103 in combination with the human-like hydrophobic residues in FR2, has a moderate antagonistic effect (IC50 ~100 nM). VHH#7B with the characteristic hydrophilic residues in FR2 does not prevent binding of TNF-α to its ligand in spite of its sensitive detection of the cytokine in ELISA (curve not shown). In contrast, VHH#3E and #3G with hydrophilic FR2 hallmark residues are very potent antagonistic VHH's (IC50 of 20 nM). VHH#3E and #3G have a high degree of homology and are clonally related (Harmsen et al., Mol. Immunol. 37, 579-590), but VHH#3E is more potent, probably due to the fact that it has a higher affinity than VHH#3G (Figure 2). The (chimeric) monoclonal antibody Remicade is very potent (IC50 of 80 pM), but its derived Fab fragment lost most of this efficacy (IC50 is 3 nM, 30 fold less than the intact mAB). This clearly shows the avidity effect of the interaction between the antibody and the cytokine: the mAB with two binding sites interacts more efficiently with the trimeric TNF molecule via cooperative binding. VHH fragments are strictly monovalent and therefore it was speculated that increasing the avidity by genetically fusing VHH genes might increase their antagonistic efficacy (see Example 4).

These experiments show that a new class of human-like VHH has bona fide binding and functional characteristics, thereby enabling their application for therapeutic purposes.

Example 2: Humanization of VHH#12B and VHH#3E by site directed mutagenesis

1) Homology between VHH#3E / VHH#12B and human germline heavy chain V-region DP-47

Alignment of VHH#12B and a human VH3 germline sequence (DP-47) revealed a high degree of homology:

- 4 AA changes in FR1 on position 1, 5, 28 and 30
- 5 AA changes in FR3 on position 74, 76, 83, 84 and 93

- 1 AA change in FR4 on position 108

as represented in the following sequence alignment:

```
DP-47    EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVS AISGSGGSTYY
VHH#12B QVQLQESGGGLVQPGGSLRLSCAASGFEFE NHWMY WVRQAPGKGLEWVS TVNTNGLITRY

DP-47    ADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK ---------- ----------
VHH#12B ADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTK VLPPYSDDSRTNAD WGQGTQVTVSS
```

A specific inhibitor for the TNF-alpha cytokine, with high homology to the human germline gene DP-47 was therefore an ideal candidate to further humanize and evaluate the influence of mutagenesis on inhibition capacity in ELISA.
Alignment of VHH#3E and a human VH3 germline (DP-47) revealed the presence of hydrophilic amino acid residues in FR2 of VHH#3E compared to hydrophobic residues in DP-47.

```
DP-47  EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS----- WVRQAPGKGLEWVS AISGSGGSTYY
VHH#3E QVQLQESGGGLVQPGGSLRLSCAASGRTFS DHSGYTYTIG WFRQAPGKEREFVA RIYWSSGNTYY

DP-47  ADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK ---------- ----------
VHH#3E ADSVKG RFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA RDGIPTSRSVESYNY WGQGTQVTVSS
```

Evaluation of the effect of substituting the hydrophilic by hydrophobic residues as present in human VH is important, since the majority of camelid VHH sequences contain hydrophilic residues.

2) Mutagenesis of VHH#12B

VHH#12B was mutated by using a non-PCR based site-directed mutagenesis method as described by Chen and Ruffner and commercialized by Stratagene (Quickchange site-directed mutagenesis). Plasmid DNA was used as template in combination with 2 mutagenic primers introducing the desired mutation(s). The 2 primers are each complementary to opposite strands of the template plasmid DNA. In a polymerase reaction using the *Pfu* DNA polymerase each strand is extended from the primer sequence during a cycling program using a limited number of cycles. This results in a mixture of wild type and mutated strands. Digestion with *DpnI* results in selection of the mutated *in vitro* synthesized DNA strand, since only the template strand is sensitive for digestion. The DNA was

precipitated and transformed to *E. coli* and analyzed for the required mutation by sequence analysis. The generated mutant VHH's and the mutagenic primers are listed in Table 2.

Plasmid was prepared from mutant clones and was transformed into WK6 electrocompetent cells. A single colony was used to start an overnight culture in LB containing 2% glucose and 100 µg/ml ampicillin. This overnight culture was diluted 100-fold in 300 ml TB medium containing 100 µg/ml ampicillin, and incubated at 37°C until OD600nm= 2, when 1 mM IPTG and 5 mM $MgSO_4$ (final concentrations) was added and the culture was incubated for 3 more hours at 37°C.

Cultures were centrifuged for 20 minutes at 4,500 rpm at 4°C. The pellet was frozen overnight or for 1 hour at -20°C. Next, the pellet was thawed at room temperature for 40 minutes, re-suspended in 20 ml PBS/1mM EDTA/1M NaCl and shaken on ice for 1 hour. Periplasmic fraction was isolated by centrifugation for 20 minutes at 4°C at 4,500 rpm. The supernatant containing the VHH was loaded on TALON (ClonTech) and purified to homogeneity. The yield of VHH was detemined using the calculated extinction coefficient.

All mutant VHH's expressed comparable to the wild type. The mutants were analyzed for their inhibition capacity in an in vitro receptor binding assay.

A microtiter plate was coated overnight at 4°C with Enbrel (Wyeth) at 2 µg/ml in PBS. The plate was washed five times with PBS-Tween and blocked for 1 hour at room temperature with PBS containing 1% casein. The plate was washed five times with PBS-Tween. Biotinylated human TNF-alpha (80 µg/ml) was pre-incubated with a dilution serie of mutant or wild type VHH#12B for 1hr at RT and the mixture was incubated for 1 hr at room temperature in the wells of the microtiterplate. The plate was washed five times with PBS-Tween. Bound human TNF-α was detected using Extravidin-AP (1/1,000 dilution) and paranitrophenylphosphate (pNPP). Signals were measured after 30 minutes at 405 nm. The results are presented in Figure 4 and 5. The IC50 increased 3-fold from 66 nM (wild type) to 200 nM (mutant Q1E+Q5L+A74S+Y76N+K83R+P84A). Mutation of position T93A resulted in loss of inhibition (data not shown). The positions that still need to be humanized are: E28, E30 and Q108. However, E28 and E30 are part of the H1 canonical structure and thus part of the CDR1 according to Chothia numbering system.

The amino acid sequences of mutant VHHs are presented in Table 4 SEQ ID NOs: 17 to 19.

3) Mutagenesis of VHH#3E

VHH#3E was mutated by using a non-PCR based site-directed mutagenesis method as described above. The obtained mutant VHH's and the mutagenic primers are listed in Table 3.

All mutant VHH's expressed comparable to the wild type. The purified mutant VHH's were analyzed for binding in ELISA and inhibition capacity in receptor binding assay identical to the method described above.

The results of the ELISA are shown in Figure 6, those from the receptor binding assay in Figure 7.

The amino acid sequences of mutant VHHs are presented in Table 4 SEQ ID NOs 21 to 24.

Example 3: Isolation of antagonistic VHH against mouse TNF-alpha

1) Selection of anti-mouse TNF-alpha VHH

In order to perform efficacy studies in mouse models for IBD or Crohn's disease mouse TNF specific VHH were selected. Therefore a llama was immunized with mouse TNF-alpha as described in Example 1. RNA was extracted from PBL's sampled 4 and 10 days after the last immunization, as well as from a biopsy taken from a lymph node after day 4. Total RNA was converted in either random primed or oligo-dT primed cDNA and used as template for the amplification of the VHH encoding gene segments using Ig derived primers or a combination of oligo-dT primer and a single Ig primer (see example 1). With the Ig primers a library containing 8.5×10^7 clones was generated from the first PBL's, and a library with 7×10^6 clones for the second PBL sample and 5.8×10^8 clones for the lymph node. Using the combination of the oligo-dT primer and the Ig primer libraries from the first PBL sample were made containing 1.2×10^8 clones, from the second sample of PBL's a library of 5.7×10^7 clones and the lymph node derived library contained 2×10^8 clones. The libraries were pooled dependent on the used combination of primers and the resulting two libraries were grown for propagation of phage as was described before. Selections were performed on biotinylated mouse TNF-alpha captured on coated streptavidin, bound phage was eluted by competition with the human receptor p75, which is known to cross-react with mouse TNF-alpha. Two distinct mouse TNF-alpha specific VHH (VHH#m3F and VHH#m9E) were selected from the library obtained by amplification with Ig derived primers, while two closely related VHH's were retrieved from the library constructed by PCR with oligo-dT primer and Ig-primer (Figure 8).

2) Determination antagonistic efficacy in cytotoxicity assay with L929 cell line (Figure 9)

The same type of assay was applied as described in Example 1, but with the murine cell line L929. VHH#m3F and VHH#m4B (Figure 9) turned out to be 10-fold more potent then the other two VHH's.

Example 4: Enhancing the antagonistic efficacy by increasing the avidity using multivalent *Camelidae* antibodies

1) Antagonistic efficacy of bi-, tri- and tetravalent VHH against human and mouse TNF-alpha

The *E. coli* production vector pAX11 (Figure 10) was designed, which allows the two-step cloning of bivalent or bispecific VHH. The carboxy terminal VHH is cloned first with *Pst*I and *Bst*EII, while in the second step the other VHH is inserted by *Sfi*I and *Not*I, which do not cut within the first gene fragment. The procedure avoids the enforcement of new sites by amplification and thus the risk of introducing PCR errors.

With this vector the bivalent derivative of the antagonistic anti- human TNF-alpha VHH#3E was generated. The plasmid vector encoding the bivalent VHH was used to generate a tri- and tetrameric derivative, which was accomplished by partial digestion of the plasmid with *Bst*EII, which occurs in both VHH gene segments. The linearized vector was purified from gel, subsequently de-phosphorylated and used as acceptor for cloning of the *Bst*EII fragment of approx. 350 bp that was obtained by complete digestion of the same plasmid. Ligation of the *Bst*EII fragment alone prior to addition to the vector enhances the insertion of multimeric VHH encoding gene segments. After transformation in *E. coli* TG1 the resulting clones were screened by PCR with M13Rev and M13Fwd primers; since *Bst*EII is an a-symmetric cutter (5 nt overhang) only correctly oriented inserts were obtained as was confirmed by digesting the plasmids with *Pst*I alone (350 bp) or double digesting with *Eco*RI and *Hin*dIII (1000 bp for bivalent (BIV 3E, SEQ ID NO: 73), 1350 bp for trivalent (TRI 3E, SEQ ID NO: 74) and 1700 bp for tetravalent (TETRA 3E, SEQ ID NO: 75), data not shown). The sequences are listed in Table 7.

The clones were grown and induced on 50 ml scale, periplasmic fractions prepared and used for IMAC purification with TALON resin. Analysis of the purified products on Coomassie stained PAGE revealed good production levels (between 2 and 10 mg per liter cell culture) of intact multivalent VHH (see Figure 11). The molecular appearance of the IMAC purified VHH was determined by gel filtration on a Superdex 75HR column and as expected the molecules with higher avidities came earlier from the column (see Figure 12).

The antagonistic efficacy was analyzed with the cell based assay using KYM cells. The cells were seeded in microtiterplates and cultured in the presence or absence of TNF-alpha (1.29 ng/ml or approx. 25 pM of trimer). The assays (Figure 13) revealed that the monovalent molecules used in this study had the poorest antagonistic characteristics,

what is reflected by their IC50 values: the Fab derived from the chimeric antibody Remicade has an IC50 of 2 nM and for VHH#3E it is 12 nM (see also Figure 3). The avidity of the used molecules turned out to have a dramatic influence on the antagonistic efficacy as was observed with the bivalent IgG molecule Remicade, which is 40-fold more effective (IC50 50 pM) than the Fab. TNF-alpha is a trimeric molecule, which interacts to a dimeric receptor and therefore it can be expected that the avidity of the IgG permits the mutual binding to two epitopes on the cytokine and supports the formation of large complexes as has been described before (Santora et al, Anal.Biochem. 299, 119-129). Surprisingly, increasing the avidity of the VHH from monomer to dimer has a far more spectacular effect than observed with Remicade, since the IC50 of the dimer (30 pM) is 400 fold lower than of the monomer. Increasing the avidity even more leads to a still better antagonistic behaviour: the trimeric VHH has an IC50 of 20 pM and the tetravalent format 6 pM. All higher avidity formats of the VHH are more efficient than Remicade, while the tetravalent format is even better than Enbrel, which consists of the extracellular domain of the receptor p75 fused to the Fc of an IgG and therefore has a bivalent binding mode.

The same unexpected effect of avidity on antagonistic behaviour was observed with VHH generated against mouse TNF (Figure 14). The same type of cytotoxicity assay was performed using MTT as substrate and mouse TNF-alpha (65 pg/ml or 1.3 pM), but with the murine cell line L929, which expresses the mouse specific receptor. Three different antagonistic (monovalent) VHH were identified coded 9E and 3F, of which the first two have IC50's of 25 nM and the latter 2 nM (see also Example 3). Conversion of 3F into the bivalent format (BIV#m3F, SEQ ID NO: 76) yielded a 1000 fold increase in IC50 (2 pM), thereby demonstrating once more that the increased avidity of the antibody leads to an unexpected improvement of the antagonistic characteristics.

2) Comparison with VHH-Fc fusion

VHH#3E, directed against human TNF, was cloned via *PstI* and *BstEII* in an adapted vector derived from pCDNA3, thereby generating a genetic fusion to the CH1 deleted Fc portion of human IgG1. After confirmation by sequencing, the plasmid construct was transfected to the myeloma cell line NS0. The obtained cell line was grown and the VHH-Fc fusion was secreted into the culture supernatant. The product was purified with an anti-human Fc VHH resin and analyzed on a Coomassie stained gel (Figure 15). In the presence of DTT the fusion was visible as a 45 kDa protein, in the absence of DTT the dimeric molecule with a molecular weight of 90 kDa could be observed. This dimeric

product results from the linkage of two chains by two disulfide bridges, which originate from cysteine residues located in the hinge region.

The VHH-fusion was tested in the bioassay with the human cell line KYM and turned out to be 5-fold less effective than the bivalent VHH in spite of the fact that both molecules have the same avidity and that they both originate from VHH#3E (Figure 16). Probably steric hindrance by the bulky Fc tail might cause this discrepancy.

Example 5: Calculation of homologies between anti-target-single domain antibodies of the invention

The degree of amino acid sequence homology between anti-target single domain antibodies of the invention was calculated using the Bioedit Sequence Alignment Editor. The calculations indicate the proportion of identical residues between all of the sequences as they are aligned by ClustalW. (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research, submitted, June 1994). Table 8 indicates the fraction homology between anti-serum albumin VHHs of the invention. Table 9 indicates the fraction homology between anti-TNF-alpha VHHs of the invention. Table 10 indicates the percentage homology between anti-IFN-gamma VHHs of the invention.

Example 6: Expression of a VHH-CDR3 fragment of VHH#3E

The CDR3 region of VHH#3E was amplified by using a sense primer located in the framework 4 region (Forward: CCCCTGGCCCCAGTAGTTATACG) and an anti-sense primer located in the framework 3 region (Reverse: TGTGCAGCAAGAGACGG).

In order to clone the CDR-3 fragment in pAX10, a second round PCR amplification was performed with following primers introducing the required restriction sites:

Reverse primer Sfi1:

GTCCTCGCAACTGCGGCCCAGCCGGCCTGTGCAGCAAGAGACGG

Forward primer Not1:

GTCCTCGCAACTGCGCGGCCGCCCCCTGGCCCCAGTAGTTATACG

The PCR reactions were performed in 50 ml reaction volume using 50pmol of each primer. The reaction conditions for the primary PCR were 11 min at 94 °C, followed by 30/60/120 sec at 94/55/72 °C for 30 cycles, and 5 min at 72°C. All reaction were performed wit 2.5 mM MgCl2 , 200 mM dNTP and 1.25U AmpliTaq God DNA Polymerase (Roche Diagnostics, Brussels, Belgium).

After cleavage with Sfi1 and Not1 the PCR product was cloned in pAX10.

Example 7: Stability testing of antibody fragments specific for human TNFα

Orally administered proteins are subject to denaturation at the acidic pH of the stomach and as well to degradation by pepsin. We have selected conditions to study the resistance of the VHH#3E to pepsin which are supposed to mimick the gastric environment. VHH#3E a VHH specific to human TNFα was produced as recombinant protein in E.coli and purified to homogeneity by IMAC and gelfiltration chromatography. The protein concentration after purification was determined spectrophotometrically by using the calculated molar exctinction coefficient at 280nm. Diluted solutions at 100 microgram/ml were prepared in McIlvaine buffer (J. Biol. Chem. 49, 1921, 183) at pH 2, pH3 and 4 respectively. These solutions were subsequently incubated for 15 minutes at 37°C, prior the addition of porcine gastric mucosa pepsin at a 1/30 w/w ratio. Sixty minutes after adding the protease a sample was collected and immediately diluted 100-fold in PBS pH7.4 containing 0.1% casein to inactivate the pepsin. Seven additional 3-fold dilutions were prepared from this sample for assessing the presence of functional antibody fragment by ELISA. Identical dilutions prepared from an aliquot collected prior the addition of the protease served as a reference. In the ELISA assay biotinylated TNFα was captured in wells of a microtiter plate coated with neutravidin. For both the pepsin-treated and reference samples similar serial dilutions of the samples were prepared and 100 microliter of those dilutions were added to the wells. After incubation for 1 hour the plates were washed. For the detection of VHH binding to of the captured TNFα a polyclonal rabbit anti-VHH antiserum (R42) and an anti-rabbit IgG alkaline phosphatase conjugate was used. After washing, the plates were developed with paranitrophenyl phosphate. The data plotted in Figure 17 shows similar curves for all of the samples exposed to digestive conditions as well as for the reference samples. This indicates that the VHH#3E essentially retains its functional activity under all of the chosen conditions.

Example 8: Oral administration of an anti-human TNFα specific VHH in mice

An antibody solution containing the anti-human TNFα specific VHH#3E (100microgram per milliliter in 100-fold diluted PBS) was prepared. Three mice which were first deprived from drinking water for 12 hours and subsequently allowed to freely access the antibody solution during the next two hours. Afterwards the mice were sacrificed and their stomachs were dissected. Immediately the content of the stomachs was collected by flushing the stomach with 500microliter PBS containing 1% BSA. This flushed material was subsequently used to prepare serial three-fold dilutions, starting at a 1/5 dilution from

the undiluted material. One hundred microliter of these samples was transferred to individual wells of a microtiter plater coated with human TNFα. After incubation for 1 hour and following extensive washing the presence of immuno-reactive material was assessed with a polyclonal rabbit anti-VHH antiserum (R42) followed by incubation with an anti-rabbit alkaline-phosphatase conjugate.The ELISA was developed with paranitrophenyl acetate. The ELISA signals obtained after 10 minutes clearly demonstrated the presence of functional VHH#3E in the gastric flushings of these mice . By comparing to the standard curve we determined the concentration of the functional antibody fragment in the gastric flushing fluid to be 1.5 , 12.6 and 8.6 microgram/ml for the three mice tested.

Example 9: Efficacy in an animal model for IBD

1) Animal model of chronic collitis

The efficacy of bivalent VHH constructs applied via various routes of administration was assessed in a DSS (dextran sodium sulfate) induced model of chronic colitis in BALB/c mice. This model was originally described by Okayasu et al. [Okayasu et al. Gastroenterology 1990; **98**: 694-702] and modified by Kojouharoff et. al. [G. Kojouharoff et al. Clin. Exp. Immunol. 1997; **107**: 353-8]. The animals were obtained from Charles River Laboratories, Germany, at an age of 11 weeks and kept in the animal facility until they reached a body weight between 21 and 22 g. Chronic colitis was induced in the animals by four DSS treatment cycles. Each cycle consisted of a DSS treatment interval (7 days) where DSS was provided with the drinking water at a concentration of 5 % (w/v) and a recovery interval (12 days) with no DSS present in the drinking water. The last recovery period was prolonged from 12 to 21 days to provide for an inflammation status rather representing a chronic than an acute inflammation at the time of the treatment. Subsequent to the last recovery interval the mice were randomly assigned to groups of 8 mice and treatment with the VHH-constructs was started. The treatment interval was 2 weeks. One week after the end of the treatment interval the animals were sacrificed, the intestine was dissected and histologically examined. The experimental setting is shown schematically in Figure 18.

2) VHH treatment schedule

During the VHH treatment period the mice (8 animals per group) were treated daily for 14 consecutive days with bivalent VHH#3F (VHH#m3F-VHH#m3F; SEQ ID No. 76) by intra-gastric or intra-venous application of 100 µg bivalent VHH 3F. An additional group of animals was treated rectally with the bivalent VHH#3F every other day for a period of 14

days. In all treatment groups a dose of 100 µg of the bivalent VHH#3F was applied at a concentration of 1 mg/ml in a buffered solution. The negative control groups received 100 µl of PBS under otherwise identical conditions. The treatment schedule is shown in Table 11.

3) Results

After the mice were sacrificed the body weight was determined and the colon was dissected. The length of the dissected colon was determined and the histology of the colon was assessed by Haematoxilin-Eosin (HE) stain (standard conditions). As compared to the negative controls (PBS treatment) the groups treated with bivalent nanobody 3F showed a prorogued colon length as well as an improved histological score [G. Kojouharoff et al. Clin. Exp. Immunol. 1997; **107**: 353-8] thereby demonstrating efficacy of the treatment.

NAME	SEQ ID NO	SEQUENCE
VHH#1A	1	QVQLQESGGGLVQPGGSLRLSCATSGFDFSVSWMYWVRQAPGKGLEWVS EINTNGLITKYVDSVKGRFTISRDNAKNTLYLQMDSLIPEDTALYYCAR SPSGSFRGQGTQVTVSS
VHH#7B	2	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVA IITSGDNLNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNAI LQTSRWSIPSNYWGQGTQVTVSS
VHH#2B	3	QVQLQESGGGLVQPGGSLRLSCATSGFTFSDYWMYWVRQAPGKGLEWVS TVNTNGLITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTK VVPPYSDDSRTNADWGQGTQVTVSS
VHH#3E	4	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKE REFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAV YYCAARDGIPTSRSVESYNYWGQGTQVTVSS
VHH#3G	5	QVQLQDSGGGLVQAGGSLRLSCAVSGRTFSAHSVYTMGWFRQAPGKERE FVARIYWSSANTYYADSVKGRFTISRDNAKNTVDLLMNSLKPEDTAVYY CAARDGIPTSRTVGSYNYWGQGTQVTVSS
VHH#10A	6	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVA IITSSDTNDTTNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYC NAVLQTSRWSIPSNYWGQGTQVTVSS
VHH#2G	7	QVQLQDSGGGLVQAGGSLRLSCTTSGRTISVYAMGWFRQAPGKEREFVA SISGSGAITPYADSVKGRFTISRDNAKNTVYLQMNSLNPEDTAVYYCAA SRYARYRDVHAYDYWGQGTQVTVSS
VHH#1F	8	QVQLQDSGGGLVQAGGSLRLSCAASTRTFSRYVVGWFRQAPGKEREFVA TISWNGEHTYYADSVKGRYTISRDNAKNTVYLQMGSLKPEDTAVYYCAA RSFWGYNVEQRDFGSWGQGTPVTVSS
VHH#9C	9	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVA IITNDTTNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNTVL QTSRWNIPTNYWGQGTQVTVSS
VHH#11E	10	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVA IISGDTTNYADAVKGRFTISTDNVKKTVYLQMNVLESEDTAVYYCNAVL QTSRWSIPSNYWGQGTQVTVSS
VHH#10C	11	QVQLQDSGGGLVQPGGSLRLACVASGSIFSIDVMGWYRQAPGQQRELVA TITNSWTTNYADSVKGRFTISRDNAKNVVYLQMNSLKLEDTAVYYCNAR RWYQPEAWGQGTQVTVSS
VHH#4B	12	QVQLQDSGGGLVQPGGSLRLSCAASGFTFSTHWMYWVRQAPGKGLEWVS TINTNGLITDYIHSVKGRFTISRDNAKNTLYLQMNSLKSEDTAVYYCAL NQAGLSRGQGTQVTVSS
VHH#10D	13	QVQLQESGGGLVQAGGSLRLSCAASRRTFSGYAMGWFRQAPGKEREFVA VVSGTGTIAYYADSVKGRFTISRDNAENTVYLQMNSLKPEDTGLYYCAV GPSSSRWYYRGASLVDYWGKGTLVTVSS
VHH#12B	14	QVQLQESGGGLVQPGGSLRLSCAASGFEFENHWMYWVRQAPGKGLEWVS TVNTNGLITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTK VLPPYSDDSRTNADWGQGTQVTVSS
VHH#m9A	79	EVQLVESGGGLVQAGGSLRLSCAASGGTLSSYITGWFRQAPGKEREFVG AVSWSSSTIVYADSVEGRFTISRDNHQNTVYLQMDSLKPEDTAVYYCAA RPYQKYNWASASYNVWGQGTQVTVSS
VHH#m9E	15	EVQLVESGGGLVQAGGSLRLSCAASGGTLSSYITGWFRQAPGKEREFVG AVSWSSSTIVYADSVEGRFTISRDNHQNTVYLQMDSLKPEDTAVYYCAA RPYQKYNWASASYNVWGQGTQVTVSS

VHH#m3F	16	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVG AVSWSGGTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAA RPYQKYNWASASYNVWGQGTQVTVSS
VHH#m4B	80	QVQLQDSGGGLVQAGGSLRLSCGVSGLSFSGYTMGWFRQAPGKEREFAA AIGWNSGTTEYRNSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA SPKYMTAYERSYDFWGQGTQVTVSS
VHH#8-29	81	QVQLVESGGGLVQPGGSLRLSCAASGFAFGDSWMYWVRQAPGKGLEWVS EINTNGLITKYKDSVTGRFTISRDNAKNTLHLEMNRLKPEDTALYYCAR DPSGKLRGPGTQVTVSS
VHH#8-41	82	QVQLVESGGGLVQPGGPLRLSCAASGFAFGDSWMYWVRQAPGKGLEWVS EINTNGLITKYKDSVTGRFTISRDNAKNTLHLEMNRLKPEDTALYYCAR DPSGKLRGPGTQVTVSS
VHH#8-42	83	QVQLVESGGGLVQPGGSLRLSCAASGFAFGDSWMYWVRQAPGKGLEWVS EINTNGLITKYKDSVTGRFTISRDNAKNTLHLEMNRLKPEDTALYYCAR DPSGKLRGPGTQVTVSS
VHH#8-44	84	QVQLVESGGGLVQPGGSLRLSCAASGFTFSDHWMYWVRQAPGKGLEWVS TINTNGLITNYIHSVKGRFTISRDNAKNTLYLQMNSLKSEDTAVYYCAL NQAGLSRGQGTQVTVSS

Table 1: Amino acid sequence listing of the peptides of aspects of present invention directed against TNF-alpha.

Mutation	Template	Primer sequence
A74S+Y76N+ K83R+P84A	Wild type	5'-AGA GAC AAC TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG-3' Arg Asp Asn **Ser** Lys **Asn** Thr Leu Tyr Leu Gln Met Asn Ser Leu **Arg Ala** Glu Asp Thr
Q1E+Q5L+A74S+Y76N+K83R+P84A	A74S+Y76N+ K83R+P84A	5'-C ATG GCT GAG GTG CAG CTG CTC GAG TCT GG-3' Met Ala **Glu** Val Gln Leu **Leu** Glu Ser
Q1E+Q5L+A74S+Y76N+K83R+P84A+T93A	Q1E+Q5L+A74S+Y76N+K83R+P84A	5'-G GAC ACG GCC GTC TAT TAC TGT GCA AAA GTA CTT C-3' Asp Thr Ala Val Tyr Tyr Cys **Ala** Lys Val Leu

Table 2: List of mutagenesis reactions, mutagenic primers and templates used for mutagenesis of VHH#12B

Muta-tion	Template	Primer sequence
F37V	Wild type	5'-ACC TAT ACC ATT GGC TGG GTC CGC CAG GCT-3' Thr Tyr Thr Ile Gly Trp **Val** Arg Gln Ala
E44G	Wild type	5'-CGC CAG GCT CCA GGG AAG GGG CGT GAG TTT-3' Arg Gln Ala Pro Gly Lys **Gly** Arg Glu Phe
R45L	Wild type	5'-A GGG AAG GAG CTT GAG TTT GTA GCG CGT AT-3' Gly Lys Glu **Leu** Glu Phe Val Ala Arg
F47W	Wild type	5'-A GGG AAG GAG CGT GAG TGG GTA GCG CGT AT-3' Gly Lys Glu Arg Glu **Trp** Val Ala Arg

Table 3: List of mutagenesis reactions, mutagenic primers and templates used for mutagenesis of VHH#3E

SEQ ID	Name	Sequence
17	VHH#12B A74S+Y76N +K83R+P84 A	QVQLQESGGGLVQPGGSLRLSCAASGFEFENHWMYWVRQAPGKGLEW VSTVNTNGLITRYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY YCTKVLPPYSDDSRTNADWGQGTQVTVSS
17	VHH#12B Q1E+Q5L+A 74S+Y76N+ K83R+P84A	EVQLLESGGGLVQPGGSLRLSCAASGFEFENHWMYWVRQAPGKGLEW VSTVNTNGLITRYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY YCTKVLPPYSDDSRTNADWGQGTQVTVSS
19	VHH#12B Q1E+Q5L+A 74S+Y76N+ K83R+P84A +T93A	EVQLLESGGGLVQPGGSLRLSCAASGFEFENHWMYWVRQAPGKGLEW VSTVNTNGLITRYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY YCAKVLPPYSDDSRTNADWGQGTQVTVSS
20	VHH#12B Wild type	QVQLQESGGGLVQPGGSLRLSCAASGFEFENHWMYWVRQAPGKGLEW VSTVNTNGLITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVY YCTKVLPPYSDDSRTNADWGQGTQVTVSS
21	VHH#3E F37V	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWVRQAPG KEREFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPE DTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS
21	VHH#3E E44G	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPG KGREFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPE DTAVYYCAARDGIPTSRSVESYNYWG

		QGTQVTVSS
23	VHH#3E R45L	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPG KELEFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPE DTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS
23	VHH#3E F47W	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPG KEREWVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPE DTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS
25	VHH#3E Wild type	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPG KEREFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPE DTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS

Table 4: Overview of humanized and wild type anti-TNF-alpha VHH

Name	SEQ ID	Sequence
		Anti-mouse serum albumin
MSA21	26	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEWV SGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYC TIGGSLNPGGQGTQVTVSS
MSA24	27	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEWV SSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVYYC TIGGSLSRSSQGTQVTVSS
MSA210	26	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSS
MSA212	26	QVQLQESGGGLVQPGGSLRLTCTASGFTFRSFGMSWVRQAPGKGLEWV SAISADGSDKRYADSVKGRFTISRDNGKKMLTLDMNSLKPEDTAVYYC VIGRGSPASQGTQVTVSS
MSAcl6	85	AVQLVESGGGLVQAGDSLRLSCVVSGTTFSSAAMGWFRQAPGKEREFV GAIKWSGTSTYYTDSVKGRFTISRDNVKNTVYLQMNNLKPEDTGVYTC AADRDRYRDRMGPMTTTDFRFWGQGTQVTVSS
MSAcl1 2	86	QVKLEESGGGLVQTGGSLRLSCAASGRTFSSFAMGWFRQAPGREREFV ASIGSSGITTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTGLCYC AVNRYGIPYRSGTQYQNWGQGTQVTVSS
MSAcl1 0	87	EVQLEESGGGLVQPGGSLRLSCAASGLTFNDYAMGWYRQAPGKERDMV ATISIGGRTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCV AHRQTVVRGPYLLWGQGTQVTVSS
MSAcl1 4	88	QVQLVESGGKLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKEREFV AGSGRSNSYNYYSDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYC AASTNLWPRDRNLYAYWGQGTQVTVSS
MSAcl1 6	89	EVQLVESGGGLVQAGDSLRLSCAASGRSLGIYRMGWFRQVPGKEREFV AAISWSGGTTRYLDSVKGRFTISRDSTKNAVYLQMNSLKPEDTAVYYC AVDSSGRLYWTLSTSYDYWGQGTQVTVSS
MSAcl1 9	90	QVQLVEFGGGLVQAGDSLRLSCAASGRSLGIYKMAWFRQVPGKEREFV AAISWSGGTTRYIDSVKGRFTLSRDNTKNMVYLQMNSLKPDDTAVYYC AVDSSGRLYWTLSTSYDYWGQGTQVTVSS
MSAcl5	91	EVQLVESGGGLVQAGGSLSLSCAASGRTFSPYTMGWFRQAPGKEREFL AGVTWSGSSTFYGDSVKGRFTASRDSAKNTVTLEMNSLNPEDTAVYYC AAAYGGGLYRDPRSYDYWGRGTQVTVSS

MScl11	92	AVQLVESGGGLVQAGGSLRLSCAASGFTLDAWPIAWFRQAPGKEREGV SCIRDGTTYYADSVKGRFTISSDNANNTVYLQTNSLKPEDTAVYYCAA PSGPATGSSHTFGIYWNLRDDYDNWGQGTQVTVSS
MSAcl1 5	92	EVQLVESGGGLVQAGGSLRLSCAASGFTFDHYTIGWFRQVPGKEREGV SCISSSDGSTYYADSVKGRFTISSDNAKNTVYLQMNTLEPDDTAVYYC AAGGLLLRVEELQASDYDYWGQGIQVTVSS
MSAcl8	94	AVQLVDSGGGLVQPGGSLRLSCTASGFTLDYYAIGWFRQAPGKEREGV ACISNSDGSTYYGDSVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYC ATADRHYSASHHPFADFAFNSWGQGTQVTVSS
MSAcl7	95	EVQLVESGGGLVQAGGSLRLSCAAYGLTFWRAAMAWFRRAPGKERELV VARNWGDGSTRYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYC AAVRTYGSATYDIWGQGTQVTVSS
MSAcl2 0	96	EVQLVESGGGLVQDGGSLRLSCIFSGRTFANYAMGWFRQAPGKEREFV AAINRNGGTTNYADALKGRFTISRDNTKNTAFLQMNSLKPDDTAVYYC AAREWPFSTIPSGWRYWGQGTQVTVSS
MSAcl4	97	DVQLVESGGGWVQPGGSLRLSCAASGPTASSHAIGWFRQAPGKEREFV VGINRGGVTRDYADSVKGRFAVSRDNVKNTVYLQMNRLKPEDSAIYIC AARPEYSFTAMSKGDMDYWGKGTLVTVSS
		Anti-mouse serum albumin/anti TNF-alpha
MSA21/ VHH#3E	30	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEWV SGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYC TIGGSLNPGGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRL SCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRF AISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVT VSS
MSA24/ VHH#3E	31	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEWV SSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVYYC TIGGSLSRSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRL SCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRF AISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVT VSS
MSA210 /VHH#3 E	31	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLS CAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRFA ISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTV SS
MSA212 /VHH#3 E	31	QVQLQESGGGLVQPGGSLRLTCTASGFTFRSFGMSWVRQAPGKGLEWV SAISADGSDKRYADSVKGRFTISRDNGKKMLTLDMNSLKPEDTAVYYC VIGRGSPASQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLS CAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRFA ISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTV SS
MSA21/ MSA21/ VHH#3E	34	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEWV SGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYC TIGGSLNPGGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGS LRLSCEASGFTFSRFGMTWVRQAPGKGVEWVSGISSLGDSTLYADSVK GRFTISRDNAKNTLYLQMNSLKPEDTAVYYCTIGGSLNPGGQGTQVTV SSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSG YTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRFAISRDIAKN TVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS
MSA210 /VHH#1 A	35	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSL

		RLSCATSGFDFSVSWMYWVRQAPGKGLEWVSEINTNGLITKYVDSVKG RFTISRDNAKNTLYLQMDSLIPEDTALYYCARSPSGSFRGQGTQVTVS S
MSA210 /VHH#7 B	36	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSL RLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITSGDNLNYADAVKGR FTISTDNVKKTVYLQMNVLKPEDTAVYYCNAILQTSRWSIPSNYWGQG TQVTVSS
MSA210 /VHH#2 B	36	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSL RLSCATSGFTFSDYWMYWVRQAPGKGLEWVSTVNTNGLITRYADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVVPPYSDDSRTNADWG QGTQVTVSS
MSA210 /VHH#3 E	36	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSL RLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYA DSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVE SYNYWGQGTQVTVSS
MSA210 /VHH#3 G	39	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQAGGSL RLSCAVSGRTFSAHSVYTMGWFRQAPGKEREFVARIYWSSANTYYADS VKGRFTISRDNAKNTVDLLMNSLKPEDTAVYYCAARDGIPTSRTVGSY NYWGQGTQVTVSS
MSA21/ VHH#12 B	40	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEWV SGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYC TIGGSLNPGGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGS LRLSCAASGFEFENHWMYWVRQAPGKGLEWVSTVNTNGLITRYADSVK GRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVLPPYSDDSRTNADW GQGTQVTVSS
MSA24/ VHH#12 B	40	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEWV SSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVYYC TIGGSLSRSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGS LRLSCAASGFEFENHWMYWVRQAPGKGLEWVSTVNTNGLITRYADSVK GRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVLPPYSDDSRTNADW GQGTQVTVSS
MSA210 /VHH#12B	40	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEWV SAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVYYC VIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSL RLSCAASGFEFENHWMYWVRQAPGKGLEWVSTVNTNGLITRYADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVLPPYSDDSRTNADWG QGTQVTVSS
MSA212 /VHH#12B	39	QVQLQESGGGLVQPGGSLRLTCTASGFTFRSFGMSWVRQAPGKGLEWV SAISADGSDKRYADSVKGRFTISRDNGKKMLTLDMNSLKPEDTAVYYC VIGRGSPASQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSL RLSCAASGFEFENHWMYWVRQAPGKGLEWVSTVNTNGLITRYADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVLPPYSDDSRTNADWG QGTQVTVSS

Table 5: Anti-mouse serum albumin, and anti-mouse serum albumin + anti TNF-alpha VHH

Seq. Family	Name	Seq. Id	Sequence
1	MP3D2SRA	44	QVQLQDSGGGTVQAGGSLRLSCAASGRTFSDYAVGWFRQA PGKEREFVARILWTGASRSYANSVDGRFTVSTDNAKNTVY LQMNSLKPEDTAIYYCAALPSNIITTDYLRVYYWGQGTQV TVSS
1	MP3A3SR	45	QVQLQDSGGGTVQAGGSLRLSCAASGRTFSNYAVGWFRQA PGKEREFVARIKWSGGSRSYANSVDGRFTVSTDNAKNTVY LQMNSLKPEDTAIYYCA?LPSNIITTDYLRVYYWGQGTQV TVSS
2	MP3C5SR	46	QVQLQESGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWY RQAPGKQRELVAGILTSGATSYAESVKGRFTISRDNAKNT VYLQMNSLSPEDTAEYYCNTYPTWVLSWGQGTQVTVSS
2	MP3C1SR	47	QVQLQDSGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWY RQAPGKQRELVAGILSSGATVYAESVKGRFTISRDNAKNT VYLQMNSLSPEDTAEYYCNTYPTWVLSWGQGTQVTVSS
2	MP3G8SR	46	QVQLQESGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWY RQAPGKQRELVAGILSSGATAYAESVKGRFTISRDNAKNT VYLQMNSLSPEDTAEYYCNTYPTWVLSWGQGTQVTVSS
3	MP3D2BR	49	QVQLQESGGGLVQPGESLRLSCAASRGIFRFNAGGWYRQA PGKQRELVAFIGVDNTTRYIDSVKGRFTISRDNAKTTVYL QMNSLQPEDTAVYYCNKVPYIDWGQGTQVTVSS
4	MP3H6SRA	50	QVQLQESGGGLVQAGGSLRLSCAASGRTFSTYNMGWFRQA PGKEREFVAGISWNGGSIYYTSSVEGRFTISRDNAENTVY LQMNSLKPEDTGVYYCASKGRPYGVPSPRQGDYDYWGQGT QVTVSS
4	MP3B4SRA	51	QVQLQESGGGLVQAGGSLRLSCAASGRTFSTYNMGWFRQA PGKEREFVAGISWNGGSIYYTSSVEGRFTISRDNAENTVY LQMNSLKPEDTGVYYCASKGRPYGVPSPRQGDYDYWGQGT QVTVSS
4	MP4E4BR	52	QVQLQESGGGLVQAGGSLRLSCAASGRTFSIYNMGWFRQA PGKEREFVAAISWNGGSIYYTSSVEGRFTISRDNAINTVY LQMNSLKPEDTGVYYCASKGRPYGVPSPRQGEYDYWGQGT QVTVSS
4	MP4H8SR	52	QVQLQESGGGLVQAGGSLRLSCAASGRTFNIYNMGWFRQA PGKERDFVAAISWNGGSIYYTSSVEGRFTISRDNAENTVY LQMNSLKPEDTGVYYCASKGRPYGVPSPRQGDYDYWGQGT QVTVSS
5	MP2F6SR	54	QVKLEESGGGLVQAGGSLRLSCAASGRTFNNYNMGWFRQA PGKEREFVAAISWNGGSTYYDDSVKGRFTISRDNANNLVY LQMNSLNFEDTAVYYCACAANPYGIPQYRENRYDFWGQGT QVTVSS
4	MP3D1BR	55	QVQLQESGGGLVQAGGSLRLSCAASGRTFDNYNMGWFRQA PGKEREFVAAISWNGGSTYYDDSVKGRFTISRDNFQKLVY LQMNSLKLEDTAVYYCACAANPYGIPQYRENRYDFWGQGT QVTVSS
5	MP2B5BR	56	QVQLVESGGRLVQAGGSLRLSCIASGRTISDYAAGWFRQA PGKEREFLASVTWGFGSTSYADSVKGRFTISRDKAKDTVY LQMNTLEPDDTSVYYCASSPRYCAGYRCYVTASEFDSWGQ GTQVTVSS
6	MP2C1BR	57	QVKLEESGGRLVQAGGSLRLSCIASGRTISDYAAGWFRQA PGKEREFLASVSWGFGSTYYADSVKGRFTISRDTAKDTVY LQMNTLEPDDTSVYYCASSPRYCAGYRCYATASEFDSWGQ GTQVTVSS

6	MP4A12SR	58	QVQLQESGGRLVQAGGSLRLSCIASGRTISDYAAGWFRQA PGKEREFLASVTWGFGSTYYADSVKGRFTISRDKAKDTVY LQMNTLEPDDTSAYYCASSPRYCAGYRCYVTASEFDSWGP GTQVTVSS
7	MP3F4SRA	59	QVQLQDSGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQA PGKEHEFVAGIWRSGVSLYYTDSVKGRFTISRDDAKMTVS LQMNSLKPEDTAVYYCAAEATFPTWSRGRFADYDYRGQGT QVTVSS
7	MP3D3BR	60	QVQLQESGGGLVQAGDSLRLSCTASGRSFSSYGMGWFRQA PGKDHEFVAGIWRSGVSLYYADSVKGRFTISRDDAKMTVS LQMNGLKPEDTAVYYCAAEATFPTWNRGTFADYDYRGQGT QVTVSS
6	MP3E5BR	61	QVQLQESGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQA PGKEHEFVAGIWRSGVSLYYADSVKGRFTISRDDAKMTVS LQMNGLKPEDTAVYYCAAEATFPTWNRGSFADYDYRGQGT QVTVSS
7	MP3C7SRA	62	QVQLQESGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQA PGKEHEFVAGIWRSGVSLYYADSVKGRFTISRDDAKMTVS LQMNSLKPEDTAVYYCAAEATFPTWNRGRFADYDYSGQGT QVTVSS
8	MP2F1BR	63	AVQLVESGGGLVQTGDSLRLSCVASGGTFSRYAMGWFRQA PGKEREFVARIGYSGRSISYATSVEGRFAISRDNAKNTVY LQMNSLKPEDTAVYYCASLVSGTLYQADYWGQGTQVTVSS
8	MP2C5BR	64	QVQLVESGGGLVQTGDSLRLSCVASGGTFSRYAMGWFRQP PGKERDFVARIGYSGQSISYATSVEGRFAISRDNAKNTVY LQMNSLKPEDTAVYYCASLVSGTLYKPNYWGQGTQVTVSS
9	MP2C10BR	65	QVKLEESGGGLVQAGGSLRLSCAASGLTYTVGWFRQAPGK EREFVAAISWSGGSALYADSVKGRFTISRDNAKNTVYLQM GSLEPEDTAYYSCAAPGTRYYGSNQVNYNYWGQGTQVTVS S
9	MP2G5SR	66	QVKLEESGGGLVQAGDSLRLSCAASGLTYTVGWFRQAPGK EREFVAAIDWSGGSALYADSVKGRFTISRDNTKNTVYLQM GSLEPEDTAVYWCAAPGTRYHGRNQVNYNYWGQGTQVTVS S
10	MP3B1SRA	67	QVQLQESGGGLVQPGGSLRLSCAASGFTSSNYAMSWVRQA PGKGLEWVSSINSRTGSITYADSVKGRFTITLDNAKNTLY LQMNSLKPEDTAVYYCASRVDDRVSRGQGTQVTVSS
11	MP2F10SR	68	QVQLVESGGGLVQAGGSLRLSCAASGRTISSFRMGWFRRA PGEEREFVAFVRSNGTSTYYADSVEGRFTITRDNAKNTVY LRMDSLKPEDTAVYYCAAATRDYGGSFDYWGQGTQVTVSS
11	MP3A7SRA	69	QVQLQDSGGGLVQAGGSLRLSCAASGRTFSSFRMGWFRRA PGEEREFVAFVRSNGTSTYYADSVEGRFTITRDNAKNTVY LRMDSLKPEDTAVYYCAAATRDYGGSFDYWGQGTQVIVSS
12	MP4C10SR	70	QVQLQESGGGLVQPGGSLRLSCAASGFTVSNYAMSWVRQP PGKGIEWVSSINNRNDHITYADSVKGRFTIARDNANNILY LQMNSLKPEDTAVYYCASRVDDRVSRGQGTQVTVSS

13	MP4D5BR	71	QVQLQDSGGGLVQPGGSLRLSCAASGRTFSSYGMAWFRQA PGKERELVVAINRSGGATSYATSVRGRFTISRDNAKNTMY LQMNSLNPEDTAVYYCAARDPTRTYSSYFEYTYWGQGTQV TVSS
13	MP3F1SRA	72	QVQLQESGGGLVQAGGSLTLSCVASGRTISDYAVGWFRQA PGKEREFVASISWGGGFTAFADSMKGRFTISRDNAKNTVY LQTHTLEPDDTSVYYCASSRRYCTGYRCYATASEFDSWGQ GTQVTVSS

Table 6: Amino acid sequence listing of VHH's directed against human IFN-gamma.

Name	SEQ ID	Sequence
		With linker sequence (underlined)
BIV 3E	73	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAVSWSG GTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQKYNWASAS YNVWGQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQAGGSLRLSCAASGGTF SSIIMAWFRQAPGKEREFVGAVSWSGGTTVYADSVLGRFEISRDSARKSVYLQMN SLKPEDTAVYYCAARPYQKYNWASASYNVWGQGTQVTVSS
TRI 3E	74	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVAR IYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTS RSVESYNYWGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLSCAA SGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRFAISRDI AKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSSEPKTP KPQPAAAQVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGK EREFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA RDGIPTSRSVESYNYWGQGTQVTVSS
TETRA 3E	75	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVAR IYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTS RSVESYNYWGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLSCAA SGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRFAISRDI AKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSSEPKTP KPQPAAAQVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGK EREFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA RDGIPTSRSVESYNYWGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGS LRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGR FAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTV SS
BIV#m 3F	76	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAVSWSG GTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQKYNWASAS YNVWGQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQAGGSLRLSCAASGGTF SSIIMAWFRQAPGKEREFVGAVSWSGGTTVYADSVLGRFEISRDSARKSVYLQMN SLKPEDTAVYYCAARPYQKYNWASASYNVWGQGTQVTVSS
		Without linker sequence
BIV#3 Edir	77	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAVSWSG GTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQKYNWASAS YNVWGQGTQVTVSSQVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAP GKEREFVGAVSWSGGTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYC AARPYQKYNWASASYNVWGQGTQVTVSS

BIV#12 Bdir	78	QVQLQESGGGLVQPGGSLRLSCAASGFEFENHWMYWVRQAPGKGLEWVSTVNTNG LITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVLPPYSDDSRTN ADWGQGTQVTVSSQVQLQESGGGLVQPGGSLRLSCAASGFEFENHWMYWVRQAPG KGLEWVSTVNTNGLITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVYYCT KVLPPYSDDSRTNADWGQGTQVTVSS

Table 7: Sequences of bivalent (BIV 3E, BIV#m3F), trivalent (TRI3E) or tetravalent (TETRA 3E) VHH directed against TNF-alpha.

SEQ	MSA21	MSA24	MSA210	MSA212
MSA21	1.000	0.834	0.800	0.782
MSA24	---	1.000	0.782	0.791
MSA210	---	---	1.000	0.903
MSA212	---	---	---	1.000

Table 8: Fractional homologies between the amino acid sequences of anti-mouse serum albumin VHHs of the invention.

SEQ	VHH#1A	VHH#7B	VHH#2B	VHH#3E	VHH#3G	VHH#10A	VHH#2G	VHH#1F	VHH#9C	VHH#11E	VHH#10C	VHH#4B	VHH#10D	VHH#12B	VHH#9E	VHH#3F
VHH#1A	1.000	0.601	0.764	0.596	0.622	0.600	0.682	0.629	0.609	0.601	0.614	0.818	0.642	0.747	0.596	0.604
VHH#7B	---	1.000	0.604	0.635	0.645	0.943	0.653	0.616	0.933	0.933	0.719	0.593	0.614	0.620	0.616	0.624
VHH#2B	---	---	1.000	0.620	0.645	0.611	0.682	0.661	0.629	0.620	0.637	0.796	0.634	0.951	0.620	0.645
VHH#3E	---	---	---	1.000	0.875	0.641	0.713	0.689	0.620	0.643	0.612	0.604	0.648	0.596	0.674	0.682
VHH#3G	---	---	---	---	1.000	0.651	0.779	0.740	0.637	0.637	0.653	0.645	0.689	0.622	0.708	0.716
VHH#10A	---	---	---	---	---	1.000	0.658	0.614	0.935	0.935	0.725	0.592	0.612	0.626	0.622	0.637
VHH#2G	---	---	---	---	---	---	1.000	0.741	0.653	0.669	0.685	0.666	0.746	0.650	0.701	0.717
VHH#1F	---	---	---	---	---	---	---	1.000	0.616	0.616	0.664	0.661	0.714	0.645	0.709	0.717
VHH#9C	---	---	---	---	---	---	---	---	1.000	0.941	0.743	0.601	0.622	0.645	0.600	0.616
VHH#11E	---	---	---	---	---	---	---	---	---	1.000	0.719	0.601	0.622	0.637	0.608	0.624
VHH#10C	---	---	---	---	---	---	---	---	---	---	1.000	0.650	0.606	0.637	0.600	0.632
VHH#4B	---	---	---	---	---	---	---	---	---	---	---	1.000	0.611	0.796	0.588	0.629
VHH#10D	---	---	---	---	---	---	---	---	---	---	---	---	1.000	0.619	0.674	0.674
VHH#12B	---	---	---	---	---	---	---	---	---	---	---	---	---	1.000	0.604	0.637
VHH#9E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1.000	0.854
VHH#3F																1.000

Table 9: Fractional homologies between anti-TNF-alpha VHHs of the invention

	% Homology																																					
	MP3D2SRA	MP3A3SR	MP3C5SR	MP3C1SR	MP3G8SR	P3D2BR	MP3H6SRA	MP3B4SRA	MP4E4BR	MP4H8SR	MP2F6SR	MP3D1BR	MP2B5BR	MP2C1BR	MP4A12SR	MP3F4SRA	MP3D3BR	MP3E5BR	MP3C7SRA	MP2F1BR	MP2C5BR	MP2C10BR	MP2G5SR	MP3B1SRA	MP2F10SR	MP3A7SRA	MP4C10SR	MP4D5BR	MP3F1SRA	MP6D6BR	MP6B1BR	MP6A8BR	MP6B12BR	MP6C11BR	MP6B10BR
MP3D2SRA	X	96	66	66	66	62	71	71	71	70	68	69	65	63	64	68	66	67	68	71	70	68	67	63	67	68	60	72	65	68	67	66	67	76	70
MP3A3SR	—	X	66	66	66	62	72	72	72	71	70	71	65	63	64	68	66	67	68	72	72	69	67	64	66	67	60	73	65	67	67	65	66	77	71
MP3C5SR	—	—	X	97	98	73	65	65	64	63	63	63	60	58	59	64	64	65	66	65	65	65	63	63	64	64	61	67	60	74	63	60	63	70	64
MP3C1SR	—	—	—	X	98	72	64	64	64	62	62	62	58	57	58	65	64	64	65	64	63	64	62	63	64	65	60	67	59	73	63	60	62	70	65
MP3G8SR	—	—	—	—	X	73	65	65	64	63	63	63	59	58	59	64	64	65	66	65	64	65	63	63	65	65	61	66	60	73	63	61	63	71	64
MP3D2BR	—	—	—	—	—	X	63	63	63	62	63	64	59	58	58	62	61	62	63	64	63	63	63	64	63	63	63	65	58	73	64	60	63	68	67
MP3H6SRA	—	—	—	—	—	—	X	100	97	97	80	81	67	68	67	75	71	73	75	73	71	73	71	66	75	75	63	71	69	71	71	68	70	82	70
MP3B4SRA	—	—	—	—	—	—	—	X	97	97	80	81	67	68	67	75	71	73	75	73	71	73	71	66	75	75	63	71	69	71	71	68	70	82	70
MP4E4BR	—	—	—	—	—	—	—	—	X	97	81	82	68	69	68	73	70	71	73	73	71	73	71	66	75	75	63	72	70	71	71	68	70	80	71
MP4H8SR	—	—	—	—	—	—	—	—	—	X	81	81	66	66	66	72	69	71	72	71	71	72	71	64	73	73	62	70	67	69	70	67	70	79	71
MP2F6SR	—	—	—	—	—	—	—	—	—	—	X	94	65	68	64	70	67	69	71	67	65	73	71	63	71	70	62	69	66	67	69	68	67	78	69
MP3D1BR	—	—	—	—	—	—	—	—	—	—	—	X	65	66	65	71	69	71	72	67	65	70	69	63	71	71	62	68	66	67	71	69	69	79	70
MP2B5BR	—	—	—	—	—	—	—	—	—	—	—	—	X	95	97	63	64	64	64	65	63	64	63	60	66	63	57	63	84	65	63	63	62	70	65
MP2C1BR	—	—	—	—	—	—	—	—	—	—	—	—	—	X	95	63	64	64	64	63	61	66	65	59	66	63	56	61	85	65	64	63	62	70	65
MP4A12SR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	63	64	64	64	62	60	63	62	59	65	63	56	61	84	64	63	63	62	70	65
MP3F4SRA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	94	96	97	69	67	68	68	62	67	69	60	72	63	67	68	65	65	76	71
MP3D3BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	98	96	70	68	67	67	62	67	67	60	70	64	66	66	64	64	75	69
MP3E5BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	98	70	68	68	69	63	68	68	60	72	64	67	68	65	66	77	71
MP3C7SRA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	71	69	69	70	63	69	69	61	72	64	68	68	66	66	78	71
MP2F1BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	94	66	67	63	68	67	61	70	64	68	65	64	64	74	67
MP2C5BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	66	67	63	67	65	62	69	63	67	64	62	63	73	67
MP2C10BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	94	62	68	66	59	67	66	69	68	64	68	74	73
MP2G5SR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	62	67	65	59	67	65	67	66	64	66	73	73
MP3B1SRA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	66	65	91	67	60	67	69	68	69	69	65
MP2F10SR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	97	61	67	65	71	66	65	67	77	68
MP3A7SRA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	61	68	63	71	65	65	67	77	69
MP4C10SR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	64	58	65	64	63	66	66	63
MP4D5BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	64	69	68	65	67	76	73
MP3F1SRA	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	65	64	64	63	71	68
MP6D6BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	70	65	70	77	73
MP6B1BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	78	81	76	71
MP6A8BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	75	74	66
MP6B12BR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	73	68
MP6C11BR																																		X	77
MP6B10BR																																			X

Table 10: Percentage homologies between anti-IFN-gamma VHHs of the invention

Group	Animals	Description	Schedule
1	8	negative control 1 ip	daily 100 µl PBS i.p. +
2	8	negative control 2 rectal	every other day 100 µl PBS rectal for 2 weeks
3	8	negative control 3 intragastric	daily 100 µl PBS intragastric for 14 consecutive days
4	8	positive control 1 dexamethasone	5 µg i.p. for 7 consecutive days
5	8	positive control 2 IL10 expressing l.lactis	applied orally once per day for 14 consecutive days
6	8	bivalent VHH 3F intra-gastric	daily 100 µg bivalent VHH $3F_2$ intragastric on 14 consecutive days
7	8	bivalent VHH 3F i.p.	daily 100 µg bivalent VHH 3F i.p. for 14 consecutive days
8	8	bivalent VHH 3F rectally	100 µg bivalent VHH 3F rectally in 100 µl PBS every other day for two weeks

Table 11: Treatment schedule

CLAIMS

1. An anti-TNF-alpha polypeptide comprising at least one anti-TNF-alpha single domain antibody.

2. An anti-TNF-alpha polypeptide according to claim 1 wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 16 and 79 to 84.

3. An anti-TNF-alpha polypeptide according to claims 1 and 2 further comprising at least one single domain antibody directed against a serum protein.

4. An anti-TNF-alpha polypeptide according to any of claims 1 to 3 wherein said serum protein is any of serum albumin, serum immunoglobulins, thyroxine-binding protein, transferring, or fibrinogen.

5. An anti-TNF-alpha polypeptide according to claims 3 and 4 wherein a single domain anti-serum protein single domain antibody correspond to a sequence represented by any of SEQ ID NOs: 26 to 29 and 85 to 97.

6. An anti-TNF-alpha polypeptide according to any of claims 3 to 5 corresponding to a sequence represented by any of SEQ ID NOs: 30 to 43.

7. An anti-TNF-alpha polypeptide according to any of claims 1 to 6 further comprising at least one single domain antibody selected from the group consisting of anti-IFN-gamma single domain antibody, anti-TNF-alpha receptor single domain antibody and anti-IFN-gamma receptor single domain antibody.

8. An anti-TNF-alpha polypeptide according to any of claims 1 and 7, wherein the number of single domain antibodies directed against TNF-alpha is at least two.

9. An anti-TNF-alpha polypeptide according to claim 8 corresponding to a sequence represented by any of SEQ ID NOs: 73 to 76.

10. An anti-TNF-alpha polypeptide according any of claims 1 to 9, wherein at least one single domain antibody is a humanized *Camelidae* VHHs.

11. An anti-TNF-alpha polypeptide according to claim 10 wherein a humanized *Camelidae* VHH corresponds to a sequence represented by any of SEQ ID NOs: 17 to 19 and 21 to 24.

12. A composition comprising an anti-TNF-alpha polypeptide according to any of claims 1 to 11 and at least one single domain antibody from the group consisting of anti-IFN-gamma single domain antibody, anti-TNF-alpha receptor single domain antibody and anti-IFN-gamma receptor single domain antibody, for simultaneous, separate or sequential administration to a subject.

13. An anti-TNF-alpha polypeptide according to any of claims 7 to 11, or a composition according to claim 12 wherein at least one anti-IFN-gamma single domain antibody correspond to a sequence represented by any of SEQ ID NOs: 44 to 72.

14. An anti-TNF-alpha polypeptide according to any of claims 1 to 11, and 13, or a composition according to claims 12 and 13, wherein said single domain antibody is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length single domain antibody.

15. An anti-TNF-alpha polypeptide according to any of claims 1 to 11 and 13 and 14, or a composition according to claims 12 to 14, wherein the anti-TNF-alpha polypeptide is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length anti-TNF-alpha polypeptide.

16. An anti-TNF-alpha polypeptide according to any of claim 1 to 11, and 13 to 15, or a composition according to claims 12 to 15 wherein at least one single domain antibody is a *Camelidae* VHH.

17. A nucleic acid encoding an anti-TNF-alpha polypeptide according to any of claims 1 to 16.

18. A method of identifying an agent that modulates the binding of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16, to Tumor Necrosis Factor-alpha comprising the steps of:

(a) contacting an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 with a target that is Tumor Necrosis Factor alpha, in the presence and absence of

a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 and Tumor Necrosis Factor-alpha.

19. A method of identifying an agent that modulates Tumor Necrosis Factor-alpha-mediated disorders through the binding of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 to Tumor Necrosis Factor-alpha comprising:

(a) contacting an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 with a target that is Tumor Necrosis Factor alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified, said candidate modulator as an agent that modulates Tumor Necrosis Factor alpha-mediated disorders.

20. A method of identifying an agent that modulates the binding of Tumor Necrosis Factor alpha to its receptor through the binding of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 to Tumor Necrosis Factor-alpha comprising:

(a) contacting an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 with a target that is Tumor Necrosis Factor-alpha, in the presence and absence of a candidate modulator under conditions permitting binding between said polypeptide and target, and

(b) measuring the binding between the polypeptide and target of step (a), wherein a decrease in binding in the presence of said candidate modulator, relative to the binding in the absence of said candidate modulator identified said candidate modulator as an agent that modulates the binding of Tumor Necrosis Factor-alpha to its receptor.

21. A kit for screening for agents that modulate Tumor Necrosis Factor-alpha-mediated disorders comprising an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 and Tumor Necrosis Factor-alpha.

22. An unknown agent that modulates the binding of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 to Tumor Necrosis Factor-alpha, identified according to the method of claim 18.

23. An unknown agent that modulates Tumor Necrosis Factor-alpha-mediated disorders, identified according to the methods of claims 19 and 20.

24. An unknown agent according to claim 23 wherein said disorders are one or more of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome and multiple sclerosis.

25. An anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16, or a nucleic acid according to claim 17, or a composition according to any of claims 12 to 16, or an agent according to any of claims 22 to 24 for treating and/or preventing and/or alleviating disorders relating to inflammatory processes.

26. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a nucleic acid according to claim 17, or a composition according to any of claims 12 to 16, or an agent according to any of claims 20 to 21 for the preparation of a medicament for treating and/or preventing and/or alleviating disorders relating to inflammatory reactions.

27. An anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the gastric environment without the substance being inactivated.

28. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the gastric environment without the substance being inactivated.

29. An anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the vaginal and/or rectal tract.

30. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the vaginal and/or rectal tract.

31. An anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the nose, upper respiratory tract and/or lung.

32. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the nose, upper respiratory tract and/or lung.

33. An anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

34. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

35. An anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for treating and/or preventing and/or alleviating

disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the tissues beneath the tongue effectively.

36. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the tissues beneath the tongue effectively.

37. An anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for treating and/or preventing and/or alleviating disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the skin effectively.

38. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 or a composition according to any of claims 12 to 16, for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a TNF-alpha modulating substance which is able pass through the skin effectively.

39. A method according to claim 19, a kit according to claim 21, a nucleic acid or agent according to claim 25, use of a nucleic acid or agent according to claim 26, a composition according to any of claims 25, 27, 29, 31, 33, 35, 37 and 39, use of a composition according to any of claims 26, 28, 30, 32, 34, 36, and 38, an anti-TNF-alpha polypeptide of any of claims 25, 27, 29, 31, 33, 35, 37 and 39, use of an anti-TNF-alpha polypeptide according to any of claims 26, 28, 30, 32, 34, 36, and 38 wherein said disorders are any of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, multiple sclerosis, Addison's disease, Autoimmune hepatitis, Autoimmune parotitis, Diabetes Type I, Epididymitis, Glomerulonephritis, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, Hemolytic anemia, Systemic lupus erythematosus, Male infertility, Multiple sclerosis, Myasthenia Gravis, Pemphigus, Psoriasis, Rheumatic fever, Rheumatoid arthritis, Sarcoidosis, Scleroderma, Sjogren's syndrome, Spondyloarthropathies, Thyroiditis, and Vasculitis.

40. A composition comprising a nucleic acid or agent according to claim 25, an anti-TNF-alpha polypeptide of any of claims 1 to 11 and 13 to 16, or a composition according to any of claims 12 to 16, and a suitable pharmaceutical vehicle.

41. A method of diagnosing a disorder characterised by the dysfunction of Tumor Necrosis Factor-alpha comprising:

(a) contacting a sample with an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of Tumor Necrosis Factor-alpha.

42. A kit for screening for a disorder cited in claim 39, using a method according to claim 38.

43. A kit for screening for a disorder cited in claim 39 comprising an isolated anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16.

44. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 for the purification of said Tumor Necrosis Factor-alpha.

45. Use of an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 for inhibiting the interaction between Tumor Necrosis Factor-alpha and one or more Tumor Necrosis Factor-alpha receptors.

46. A method for producing an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 comprising the steps of:

(a) obtaining double stranded DNA encoding a *Camelidae* VHH directed to Tumor Necrosis Factor alpha,

(b) cloning and expressing the DNA selected in step (b).

47. A method of producing an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16 comprising:

(a) culturing host cells comprising nucleic acid capable of encoding an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16, under conditions allowing the expression of the polypeptide, and,

(b) recovering the produced polypeptide from the culture.

48. A method according to claim 47, wherein said host cells are bacterial or yeast.

49. A kit for screening for any of inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome or multiple sclerosis comprising an anti-TNF-alpha polypeptide of any of claims 1 to 11, and 13 to 16.

```
                    FR1                          CDR1          FR2
VHH#3G      QVQLQDSGGGLVQAGGSLRLSCAVSGR TFSAHS--VYTMG WFRQAPGKEREFVA
VHH#3E      QVQLQESGGGLVQPGGSLRLSCAASGR TFSDHSGYTYTIG WFRQAPGKEREFVA
VHH#1A      QVQLQESGGGLVQPGGSLRLSCATSGF DFS--VSWMY--- WVRQAPGKGLEWVS
VHH#2B      QVQLQESGGGLVQPGGSLRLSCATSGF TFS--DYWMY--- WVRQAPGKGLEWVS
VHH#12B     QVQLQESGGGLVQPGGSLRLSCAASGF EFE--NHWMY--- WVRQAPGKGLEWVS
VHH#7B      QVQLQESGGGLVQPGGSLRLSCAASGS IFRVNA-----MG WYRQVPGNQREFVA
            ***** ******* ********* **   *            * ** **   * *

                 CDR2                          FR2
VHH#3G      RIYWSSANTYYADSVKG RFTISRDNAKNTVDLLMNSLKPEDTAVYYCAA
VHH#3E      RIYWSSGNTYYADSVKG RFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA
VHH#1A      EINTNGLITKYVDSVKG RFTISRDNAKNTLYLQMDSLIPEDTALYYCAR
VHH#2B      TVNTNGLITRYADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTK
VHH#12B     TVNTNGLITRYADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTK
VHH#7B      -IITSGDNLNYADAVKG RFTISTDNVKKTVYLQMNVLKPEDTAVYYCNA
                  * * *** ** ** *  * *  * * *  *  **** ***

                 CDR3            FR4       Hinge
VHH#3G      RDGIPTSRTVGSYNY WGQGTQVTVSS EPKTPKPQP
VHH#3E      RDGIPTSRSVESYNY WGQGTQVTVSS EPKTPKPQP
VHH#1A      ---------SPSGSF RGQGTQVTVSS EPKTPKPQP
VHH#2B      -VVPPYSDDSRTNAD WGQGTQVTVSS EPKTPKPQP
VHH#12B     -VLPPYSDDSRTNAD WGQGTQVTVSS EPKTPKPQP
VHH#7B      --ILQTSRWSIPSNY WGQGTQVTVSS EPKTPKPQP
                            ********** *********
```

Figure 1

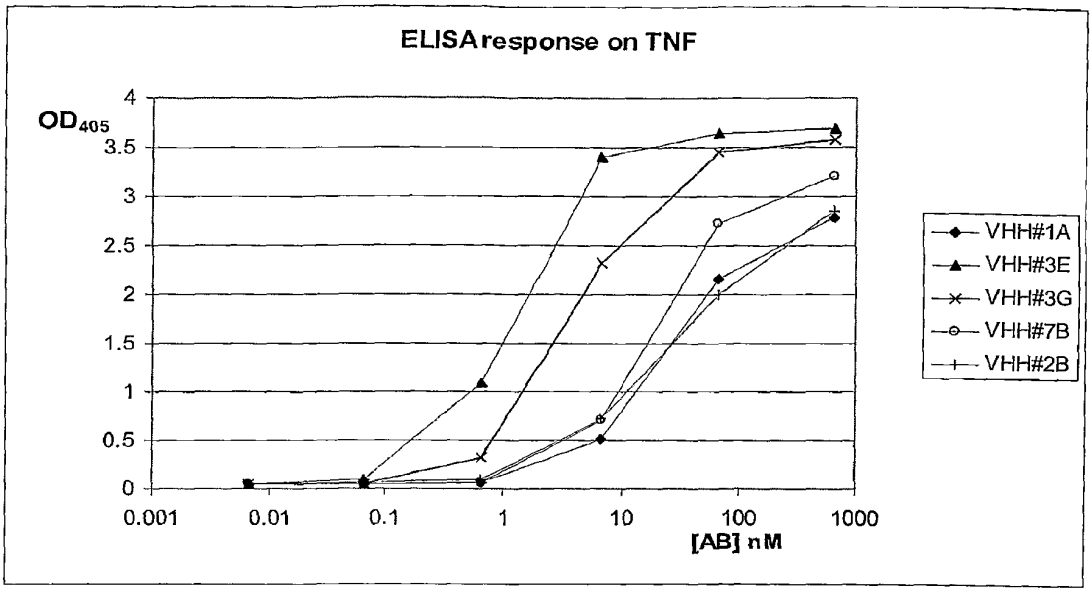


Figure 2

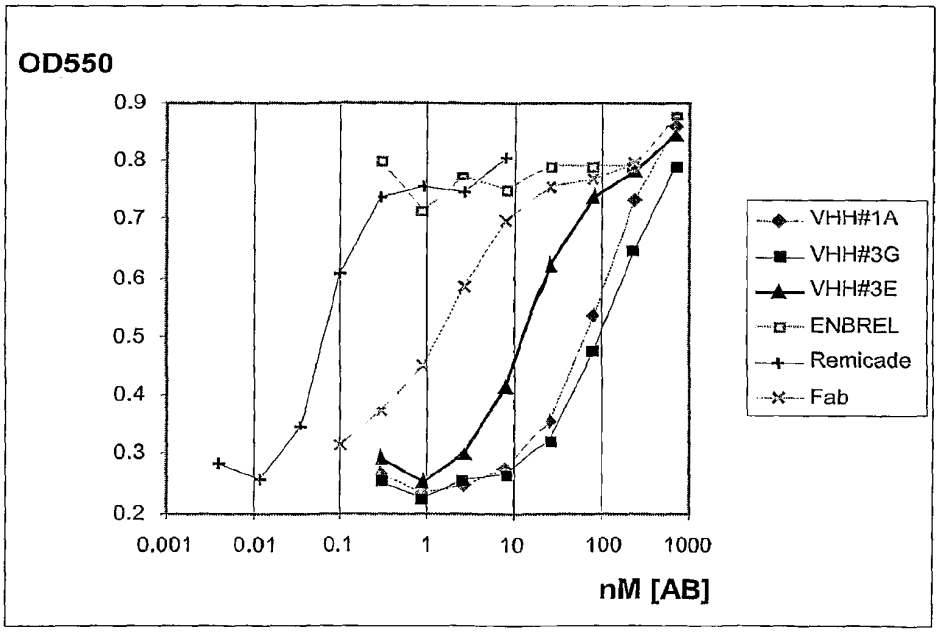


Figure 3

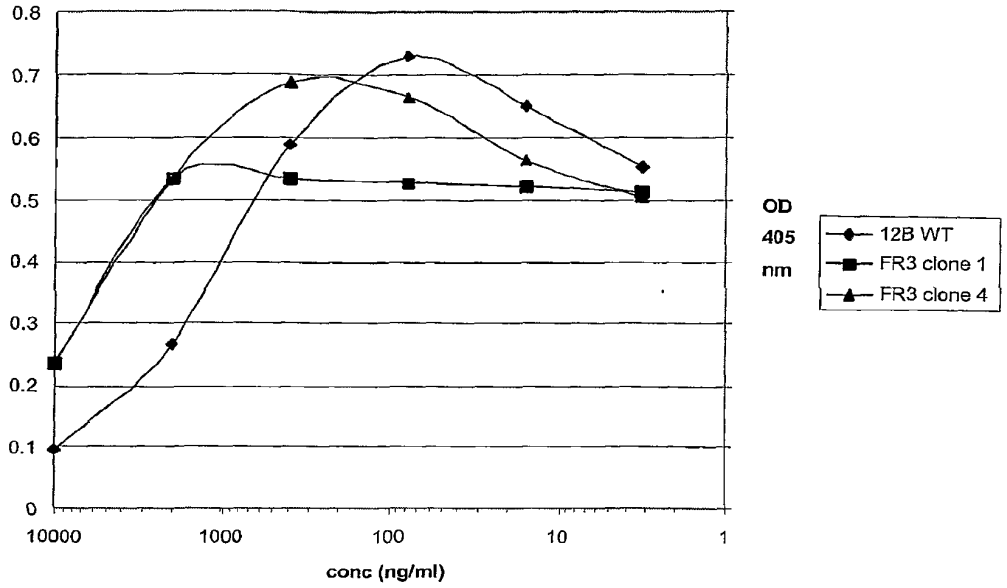


Figure 4

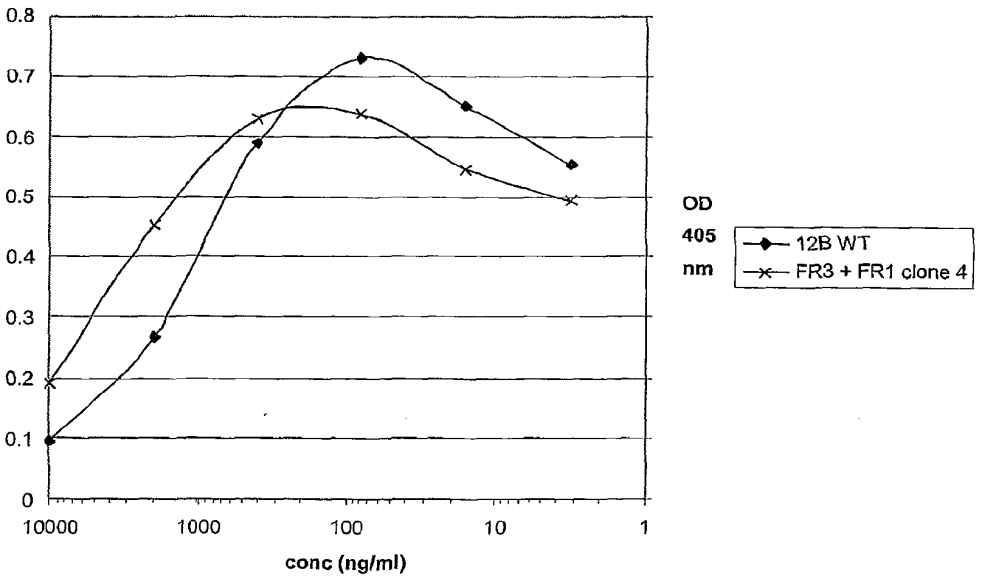


Figure 5

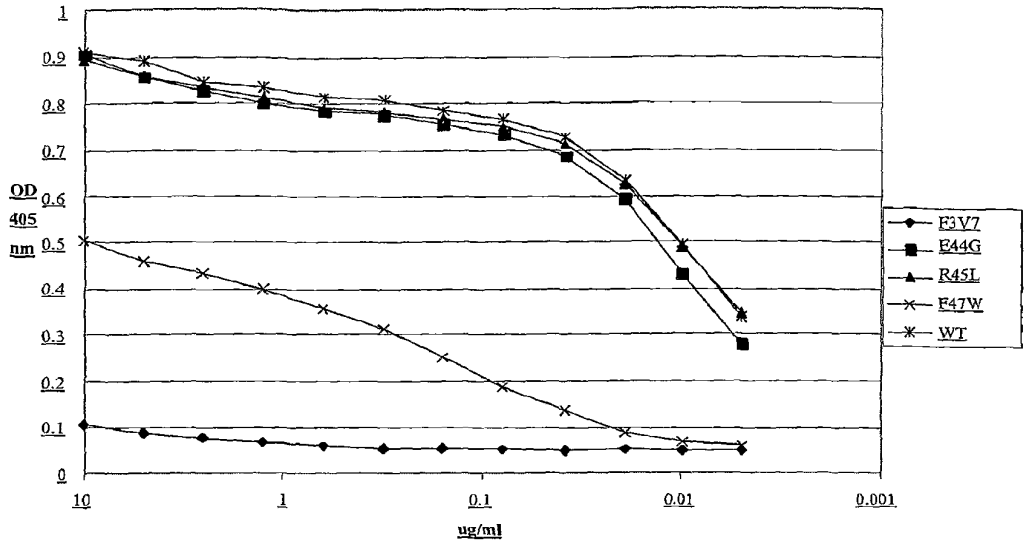


Figure 6

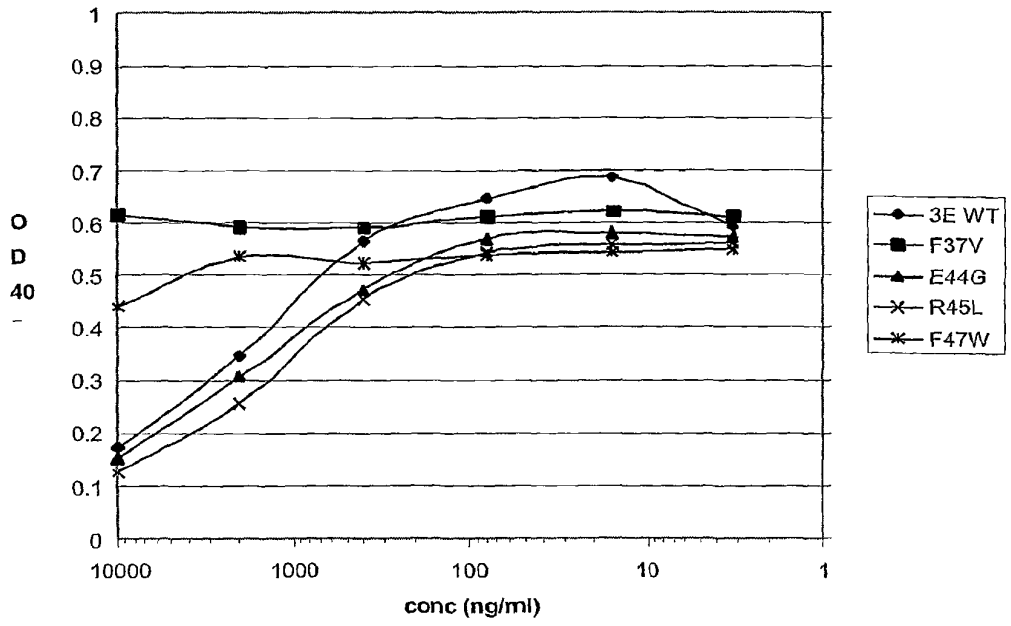


Figure 7

```
                          FR1                                CDR1     FR2
VHH#m3F     QVQLQDSGGGLVQAGGSLRLSCAASGG TFSSIIMA WFRQAPGKEREFVGA
VHH#m4B     QVQLQDSGGGLVQAGGSLRLSCGVSGL SFSGYTMG WFRQAPGKEREFAAA
VHH#m9A     EVQLVESGGGLVQAGGSLRLSCAASGG TLSSYITG WFRQAPGKEREFVGA
VHH#m9E     QVQLVESGGGLVQAGGSLRLSCAASEG TLSGYILG WFRQAPGKEREFVGA
             ***  ****************  *     *       ************  *

                CDR2                              FR2
VHH#m3F     VSWSGGTTVYADSVLG RFEISRDSARKSVYLQMNSLKPEDTAVYYCAA
VHH#m4B     IGWNSGTTEYRNSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA
VHH#m9A     VSWSSSTIVYADSVEG RFTISRDNHQNTVYLQMDSLKPEDTAVYYCAA
VHH#m9E     VSWSGGTIVYADSVKG RFEISRDNARNTVYLQMDSLKSEDTAVYYCAA
              *   *  *  ** * ** ****     ***** *** **********

                CDR3             FR4       Hinge
VHH#m3F     RPYQKYNWA-SASYNV WGQGTQVTVSS EPKTPKPQP
VHH#m4B     SP--KYMTAYERSYDF WGQGTQVTVSS EPKTPKPQP
VHH#m9A     RPYQKYNWA-SASYNV WGQGTQVTVSS ---------
VHH#m9E     RPYQRFNWA-SASYNV WGRGTQVTVSS ---------
             *    * *   **   ** ********
```

Figure 8

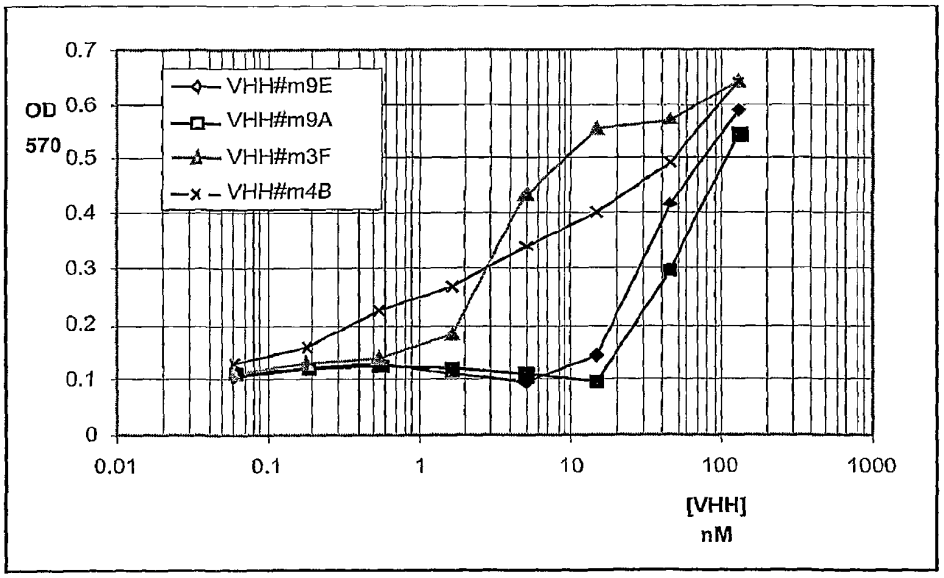


Figure 9


```
    HindIII
  1 aagcttgcat gcaaattcta tttcaaggag acagtcataa tgaaatacct attgcctacg gcagccgctg gattgttatt
                                                M  K  Y   L  L  P  T   A  A  A   G  L  L  L
                                                     <                  pelB-leader

                  SfiI    NcoI                NotI                PstI
 81 actcgcggcc cagccggcca tggggcctaa taggcggccg cacaggtgca gctgcaggag tcataatgag ggacccaggt
      L  A  A   Q  P  A   M  G  P -   - A  A   A  Q  V   Q  L  Q  E   S  -  -   G  T  Q  V
            Leader            ><     VHH#1 >        <                          VHH#2

    BstEII
161 caccgtctcc tcagaacaaa aactcatctc agaagaggat ctgaatgggg ccgcacatca tcatcatcat cattaatgag
      T  V  S   S  E  Q   K  L  I   S  E  E  D   L  N  G   A  A  H   H  H  H  H  H   -  -
               ><       C-MYC                      >           <      His6          >

    EcoRI
241 aattcactgg ccg
```

Figure 10

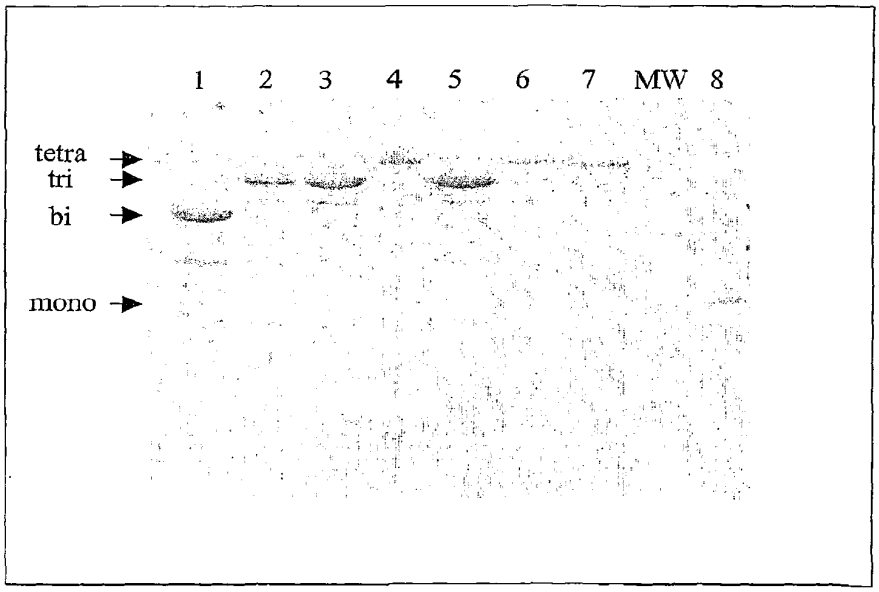


Figure 11

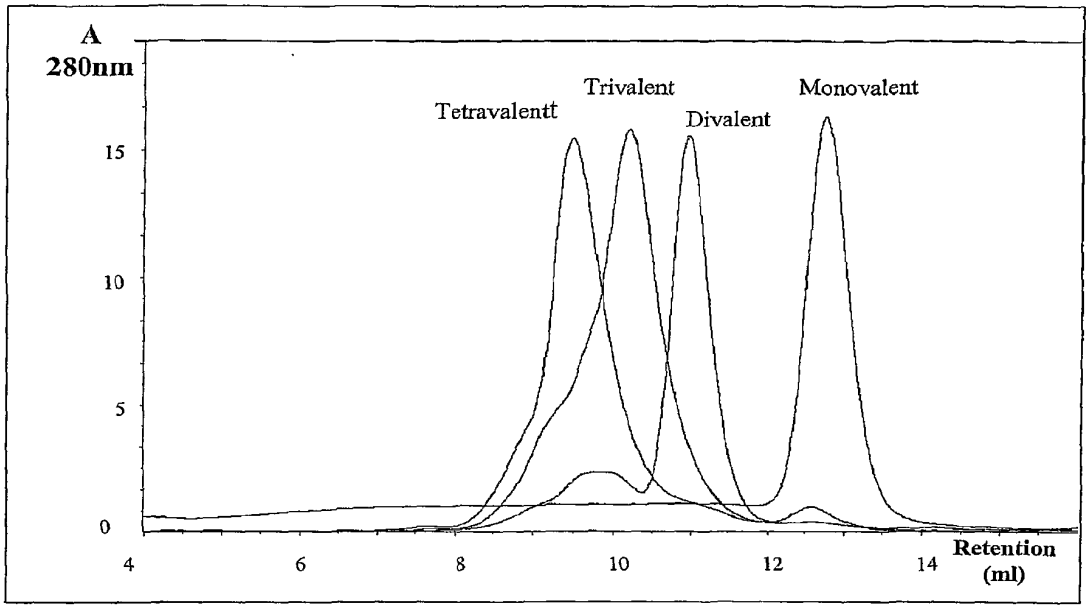


Figure 12

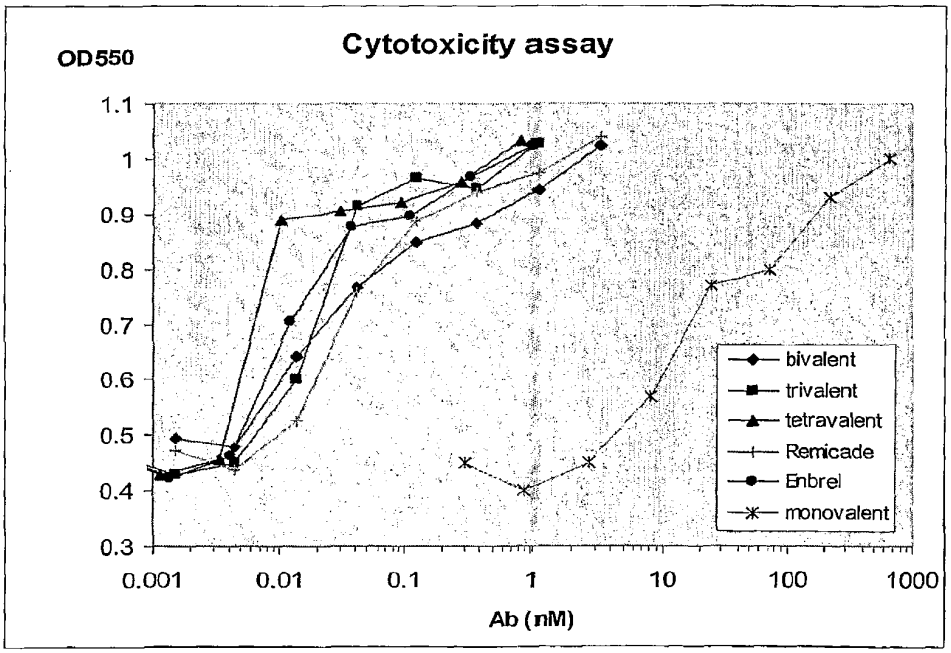


Figure 13

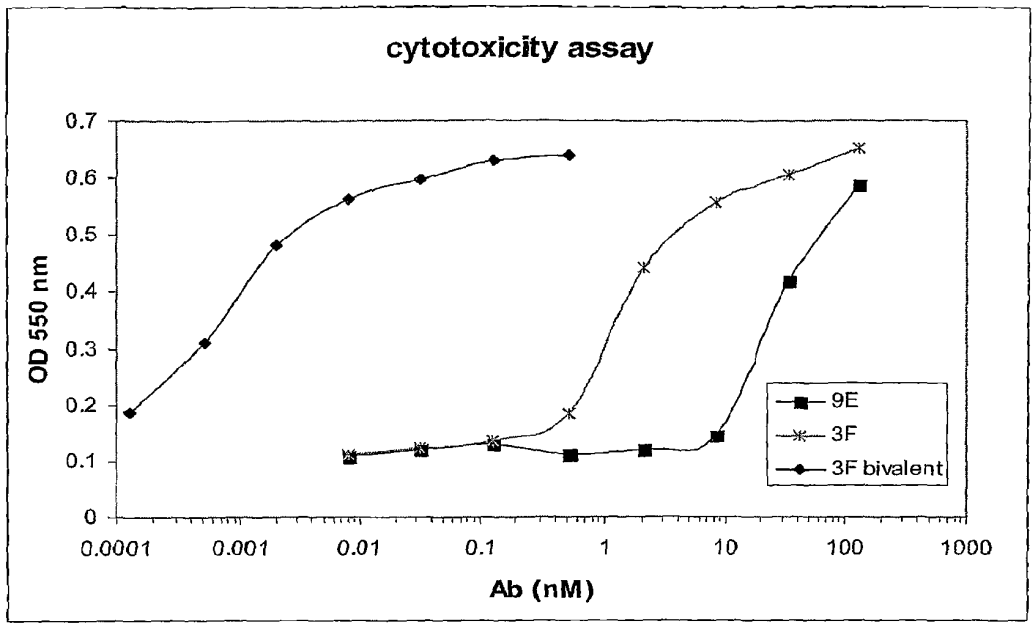


Figure 14

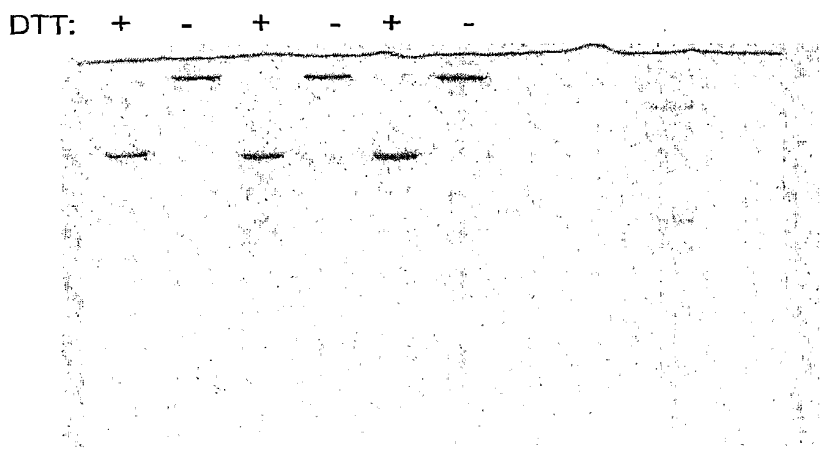


Figure 15

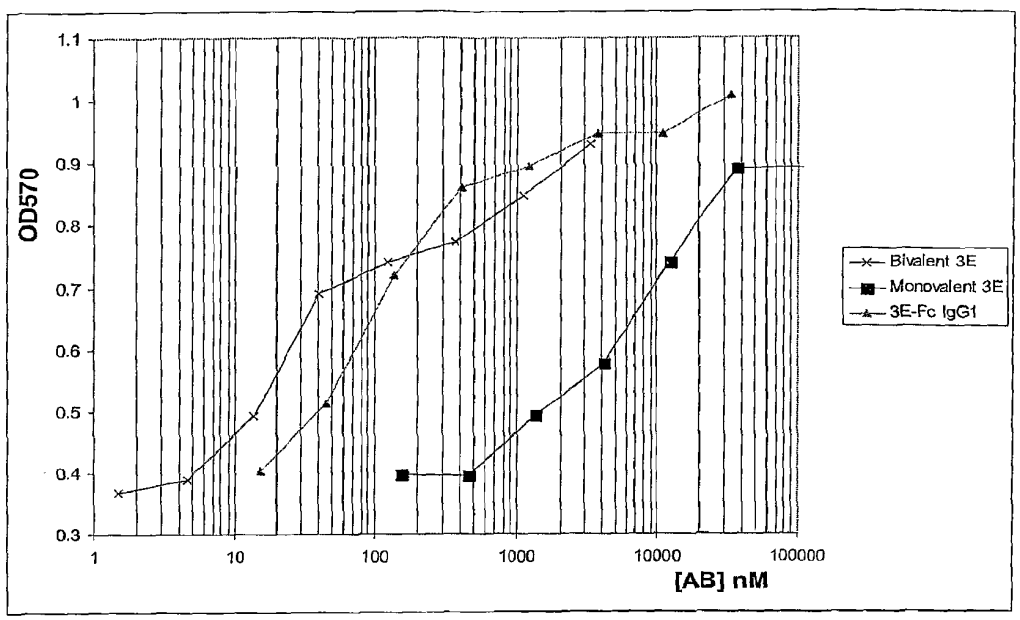


Figure 16

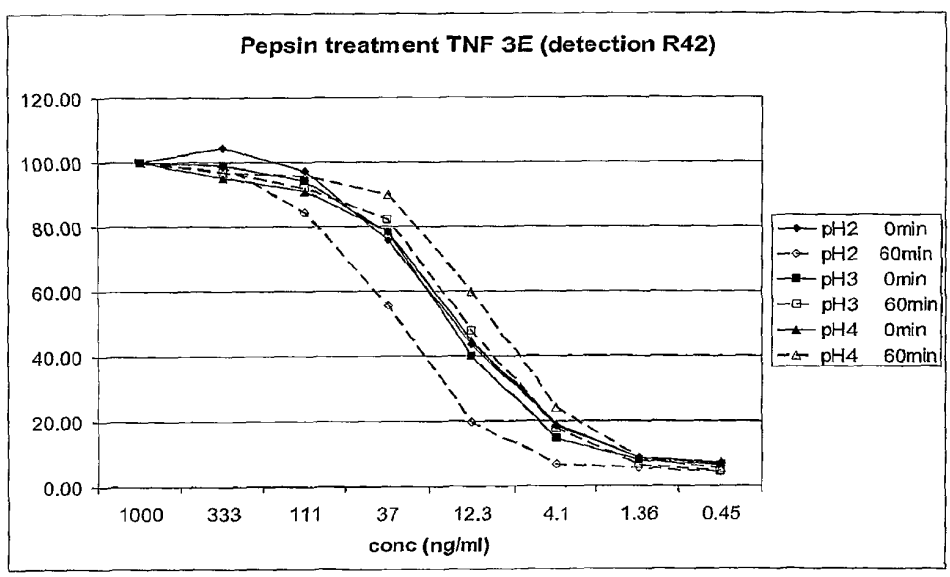


Figure 17

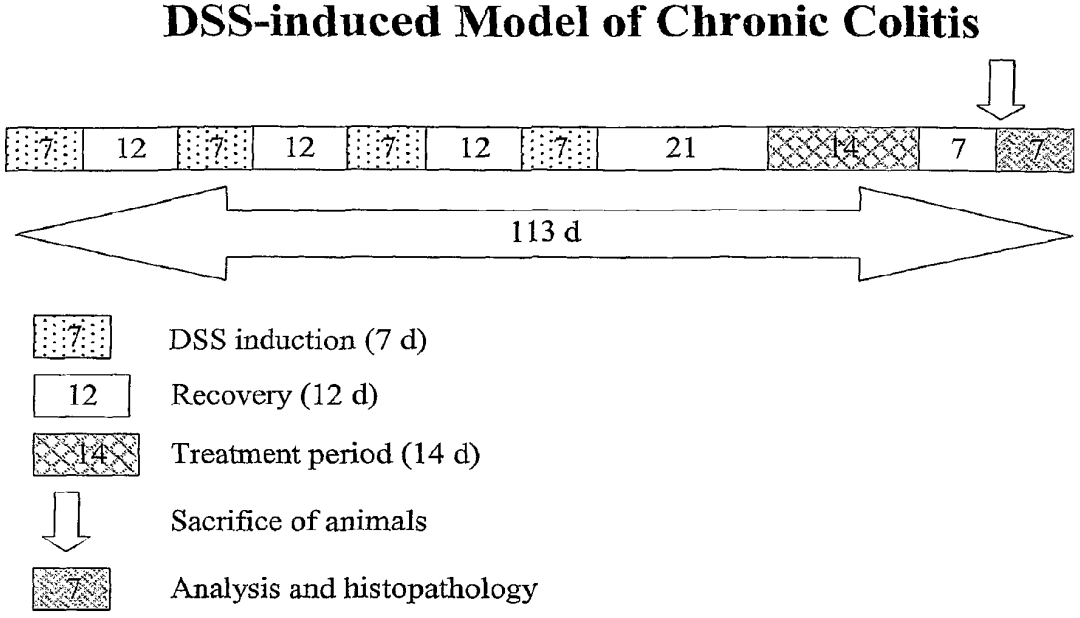

DSS-induced Model of Chronic Colitis
7
12
7
12
7
12
7
21
14
7
7
113 d
7
DSS induction (7 d)
12
Recovery (12 d)
14
Treatment period (14 d)
Sacrifice of animals
7
Analysis and histopathology

Figure 18

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
01 September 2022 (01.09.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/183072 A1

(51) International Patent Classification:
C07K 14/145 (2006.01) *C12N 15/867* (2006.01)

(21) International Application Number:
PCT/US2022/018027

(22) International Filing Date:
25 February 2022 (25.02.2022)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/154,639 26 February 2021 (26.02.2021) US

(71) Applicant: **KELONIA THERAPEUTICS, INC.** [US/US]; 5 Channel Center, Suite 501, Boston, MA 02210 (US).

(72) Inventors: **PERKINS, Molly R.**; 64 Bradlee Rd., Milton, Massachusetts 02186 (US). **FRIEDMAN, Kevin M.**; 4 Clover Circle, Melrose, Massachusetts 02176 (US).

(74) Agent: **SUN, Eileen, S.** et al.; Seed Intellectual Property Law Group LLP, Suite 5400, 701 Fifth Avenue, Seattle, Washington 98104-7064 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2022/183072 A1

(54) Title: LYMPHOCYTE TARGETED LENTIVIRAL VECTORS

(57) Abstract: Provided herein are lentiviral vectors comprising a mutated, heterologous envelope protein, a targeting protein, and at least one transgene for delivery to and expression by a cell characterized by the targeting protein. Also provided are methods and materials for producing the lentiviral vectors described herein, methods for transducing target cells, and cells transduced by lentiviral vectors according to the present disclosure.

LYMPHOCYTE TARGETED LENTIVIRAL VECTORS

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 930207_401WO_SEQUENCE_LISTING.txt. The text file is 158 KB, was created on February 25, 2022, and is being submitted electronically via EFS-Web.

BACKGROUND

Lentiviral vectors play a critical role in gene-modified cell therapies, particularly T cell therapies. Recently approved T cell therapies rely on retroviral vectors to transduce the therapeutic molecule (e.g., chimeric antigen receptor (CAR)) into T lymphocytes. An associated risk to CAR T cell production is the transduction of other cell types with the transgene. The use of integrating vectors with broad cell tropism, e.g., lentiviral vectors pseudotyped with a VSV-G envelope protein, can represent a serious, though rare, safety concern.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 is a schematic representation of helper plasmids suitable for use in a third generation LVV production system.

FIGS. 2A-2B depict graphs (FIG. 2A) and FACS plots (FIG. 2B) showing on-target and off-target entry of Jurkat T cells and Raji B cells by lentiviral vectors bearing mutated VSV-G envelope to abolish LDL receptor binding (Trop-002, Trop-051, Trop-052, Trop-055, and Trop-061) and T cell targeting protein CD80. In FIG. 2A, on-target entry is the left bar and off-target is the right bar of each sample. Binding of the T cell targeting protein to its cognate ligand on T cells leads to entry of the lentiviral vector, and subsequent expression of reporter green fluorescent protein (GFP) is measured.

FIGS. 3A-3B depicts graphs showing: (FIG. 3A) T cell targeting protein CD80 expressed from the VSV-G packaging plasmid is expressed at relatively equivalent levels as the mutated VSV-G on the surface of HEK293 producer cells; and (FIG. 3B) LVV generated with this approach can transduce targeted Jurkat T cells but do not transduce Raji B cells.

FIG. 4 depicts graphs showing: (top row) expression levels of CD80 and mutated VSV-G on the surface of HEK293T producer cells using LVV generated by cloning CD80 targeting protein into the Rev packaging plasmid or into the mutated VSV-G packaging plasmid; and (bottom) LVV generated by cloning CD80 targeting protein into the Rev packaging plasmid or into the mutated VSV-G packaging plasmid transduce targeted Jurkat T cells.

FIG. 5 depicts (left) CD80 targeting protein expression on surface of HEK293T producer cells using a five plasmid packaging system as a function of CD80 plasmid concentration; and (right) LVV generated with the five plasmid packaging system transduce targeted Jurkat T cells and transduction efficiency was associated with CD80 packaging plasmid concentration.

FIGS. 6A-6D depict titers of lymphocyte targeting LVV produced in adherent HEK293 or suspension HEK293 producer cells. LVV harvested from HEK293 adherent cell culture medium by centrifugation (FIG. 6A) or by anion exchange chromatography followed by tangential flow filtration (FIG. 6B). LVV harvested from HEK293 suspension cell culture medium by anion exchange chromatography (FIG. 6C). Concentration by AEX/TFF resulted in LVV preparations with a high level of purity and recovery (FIG. 6D).

FIG. 7 depicts a schematic for testing T cell transduction in PBMCs from healthy human donors with LVV comprising a BCMA CAR transgene or T cell targeting LVV (anti-CD3 and CD80) comprising a BCMA CAR trasngene. Graphs shown on lower right indicate that even at low MOI, the T cell targeting LVV transduced T cells at a higher level than standard LVV and that T cell targeting LVV is capable of transducing T cells without the presence of IL-2 and exogenous activating antibodies (anti-CD3 and anti-CD28) in contrast to standard LVV.

FIG. 8 depicts graphs showing T cell expansion from PBMCs obtained from three different donors and transduced using standard LVV or T cell redirected LVV (anti-CD3 and CD80) in the presence or absence of exogenous activating anti-CD3 and anti-CD28 antibodies

FIG. 9 depcits a schematic for testing T cell transduction in PBMCs from healthy human donors with LVV comprising a CD19 CAR transgene or T cell targeting LVV (anti-CD3 and CD80) comprising a CD19 CAR trasngene. Graphs shown on lower right show that the T cell targeting LVV transduced T cells at a higher level than standard LVV and that T cell targeting LVV is capable of transducing T cells without the presence of exogenous activating antibodies (anti-CD3 and anti-CD28) in contrast to standard LVV.

FIG. 10 depicts graphs showing levels of T cell transduction efficiency and T cell activation with anti-CD3 targeting proteins (12F6 in VH-VL orientation and VL-VH orientation) used to generate T cell targeting LVV.

FIG. 11 depicts graphs showing that BCMA CAR T cells exhibited increased expression of T cell effector cytokines (TNFα – left; TNFα and IFNγ – right) after culture with BCMA-positive cell lines that is not observed with BCMA-negative cell lines whether generated by standard LVV or T cell-redirected LVV (anti-CD3 and CD80).

FIGS. 12A-12B depict graphs showing *in vivo* delivery of transgene using T cell targeting LVV: (FIG. 12A) T cell targeting LVV (anti-CD3 and CD80) specificly transduce human T cells (CD3+) and not human B cells (CD20+) in humanized mouse model (n=5); and (FIG. 12B) T cell targeting LVV transduce both CD8+ and CD8- (CD4) T cells compared to standard LVV which did not.

FIG. 13 depicts graphs showing that CD80 targeting LVVs enhance transduction of CD4 T cells compared to standard LVVs.

FIG. 14 depicts graphs showing that T cell targeting (anti-CD3 and CD80) LVVs transduce target Jurkat T cells but do not transduce off-target tumor cells (Raji, Ramos, Jeko-1, and NALM-6) compared to standard LVV.

DETAILED DESCRIPTION

Engineered lentiviral vectors are described herein. The lentiviral vectors include a mutated, heterologous envelope protein, a targeting protein, and at least one transgene for delivery to and expression by a cell characterized by the targeting protein. In some embodiments, the targeting protein is selected to target an immune cell, including, for example a lymphocyte or a T cell. In certain such embodiments, the lentiviral vectors described herein are capable of selectively targeting and efficiently transducing resting lymphocytes, e.g., T cells. In some embodiments, lentiviral vectors described herein are capable of transducing and/or activating T cells in the absence of an exogenous T cell stimulating agent. In some embodiments, lentiviral vectors described herein enhance transduction of CD4 T cells compared to standard lentiviral vectors.

In some embodiments, the lentiviral vectors incorporating a mutated env and a targeting protein as described herein are capable of producing a high titer LVV product, as compared to standard LVV incorporating another fusogenic env protein (e.g., cocal env, paramyxovirus env, truncated VSV-G env).

Also provided are methods and materials for producing the lentiviral vectors described herein, methods for transducing target cells, and cells transduced by lentiviral vectors according to the present disclosure. In some embodiments, a lentiviral vector as described herein and/or cells transduced by such a vector may be used in treating a disease or disorder responsive to the presence of cells expressing the transgene delivered by the vector.

Definitions

Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms to be used herein.

In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. Also, any number range recited

herein relating to any physical feature, such as polymer subunits, size or thickness, are to be understood to include any integer within the recited range, unless otherwise indicated. As used herein, the term "about" means ± 20% of the indicated range, value, or structure, unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components. The use of the alternative (*e.g.*, "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the terms "include," "have" and "comprise" are used synonymously, which terms and variants thereof are intended to be construed as non-limiting.

Terms understood by those in the art of antibody technology are each given the meaning acquired in the art, unless expressly defined differently herein. The term "antibody" is used in the broadest sense and includes polyclonal and monoclonal antibodies. An "antibody" may refer to an intact antibody comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as an antigen-binding portion (or antigen-binding domain) of an intact antibody that has or retains the capacity to bind a target molecule. An antibody may be naturally occurring, recombinantly produced, genetically engineered, or modified forms of immunoglobulins, for example intrabodies, peptibodies, nanobodies, single domain antibodies, SMIPs, multispecific antibodies (*e.g.*, bispecific antibodies, diabodies, triabodies, tetrabodies, tandem di-scFv, tandem tri-scFv, ADAPTIR). A monoclonal antibody or antigen-binding portion thereof may be non-human, chimeric, humanized, or human, preferably humanized or human. Immunoglobulin structure and function are reviewed, for example, in Harlow *et al.*, Eds., Antibodies: A Laboratory Manual, Chapter 14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988). "Antigen-binding portion" or "antigen-binding domain" of an intact antibody is meant to encompass an "antibody fragment," which indicates a portion of an intact antibody and refers to the antigenic determining variable regions or complementary determining regions of an intact antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab′, $F(ab')_2$, and Fv fragments, Fab'-SH, $F(ab')_2$, diabodies, linear antibodies, scFv antibodies, VH, and multispecific antibodies formed from antibody fragments. A "Fab" (fragment antigen binding) is a portion of an antibody that binds to antigens and includes the variable region and CH1 of the heavy chain linked to the light

chain via an inter-chain disulfide bond. An antibody may be of any class or subclass, including IgG and subclasses thereof (IgG_1, IgG_2, IgG_3, IgG_4), IgM, IgE, IgA, and IgD.

The term "variable region" or "variable domain" in the context of an antibody refers to the domain of an antibody heavy or light chain that is involved in binding of the antibody to antigen. The variable domains (or regions) of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three complementary determining regions (CDRs). (*See, e.g.*, Kindt et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007)). A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. *See, e.g.*, Portolano et al., J. Immunol. 150:880-887 (1993); Clarkson et al., Nature 352:624-628 (1991).

The terms "complementarity determining region" and "CDR," which are synonymous with "hypervariable region" or "HVR," are known in the art to refer to non-contiguous sequences of amino acids within antibody variable regions, which confer antigen specificity and/or binding affinity. In general, there are three CDRs in each heavy chain variable region (HCDR1, HCDR2, HCDR3) and three CDRs in each light chain variable region (LCDR1, LCDR2, LCDR3).

As used herein, the terms “binding domain”, “binding region”, and “binding moiety" refer to a molecule, such as a peptide, oligopeptide, polypeptide, or protein that possesses the ability to specifically and non-covalently bind, associate, unite, recognize, or combine with a target molecule (*e.g.*, tumor antigen). A binding domain includes any naturally occurring, synthetic, semi-synthetic, or recombinantly produced binding partner for a biological molecule or other target of interest. In some embodiments, the binding domain is an antigen-binding domain, such as an antibody or functional binding domain or antigen-binding portion thereof. Exemplary binding domains include single chain antibody variable regions (*e.g.*, domain antibodies, sFv, scFv, Fab), receptor ectodomains (*e.g.*, TNF-α), ligands (*e.g.*, cytokines, chemokines), or synthetic polypeptides selected for the specific ability to bind to a biological molecule.

"Major histocompatibility complex molecule" (MHC molecule) refers to a glycoprotein that delivers a peptide antigen to a cell surface. MHC class I molecules

are heterodimers composed of a membrane spanning α chain (with three α domains) and a non-covalently associated β2 microglobulin. MHC class II molecules are composed of two transmembrane glycoproteins, α and β, both of which span the membrane. Each chain has two domains. MHC class I molecules deliver peptides originating in the cytosol to the cell surface, where peptide:MHC complex is recognized by $CD8^+$ T cells. MHC class II molecules deliver peptides originating in the vesicular system to the cell surface, where they are recognized by $CD4^+$ T cells. An MHC molecule may be from various animal species, including human, mouse, rat, or other mammals.

"Chimeric antigen receptor" (CAR) refers to a chimeric fusion protein comprising two or more distinct domains linked together in a way that does not occur naturally in a host cell and can function as a receptor when expressed on the surface of a cell. CARs are generally composed of an extracellular domain comprising a binding domain that binds a target antigen, an optional extracellular spacer domain, a transmembrane domain, and an intracellular signaling domain (*e.g.*, comprising an immunoreceptor tyrosine-based activation motif (ITAM)), and optionally an intracellular costimulatory domain). In certain embodiments, an intracellular signaling domain of a CAR has an ITAM (*e.g.*, CD3ζ) containing intracellular signaling domain and an intracellular costimulatory domain (*e.g.*, 4-1BB). In certain embodiments, a CAR is synthesized as a single polypeptide chain or is encoded by a nucleic acid molecule as a single chain polypeptide.

A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, as well as determining binding domain affinities, such as Western blot, ELISA, analytical ultracentrifugation, spectroscopy, surface plasmon resonance (BIACORE®) analysis, and MHC tetramer analysis (*see also, e.g.*, Scatchard *et al.*, *Ann. N.Y. Acad. Sci. 51*:660, 1949; Wilson, *Science 295*:2103, 2002; Wolff *et al.*, *Cancer Res. 53*:2560, 1993; Altman *et al.*, Science 274:94-96, 1996; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent). As used herein, "specifically binds" refers to an association or union of a binding domain, or a fusion protein thereof, to a target molecule with an affinity or K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of 1/M) equal to or greater than 10^5 M^{-1}, while not significantly associating or uniting with any other molecules or components in a sample.

The terms "antigen" and "Ag" refer to a molecule that is capable of inducing an immune response. The immune response that is induced may involve antibody production, the activation of specific immunologically-competent cells, or both. Macromolecules, including proteins, glycoproteins, and glycolipids, can serve as an antigen. Antigens can be derived from recombinant or genomic DNA. As contemplated herein, an antigen need not be encoded (i) solely by a full-length nucleotide sequence of a gene or (ii) by a "gene" at all. An antigen can be generated or synthesized, or an antigen can be derived from a biological sample. Such a biological sample can include, but is not limited, to a tissue sample, a tumor sample, a cell, or a biological fluid.

The term "epitope" or "antigenic epitope" includes any molecule, structure, amino acid sequence or protein determinant within an antigen that is specifically bound by a cognate immune binding molecule, such as an antibody or fragment thereof (*e.g.*, scFv), T cell receptor (TCR), CAR, or other binding molecule, domain or protein. Epitopic determinants generally contain chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics. An epitope may be a linear epitope or a conformational epitope.

As used herein, an "effector domain" is an intracellular portion of a fusion protein or chimeric receptor that can directly or indirectly promote a biological or physiological response in a cell expressing the effector domain when receiving the appropriate signal. In certain embodiments, an effector domain is part of a protein or protein complex that receives a signal when bound. In other embodiments, the effector domain is part of a protein or protein complex that binds directly to a target molecule, which triggers a signal from the effector domain. For example, in response to binding of a CAR to a target molecule, the effector domain may transduce a signal to the interior of the host cell, eliciting an effector function. An effector domain may directly promote a cellular response when it contains one or more signaling domains or motifs. In other embodiments, an effector domain will indirectly promote a cellular response by associating with one or more other proteins that directly promote a cellular response.

"Junction amino acids" or "junction amino acid residues" refer to one or more (*e.g.*, about 2-20) amino acid residues between two adjacent motifs, regions or domains of a polypeptide. Junction amino acids may result from the construct design of a

chimeric protein (*e.g.*, amino acid residues resulting from the use of a restriction enzyme site during the construction of a nucleic acid molecule encoding a fusion protein).

A "disease" is a state of health of a subject wherein the subject cannot maintain homeostasis, and wherein, if the disease is not ameliorated, then the subject's health continues to deteriorate. In contrast, a "disorder" or "undesirable condition" in a subject is a state of health in which the subject is able to maintain homeostasis, but in which the subject's state of health is less favorable than it would be in the absence of the disorder or undesirable condition. Left untreated, a disorder or undesirable condition does not necessarily result in a further decrease in the subject's state of health.

"Nucleic acid molecule" and "polynucleotide" can be in the form of RNA or DNA, which includes cDNA, genomic DNA, and synthetic DNA. A nucleic acid molecule may be composed of naturally occurring nucleotides (such as deoxyribonucleotides and ribonucleotides), analogs of naturally occurring nucleotides (*e.g.*, α-enantiomeric forms of naturally occurring nucleotides), or a combination of both. Modified nucleotides can have modifications in or replacement of sugar moieties, or pyrimidine or purine base moieties. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. A nucleic acid molecule may be double stranded or single stranded, and if single stranded, may be the coding strand or non-coding (anti-sense strand). A coding molecule may have a coding sequence identical to a coding sequence known in the art or may have a different coding sequence, which, as the result of the redundancy or degeneracy of the genetic code, or by splicing, can encode the same polypeptide.

"Encoding" refers to the inherent property of specific polynucleotide sequences, such as DNA, cDNA, and mRNA sequences, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a polynucleotide encodes a protein if transcription and translation of mRNA corresponding to that polynucleotide produces the protein in a cell or other biological system. Both a coding strand and a

non-coding strand can be referred to as encoding a protein or other product of the polynucleotide. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence.

As used herein, the term "endogenous" or "native" refers to a gene, protein, compound, molecule or activity that is normally present in a host or host cell, including naturally occurring variants of the gene, protein, compound, molecule, or activity.

As used herein, "homologous" or "homolog" refers to a molecule or activity from a host cell that is related by ancestry to a second gene or activity, *e.g.*, from the same host cell, from a different host cell, from a different organism, from a different strain, from a different species. For example, a heterologous molecule or heterologous gene encoding the molecule may be homologous to a native host cell molecule or gene that encodes the molecule, respectively, and may optionally have an altered structure, sequence, expression level or any combination thereof.

As used herein, "heterologous" nucleic acid molecule, construct or sequence refers to a nucleic acid molecule or portion of a nucleic acid molecule that is not native to a host cell, but can be homologous to a nucleic acid molecule or portion of a nucleic acid molecule from the host cell. The source of the heterologous nucleic acid molecule, construct or sequence can be from a different genus or species. In some embodiments, the heterologous nucleic acid molecules are not naturally occurring. In certain embodiments, a heterologous nucleic acid molecule is added (*i.e.*, not endogenous or native) into a host cell or host genome by, for example, conjugation, transformation, transfection, transduction, electroporation, or the like, wherein the added molecule can integrate into the host cell genome or exist as extra-chromosomal genetic material (*e.g.*, as a plasmid or other form of self-replicating vector), and can be present in multiple copies. In addition, "heterologous" refers to a non-native enzyme, protein or other activity encoded by a non-endogenous nucleic acid molecule introduced into the host cell, even if the host cell encodes a homologous protein or activity.

As used herein, the term "engineered," "recombinant," "mutant," "modified" or "non-natural" refers to an organism, microorganism, cell, nucleic acid molecule, or vector that has been modified by introduction of a heterologous nucleic acid molecule, or refers to a cell or microorganism that has been genetically engineered by human intervention—that is, modified by introduction of a heterologous nucleic acid

molecule, or refers to a cell or microorganism that has been altered such that expression of an endogenous nucleic acid molecule or gene is controlled, deregulated or constitutive, where such alterations or modifications can be introduced by genetic engineering. Human-generated genetic alterations can include, for example, modifications introducing nucleic acid molecules (which may include an expression control element, such as a promoter) encoding one or more proteins, chimeric receptors, or enzymes, or other nucleic acid molecule additions, deletions, substitutions, or other functional disruption of or addition to a cell's genetic material. Exemplary modifications include those in coding regions or functional fragments thereof heterologous or homologous polypeptides from a reference or parent molecule. Additional exemplary modifications include, for example, modifications in non-coding regulatory regions in which the modifications alter expression of a gene or operon.

As used here, the term "transgene" refers to a gene or polynucleotide encoding a protein of interest (*e.g.*, a CAR) whose expression is desired in a host cell and that has been transferred by genetic engineering techniques into a cell. A transgene may encode proteins of therapeutic interest as well as proteins that are reporters, tags, markers, suicide proteins, etc. A transgene may be from a natural source, modification of a natural gene, or a recombinant or synthetic molecule. In certain embodiments, a transgene is a component of a vector.

The term "overexpressed" or "overexpression" of an antigen refers to an abnormally high level of antigen expression in a cell. Overexpressed antigen or overexpression of antigen is often associated with a disease state, such as in hematological malignancies and cells forming a solid tumor within a specific tissue or organ of a subject. Solid tumors or hematological malignancies characterized by overexpression of a tumor antigen can be determined by standard assays known in the art.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as

peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

As used herein, the term "mature polypeptide" or "mature protein" refers to a protein or polypeptide that is secreted or localized in the cell membrane or inside certain cell organelles (*e.g.*, the endoplasmic reticulum, golgi, or endosome) and includes a partially cleaved N-terminal signal sequence (e.g., one or more amino acids of the signal sequence remaining but less than the whole signal sequence) or does not include an N-terminal signal sequence (*i.e.*, the N-terminal signal sequence has been entirely removed, such as by an endogenous cleavage process, from the protein or polypeptide).

A "signal sequence", also referred to as "signal peptide", "leader sequence", "leader peptide", "localization signal" or "localization sequence", is a short peptide (usually 13-36 amino acids in length) present at the N-terminus of newly synthesized proteins that are destined for the plasma membrane or a secretory pathway. A signal sequence typically comprises a short stretch of hydrophilic, positively charged amino acids at the N-terminus, a central hydrophobic domain of 5-15 residues, and a C-terminal region with a cleavage site for a signal sequence. In eukaryotes, a signal sequence prompts translocation of the newly synthesized protein to the endoplasmic reticulum where it is cleaved by the signal peptidase, creating a mature protein that then proceeds to its appropriate destination. The diversity of signal sequence length and amino acid composition makes it difficult to precisely predict the cleavage site. For polypeptide sequences disclosures herein, where a signal sequence is noted, the polypeptide sequence absent the signal sequence or having a partial signal sequence is also contemplated.

The "percent identity" between two or more nucleic acid or amino acid sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = number of identical positions/total number of positions x 100), taking into account the number of gaps, and the length of each gap that needs to be introduced

to optimize alignment of two or more sequences. The comparison of sequences and determination of percent identity between two or more sequences can be accomplished using a mathematical algorithm, such as BLAST and Gapped BLAST programs at their default parameters (*e.g.*, Altschul *et al.*, *J. Mol. Biol. 215*:403, 1990; see also BLASTN at www.ncbi.nlm.nih.gov/BLAST).

A "conservative substitution" is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well known in the art (*see, e.g.*, WO 97/09433, page 10, published March 13, 1997; Lehninger, Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge, MA (1990), p. 8).

The term "chimeric" refers to any nucleic acid molecule or protein that is not endogenous and comprises a combination of sequences joined or linked together that are not naturally found joined or linked together in nature. For example, a chimeric nucleic acid molecule may comprise nucleic acids encoding various domains from multiple different genes. In another example, a chimeric nucleic acid molecule may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences that are derived from the same source but arranged in a manner different than that found in nature.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may include an enhancer sequence and other regulatory elements that are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one that expresses the gene product in a tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

The phrase "under transcriptional control" or "operatively linked" as used herein means that a promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide.

A "vector" is a nucleic acid molecule that is capable of transporting another nucleic acid. Vectors may be, for example, plasmids, cosmids, viruses, or phage. The term should also be construed to include non-plasmid and non-viral compounds that facilitate transfer of nucleic acid into cells. An "expression vector" is a vector that is capable of directing the expression of a protein encoded by one or more transgenes carried by the vector when it is present in the appropriate environment. The one or more transgenes to be expressed by a vector as described herein are encoded in an expression cassette.

A "lentiviral vector" is a vector derived from a lentivirus and includes one or more lentiviral packaging proteins and/or one or more lentiviral proteins necessary for expression of the one or more genes carried by the vector. The abbreviation "LVV" is used herein to refer to a lentiviral vector (singular), as well as multiple lentiviral vectors (plural).

"Lentivirus" refers to a genus of retroviruses that are capable of infecting dividing and non-dividing cells. Examples of lentiviruses include, but are not limited to HIV (human immunodeficiency virus, including HIV type 1 and HIV type 2, equine infectious anemia virus, feline immunodeficiency virus (FIV), bovine immune deficiency virus (BIV), and simian immunodeficiency virus (SIV).

"Retrovirus" refers to are RNA viruses with a single strand positive-sense RNA molecule. Retroviruses comprise a reverse transcriptase enzyme and an integrase enzyme. Upon entry into a target cell, retroviruses utilize their reverse transcriptase to

transcribe their RNA molecule into a DNA molecule. Subsequently, the integrase enzyme is used to integrate the DNA molecule into the host cell genome. Upon integration into the host cell genome, the sequence from the retrovirus is referred to as a provirus (e.g., proviral sequence or provims sequence).

As used herein, the term "expression cassette" refers to a distinct component of a vector nucleic acid comprising at least one transgene and regulatory sequences controlling its expression (*e.g.*, promoter, 3'UTR) in a host cell. A tandem expression cassette refers to a component of a vector nucleic acid comprising at least two transgenes under the control of the same set of regulatory sequences for tandem expression of the at least two transgenes. In certain embodiments, the tandem expression cassette comprises at least two transgenes under the control of the same promoter. In certain embodiments, the first transgene and second transgene are separated by an internal ribosome entry site (IRES), furin cleavage site, or self-cleaving viral 2A peptide to allow for co-expression of two proteins from a single mRNA.

The term "immune system cell" or "immune cell" means any cell of the immune system that originates from a hematopoietic stem cell in the bone marrow. Hematopoietic stem cells give rise to two major lineages: myeloid progenitor cells (which give rise to myeloid cells such as monocytes, macrophages, dendritic cells, megakaryocytes and granulocytes) and lymphoid progenitor cells (which give rise to lymphoid cells such as T cells, B cells and natural killer (NK) cells). Exemplary immune system cells include a CD4+ T cell, a CD8+ T cell, a CD4- CD8- double negative T cell, a γδ T cell, a regulatory T cell, a natural killer cell, and a dendritic cell. Macrophages and dendritic cells may also be referred to as "antigen presenting cells" or "APCs," which are specialized cells that can activate T cells when a major histocompatibility complex (MHC) receptor on the surface of the APC complexed with a peptide interacts with a TCR on the surface of a T cell.

The term "lymphocyte" refers to immune cells of lymphoid origin, which are cells that show at least one phenotype characteristic of a lymphocyte or a precursor or progenitor thereof that distinguishes the cells from cells of erythroid or myeloid lineages. The term "lymphocytes" encompasses T cells, B cells, and natural killer (NK) cells.

The term "T cells" refers to cells of T cell lineage. "Cells of T cell lineage" refer to cells that show at least one phenotypic characteristic of a T cell or a precursor or

progenitor thereof that distinguishes the cells from other lymphoid cells, and cells of the erythroid or myeloid lineages. Such phenotypic characteristics can include expression of one or more proteins specific for T cells (*e.g.* , $CD3^+$, $CD4^+$, $CD8^+$), or a physiological, morphological, functional, or immunological feature specific for a T cell. For example, cells of the T cell lineage may be progenitor or precursor cells committed to the T cell lineage; $CD25^+$ immature and inactivated T cells; cells that have undergone CD4 or CD8 linage commitment; thymocyte progenitor cells that are $CD4^+CD8^+$ double positive; single positive $CD4^+$ or $CD8^+$; TCRαβ or TCR γδ; or mature and functional or activated T cells. The term "T cells" encompasses naïve T cells (CD45RA+, CCR7+, CD62L+, CD27+, CD45RO-), central memory T cells (CD45RA-, $CD45RO^+$, $CD62L^+$, CCR7+, CD27+), effector memory T cells (CD45RA-, CD45RO+, CCR7-, CD62L-, CD27-), mucosal-associated invariant T (MAIT) cells, γδ T cells, Tregs, natural killer T cells, and tissue resident T cells.

The term "natural killer cells" or "NK cells" refers to large granular lymphocytes (LGL) and constitute the third kind of cells differentiated from the common lymphoid progenitor-generating B and T lymphocytes. NK cells are known to differentiate and mature in the bone marrow, lymph nodes, spleen, tonsils, and thymus, where they then enter into the circulation. NK cells differ from natural killer T cells (NKTs) phenotypically, by origin and by respective effector functions; often, NKT cell activity promotes NK cell activity by secreting IFNγ. In contrast to NKT cells, NK cells do not express T-cell antigen receptors (TCR) or pan T marker CD3 or surface immunoglobulins (Ig) B cell receptors, but they usually express the surface markers CD16 (FcγRIII) and CD56 in humans, NK1.1 or NK1.2 in C57BL/6 mice. Up to 80% of human NK cells also express CD8.

The term "B cells" refers to cells of the B cell lineage. "Cells of B cell lineage" refers to cells that show at least one phenotypic characteristic of a B cell or a precursor or progenitor thereof that distinguishes the cells from other lymphoid cells, and cells of the erythroid or myeloid lineages. Such phenotypic characteristics can include expression of one or more proteins specific for B cells (e.g. , cells positive for one or more of CD19+, CD72+, CD24+, CD20+, CD21+, CD22+, CD38+, CD40+, CD72+, CD32b+, CD268+, CD269+, CD267+, CD86+, CD80+, CD40+, CD52+, CD138+, CD27+, CD28+, CD21+, CD23+, CD84+, CD257+, CD270+, CD37+, and CD74+), or a physiological, morphological, functional, or immunological feature specific for a B

cell. For example, cells of the B cell lineage may be progenitor or precursor cells committed to the B cell lineage (e.g., pre-pro-B cells, pro-B cells, and pre-B cells); immature and inactivated B cells or mature and functional or activated B cells. Thus, "B cells" encompass naïve B cells, plasma cells, regulatory B cells, marginal zone B cells, follicular B cells, lymphoplasmacytoid cells, plasmablast cells, and memory B cells (e.g., CD27+, IgD-).

The term "cytotoxic activity," also referred to as "cytolytic activity," with respect to a cell (*e.g.*, T cell) that expresses an immune receptor (*e.g.*, a CAR) on its surface, means that upon antigen-specific signaling (*e.g.*, via the CAR) the cell induces a target cell to undergo apoptosis. In some embodiments, a cytotoxic cell may induce apoptosis in a target cell via the release of cytotoxins, such as perforin, granzyme, and granulysin, from granules. Perforins insert into the target cell membrane and form pores that allow water and salts to rapidly enter the target cell. Granzymes are serine proteases that induce apoptosis in the target cell. Granulysin is also capable of forming pores in the target cell membrane and is a proinflammatory molecule. In some embodiments, a cytotoxic cell may induce apoptosis in a target cell via interaction of Fas ligand, which is upregulated on T cell following antigen-specific signaling, with Fas molecules expressed on the target cell. Fas is an apoptosis-signaling receptor molecule on the surface of a number of different cells.

A "disease" is a state of health of a subject wherein the subject cannot maintain homeostasis, and wherein, if the disease is not ameliorated, then the subject's health continues to deteriorate. In contrast, a "disorder" or "undesirable condition" in a subject is a state of health in which the subject is able to maintain homeostasis, but in which the subject's state of health is less favorable than it would be in the absence of the disorder or undesirable condition. Left untreated, a disorder or undesirable condition does not necessarily result in a further decrease in the subject's state of health.

The term "cancer" as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. The aberrant cells may form solid tumors or constitute a hematological malignancy. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include, but are not limited to, breast cancer, prostate cancer, ovarian cancer,

cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like.

The term "subject," "patient" and "individual" are used interchangeably herein and are intended to include living organisms in which an immune response can be elicited (*e.g.*, mammals). Examples of subjects include humans, primates, cows, horses, goats, sheep, dogs, cats, mice, rats, rabbits, guinea pigs, pigs, and transgenic species thereof.

"Adoptive cellular immunotherapy" or "adoptive immunotherapy" refers to the administration of naturally occurring or genetically engineered, disease antigen-specific immune cells (*e.g.*, T cells). Adoptive cellular immunotherapy may be autologous (immune cells are from the recipient), allogeneic (immune cells are from a donor of the same species) or syngeneic (immune cells are from a donor genetically identical to the recipient).

"Autologous" refers to a graft (*e.g.*, organ, tissue, cells) derived from the same subject to which it is later to be re-introduced.

"Allogeneic" refers to a graft derived from a different subject of the same species.

A "therapeutically effective amount" or "effective amount" of a lentiviral vector or a cell transduced by a lentiviral vector as described herein (*e.g.*, a T cell expressing a CAR as encoded by the transgene of the lentiviral vector) refers to that amount of lentiviral particles or cells sufficient to result in amelioration of one or more symptoms of the disease, disorder, or undesired condition being treated.

"Treat" or "treatment" or "ameliorate" refers to medical management of a disease, disorder, or undesired condition of a subject. In general, an appropriate dose or treatment regimen comprising a lentiviral vector or a cell expressing a CAR of this disclosure is administered in an amount sufficient to elicit a therapeutic or prophylactic benefit. Therapeutic or prophylactic/preventive benefit includes improved clinical outcome; lessening or alleviation of symptoms associated with a disease, disorder, or undesired condition; decreased occurrence of symptoms; improved quality of life; longer disease-free status; diminishment of extent of disease, disorder, or undesired condition; stabilization of disease state; delay of disease progression; remission; survival; prolonged survival; or any combination thereof.

The term "anti-tumor effect" refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with a cancerous condition. An "anti-tumor effect" can also be manifested by prevention of a hematological malignancy or tumor formation.

Additional definitions are provided throughout the present disclosure.

Lentiviral Vector

Lentivirus is a genus of retroviruses that typically gives rise to slowly developing diseases due to their ability to incorporate into a host genome. Modified lentiviral genomes are useful as viral vectors for the delivery of a nucleic acids to a host cell.

The present disclosure provides self-inactivating lentiviral vectors ("LVV") that include a viral envelope comprising a mutated, heterologous envelope protein and a tropism defining molecule (also referred to as a "targeting protein"). The LVV described herein further includes a transgene, with the LVV being capable of specifically binding to a target immune cell and transducing the target immune cell such that the transgene is expressed by the immune cell. In some embodiments, the LVV may carry more than one transgene. In further specific embodiments, the transgene encodes a chimeric antigen receptor (CAR).

Mutated Viral Envelope Protein

Lentiviral vectors as described herein are pseudotyped with a mutated heterologous viral envelope protein that, in the absence of mutation, mediates both cellular attachment and membrane fusion. In particular embodiments, the mutated envelope protein includes at least one mutation that inhibits the envelope protein's ability to bind its native target, while preserving the envelope protein's fusogenic properties. In specific embodiments, the heterologous envelope protein is a vesicular stomatitis virus G protein ("VSV-G", "VSV-G envelope protein" or "VSV-G protein") that includes one or more mutations that inhibit binding of VSV-G to the low-density

lipoprotein receptor ("LDL-R"), while preserving the VSV-G protein's fusogenic function.

When referring to the envelope protein's ability to bind its native target, the terms "inhibit" and "inhibits" encompass both complete elimination of binding by the envelope protein to its native target, as well as a significant reduction in binding by the envelope protein to its native target. In particular embodiments, "significant reduction" refers to a reduction selected from a reduction of at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, at least 50%, at least 45%, at least 40%, at least 35%, at least 30%,at least 25%, at least 20%, at least 15%, and at least 10% in binding to the native target.

The nucleic acid and amino acid sequences set forth in Table 10 provide references sequences for a wild type VSV-G envelope protein and examples of mutated VSV-G envelope proteins according to the present disclosure. SEQ ID NO: 77 is a reference nucleic acid sequence encoding the VSV-G envelope protein, and SEQ ID NO: 78 is an amino acid sequence of a reference VSV-G envelope protein. SEQ ID NO: 87 is a nucleic acid sequence encoding a cleavable signal peptide of the reference VSV-G envelope protein. SEQ ID NO: 88 is an amino acid sequence of the cleavable signal peptide of the reference VSV-G envelope protein. SEQ ID NO: 89 is a reference nucleic acid sequence encoding the VSV-G envelope protein, absent the signal sequence. SEQ ID NO: 90 is an amino acid sequence of a reference VSV-G envelope protein absent the sequence. The position and nature of VSV-G mutations disclosed herein are described in reference to the nucleic acid sequence and the amino acid sequence provided by SEQ ID NO: 77 absent the N-terminal signal sequence of SEQ ID NO: 87 and SEQ ID NO: 78 absent the N-terminal signal sequence of SEQ ID NO: 88, respectively. Thus, reference to a position 1 of the VSV-G env amino acid sequence for the purpose of identifying mutation position refers to position 17 of the wildtype VSV-G amino acid sequence of SEQ ID NO: 78, which is a lysine (K), or position 1 of the wildtype VSV-G amino acid sequence of SEQ ID NO: 90.

In some embodiments, the mutated VSV-G envelope protein includes a mutation at amino acid position H8, N9, Q10, K47, K50, A51, S183, S179, N180, I182, M184, Y209, I347, T350, T352, E353, R354, an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an

insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, an insertion of GGS between N208 and Y209, and/or a deletion of residues 1-8. In certain embodiments, the VSV-G envelope protein includes two or more mutations at amino acid positions selected from H8, N9, Q10, K47, K50, A51, S183, S179, N180, I182, M184, Y209, I347, T350, T352, E353, R354, an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, an insertion of GGS between N208 and Y209, and a deletion of residues 1-8. In other embodiments, the VSV-G envelope protein includes three or more mutations at amino acid positions selected from H8, N9, Q10, K47, K50, A51, S183, S179, N180, I182, M184, Y209, I347, T350, T352, E353, R354, an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, an insertion of GGS between N208 and Y209, and a deletion of residues 1-8. In other embodiments, the mutated VSV-G envelope protein comprises a H8A, K47A, K47Q, Y209A, R354A, and/or R354Q mutation. In still other embodiments, the VSV-G envelope protein includes one or more mutations selected from N9, Q10, K50, A51, S183, S179, N180, I182, M184, I347, T350, T352, and E353, an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, an insertion of GGS between N208 and Y209, and a deletion of residues 1-8. In yet other embodiments, the VSV-G envelope protein includes two or more mutations selected from a mutation at one or more of N9, Q10, K50, A51, S183, S179, N180, I182, M184, I347, T350, T352, and E353, an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, an insertion of GGS between N208 and Y209, and a deletion of residues 1-8. In some embodiments, a mutated VSV-G envelope protein is selected from one or more of an H8A, N9A, Q10A, K47A, K47Q, N180A, I182A, Y209A, T352A, T352W, E353A, R354A, and R354Q mutation. In some embodiments, a mutated VSV-G envelope protein is selected from one or more of an H8A, K47A, K47Q, Y209A, R354A, and R354Q mutation. In some embodiments, the mutated VSV-G envelope protein comprises a K47 mutation and a R354 mutation. In some embodiments, the

mutated VSV-G envelope protein comprises a K47Q mutation and a R354A mutation. In some embodiments, the mutated VSV-G envelope protein comprises a N180 mutation, a I182 mutation, a T352 mutation, and a E353 mutation. In some embodiments, the mutated VSV-G envelope protein comprises a N180A mutation, a I182A mutation, a T352A mutation, and a E353A mutation. In some embodiments, the mutated VSV-G envelope protein comprises a T352 mutation and E353 mutation. In further embodiments, the mutated VSV-G envelope protein comprises a T352W mutation and E353A mutation. In some embodiments, the mutated VSV-G envelope protein comprises a N9 mutation, a Q10 mutation, and a N180 mutation. In some embodiments, the mutated VSV-G envelope protein comprises a N9A mutation, a Q10A mutation, and a N180A mutation. In some embodiments, the mutated VSV-G envelope protein comprises an insertion of GGS between H8 and N9 and an insertion of GGS between N9 and Q10. In some embodiments, the mutated VSV-G envelope protein comprises an insertion of TT between N9 and Q10. In some embodiments, the mutated VSV-G envelope protein comprises an insertion of GGS between P46 and K47. In other embodiments, the mutated VSV-G envelope protein is as described in Nikolic et al., "Structural basis for the recognition of LDL-receptor family members by VSV glycoprotein." *Nature Comm.*, 2018, 9:1029, the relevant disclosures of which are incorporated by reference herein.

In specific embodiments, the mutant VSV envelope protein comprises a K47Q mutation and R354A mutation. In specific embodiments, the mutant VSV-G envelope protein has an amino acid sequence according to SEQ ID NO: 74.

In specific embodiments, the mutant VSV envelope protein comprises a N180A mutation, an I182A mutation, a T352A mutation, and an E353A mutation. In specific embodiments, the mutant VSV-G envelope protein has an amino acid sequence according to SEQ ID NO: 93.

In specific embodiments, the mutant VSV envelope protein comprises a T352W mutation and an E353A mutation. In specific embodiments, the mutant VSV-G envelope protein has an amino acid sequence according to SEQ ID NO: 95.

In specific embodiments, the mutant VSV envelope protein comprises a N9A mutation, a Q10A mutation, and a N180A mutation. In specific embodiments, the

mutant VSV-G envelope protein has an amino acid sequence according to SEQ ID NO: 97.

In specific embodiments, the mutant VSV envelope protein comprises an insertion of TT between N9 and Q10 mutation. In specific embodiments, the mutant VSV-G envelope protein has an amino acid sequence according to SEQ ID NO: 99.

In specific embodiments, the mutant VSV envelope protein comprises an insertion of GGS between P46 and K47 mutation. In specific embodiments, the mutant VSV-G envelope protein has an amino acid sequence according to SEQ ID NO: 101.

In specific embodiments, the mutant VSV envelope protein comprises an insertion of GGS between H8 and N9 and an insertion of GGS between N9 and Q10. In specific embodiments, the mutant VSV-G envelope protein has an amino acid sequence according to SEQ ID NO: 103.

Targeting Protein

The targeting protein is a membrane-bound protein displayed on the viral envelope and includes an extracellular domain that includes a lymphocyte targeting domain and a transmembrane domain. A lymphocyte targeting domain is any protein or peptide that has an amino acid sequence and is a binding partner for a target molecule or ligand (*e.g.*, a cognate protein or ligand) on a target lymphocyte cell surface. In some embodiments, a lymphocyte targeting domain is a T cell targeting domain. In some embodiments, a lymphocyte targeting domain is a NK cell targeting domain. In some embodiments, a lymphocyte targeting domain is a B cell targeting domain. For example, in certain embodiments, the extracellular domain of the targeting protein includes a lymphocyte targeting domain that specifically binds a protein or ligand on the surface of a defined population of cells, such as a population of lymphocytes characterized by the presence of the targeted protein or ligand on the cell surface. In some embodiments, a lymphocyte targeting domain targets a specific type of lymphocyte, such as for example, T cells, B cells, or natural killer (NK) cells. In some embodiments, the targeting protein additionally includes an extracellular linker or hinge domain positioned between the transmembrane domain and the extracellular domain.

In some embodiments, the targeting protein is a full-length cell surface protein or receptor, wherein each component (*e.g.*, each of the extracellular domain, transmembrane domain, and intracellular domain) derived from a single protein or receptor. In other embodiments, the targeting protein is a chimeric protein, having at least two components derived from a different protein or receptor. In some embodiments, the targeting protein is obtained from a mammalian species, including humans, primates, cows, horses, goats, sheep, dogs, cats, mice, rats, rabbits, guinea pigs, and pigs. In some embodiments, the targeting protein is a fully human sequence. In some embodiments, the targeting protein is a chimeric sequence comprising human sequence component(s) and component(s) from another species or synthetic component(s). In some embodiments, a chimeric targeting protein may be a fully synthetic protein. In still other embodiments, a chimeric targeting protein may include one or more of an extracellular domain, an extracellular binding domain, a transmembrane domain, an intracellular domain, a hinge domain, or a linker that is a fully synthetic protein.

In some embodiments, the lymphocyte targeting domain includes an antibody. In such embodiments, the extracellular targeting domain may be a full-length antibody, an antibody fragment, a nanobody, or a single chain Fv fragment (scFv).

In particular embodiments, the targeting molecule includes a T cell targeting domain that specifically binds to a T cell surface marker, including, for example, a T cell antigen, a T cell surface receptor, or any other protein present on the surface of a targeted T cell. In some embodiments, the targeting protein specifically binds to a T cell marker selected from CD3, CD28, CD80, 4-1BB, AhR, CD3, CD2, CD7, CD4, CD8, CD25, CD44, CD45RA, CD47, CD62L, CD69, CD94, CD95, CD127, CD161, CD183 (CXCR3), CD184 (CXCR4), CD185 (CXCR5), CD193 (CCR3), CD194 (CCR4), CD195 (CCR5), CD196 (CCR6), CD197 (CCR7), CCR10, PD-1, TCRa/b, CD5, CD27, CD45RO, CD45RB, CD57, CD103, CD122, P2RX7, TIGIT, LAG-3, TIM-3, IL6ST, and any combination thereof.

In further embodiments, the targeting protein specifically binds to a γδ T cell marker selected from γδ TCR, Vdelta1, Vdelta2, NKG2D (KLRK1, CD314), and any combination thereof.

In other embodiments, the targeting protein specifically binds to an NK T cell marker selected from Invariant TCR (Va24-Ja18), CD185 (CXCR5), CXCR6, IL-21R, and any combination thereof.

In other embodiments, the targeting protein specifically binds to a MAIT cell marker selected from Va7.2, Ja33, CXCR6, IL-18R, KLRB1 (CD161), VLA4 (alpha4beta1 integrin), and any combination thereof.

In other embodiments, the targeting protein specifically binds to an NK cell marker selected from CD56, NKp46, CD16, KIR(s), NKG2 proteins (e.g., NKG2D (KLRK1, CD314)), KLRB1 (CD161), KLRD1 (cd94), IL2Rb (CD122), IL-21R, SLAMF6 (CD352), SLAMF7 (CD319), IL-18R, and any combination thereof.

In other embodiments, the targeting protein specifically binds to a B cell marker selected from CD19, CD20, CD21, CD22, CD24, CD38, CD40, CD72, CD32b, CD268, CD269, CD267, CD86, CD80, CD52, CD138, CD27, CD28, CD23, CD84, CD257, CD270, CD37, CD74, and CD269, and any combination thereof.

In embodiments, the targeting protein specifically binds to a lymphocyte marker (e.g., B cell and T cells) selected from CD80, CD27, CD28, and any combination thereof.

In some embodiments of the targeting protein, a linker is positioned between the transmembrane domain and the lymphocyte targeting domain. A linker is an amino acid linker and may be a rigid linker, a flexible linker, or an oligomerized linker. A rigid linker is an amino acid sequence that lacks flexibility (*e.g.*, may comprise at least one proline). In some embodiments, a rigid linker comprises a platelet-derived growth factor receptor (PDGFR) stalk or a CD8a stalk. In some embodiments, a PDGFR stalk comprises an amino acid sequence comprising AVGQDTQEVIVVPHSLPFK (SEQ ID NO: 104). In some embodiments, a PDGFR stalk comprises an amino acid sequence comprising ASAKPTTTPAPRPPTPAPTIASQPLSLRPEAARPAAGGAVHTRGLDFAK (SEQ ID NO: 105).

A flexible linker is an amino acid sequence that has many degrees of freedom (*e.g.*, may comprise a plurality of amino acids with small side chains, *e.g.*, glycine or alanine). In some embodiments, a flexible linker comprises an amino acid sequence

comprising GAPGAS. In some embodiments, a flexible linker comprises an amino acid sequence consisting of GAPGSGGGGSGGGGSAS (SEQ ID NO: 106). In some embodiments, a flexible linker comprises an amino acid sequence comprising GGGGS. In some embodiments, a flexible linker comprises an amino acid sequence comprising GGGS. In some embodiments, a flexible linker comprises an amino acid sequence comprising $(GAPGAS)_N$, $(G_3S)_N$, or $(G_4S)_N$, wherein N is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more.

An oligomerized linker is an amino acid that can oligomerize to another related amino acid. In some embodiments, an oligomerized linker is an amino acid sequence that can form a dimer, trimer, or tetramer. In some embodiments, an oligomerized linker comprises an IgG4 hinge domain (*e.g.*, ESKYGPPCPPCPAVGQDTQEVIVVPHSLPFK (SEQ ID NO: 107)). In some embodiments, an oligomerized linker comprises an amino acid sequence that can form a tetrameric coiled coil (*e.g.*, ASGGGGSGELAAIKQELAAIKKELAAIKWELAAIKQGAG (SEQ ID NO: 108)). In some embodiments, an oligomerized linker comprises an amino acid sequence that can form a dimeric coiled coil (*e.g.*, ASESKYGPPCPPCP (SEQ ID NO: 109)).

The targeting protein will typically include a signal sequence (also referred to as a signal peptide of localization sequence). The signal sequence can be located at the N- or C-terminal ends of the targeting protein. A signal sequence functions to translocate the targeting protein to the membrane that serves as the envelope of the lentiviral vector. Non-limiting examples of signal sequences that might be included in a targeting protein as described herein include an Ig Kappa leader sequence (*e.g.*, a murine Ig Kappa leader sequence comprising: METDTLLLWVLLLWVPGSTG (SEQ ID NO: 110)), CD8a signal peptide sequence (e.g., a CD8a signal peptide comprising: MALPVTALLLPLALLLHAARP (SEQ ID NO: 12)), and a B2M signal peptide sequence (*e.g.*, a B2M signal peptide sequence comprising: MSRSVALAVLALLSLSGLEA (SEQ ID NO: 123)). In certain embodiments, a mature targeting protein has a partially cleaved signal sequence that retains one or more amino acids of the full-length signal sequence. In other embodiments, cleavage by a signal peptidase results in complete removal of the signal sequence, resulting in a mature targeting protein that lacks a signal sequence entirely.

In some embodiments, the targeting protein is a CD80 protein. Where the targeting protein is a CD80 protein, it may include an extracellular domain according to SEQ ID NO: 6. In such embodiments, a CD80 protein may include a signal peptide according to SEQ ID NO: 4 and a transmembrane and intracellular domain according to SEQ ID NO: 8. In a specific embodiment, a CD80 targeting protein included in a lentiviral vector as described herein includes an amino acid sequence according to SEQ ID NO: 2 or SEQ ID NO: 2 absent the signal peptide of SEQ ID NO: 4.

In some embodiments, where the targeting protein is a CD80 protein, it may include an extracellular domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 6. In such embodiments, a CD80 protein may include a signal peptide that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 4, and/or a transmembrane and intracellular domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 8. In a specific embodiment, a CD80 targeting protein included in a lentiviral vector as described herein includes an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 2 or SEQ ID NO: 2 absent the signal peptide of SEQ ID NO: 4.

TABLE 1: CD80 T cell targeting molecule

Full molecule	
Nucleotide	**Amino acid (signal peptide underlined)**
ATGGGTCATACACGCCGCCAAGGA ACCTCACCATCTAAGTGCCCATAT CTGAATTTCTTTCAACTTCTCGTGC TGGCGGGGCTCAGTCATTTCTGCA GTGGGGTCATTCACGTTACTAAAG AGGTCAAGGAGGTCGCAACATTGA GTTGTGGCCATAACGTATCAGTTG AAGAACTCGCGCAGACACGGATTT ACTGGCAAAAGGAAAAGAAGATG GTGTTGACAATGATGAGCGGTGAC ATGAACATTTGGCCAGAGTACAAA AATCGAACGATATTCGATATAACC AATAACTTGTCCATAGTAATACTT GCCTTGCGACCTTCTGACGAGGGA	MGHTRRQGTSPSKCPYLNFFQLLVL AGLSHFCSGVIHVTKEVKEVATLSC GHNVSVEELAQTRIYWQKEKKMVL TMMSGDMNIWPEYKNRTIFDITNNL SIVILALRPSDEGTYECVVLKYEKDA FKREHLAEVTLSVKADFPTPSISDFEI PTSNIRRIICSTSGGFPEPHLSWLENG EELNAINTTVSQDPETELYAVSSKLD FNMTTNHSFMCLIKYGHLRVNQTFN WNTTKQEHFPDNLLPSWAITLISVN GIFVICCLTYCFAPRCRE (SEQ ID NO: 2)

ACGTATGAATGTGTAGTGCTTAAG TATGAAAAAGATGCCTTTAAGCGG GAACACTTGGCTGAGGTTACACTC TCCGTTAAGGCGGACTTTCCTACG CCGTCTATATCCGACTTCGAGATA CCCACTTCTAACATTCGACGCATC ATTTGCTCAACCTCAGGTGGTTTCC CAGAGCCTCACTTGAGCTGGCTGG AGAATGGCGAAGAACTTAACGCA ATCAATACCACGGTGTCCCAAGAC CCGGAGACAGAGCTGTACGCCGTG TCATCCAAACTGGATTTTAACATG ACGACAAATCATAGTTTCATGTGT CTGATCAAATATGGGCATCTCAGG GTGAATCAGACTTTTAATTGGAAC ACTACCAAACAAGAGCACTTCCCA GATAATCTGTTGCCAAGCTGGGCG ATAACTCTTATCTCCGTCAACGGT ATCTTCGTAATTTGCTGCCTCACCT ATTGTTTCGCGCCTCGATGCCGAG AA (SEQ ID NO: 1)	
CD80 signal peptide	
Nucleotide	**Amino acid**
ATGGGTCATACACGCCGCCAAGGA ACCTCACCATCTAAGTGCCCATAT CTGAATTTCTTTCAACTTCTCGTGC TGGCGGGGCTCAGTCATTTCTGCA GTGGGGTC (SEQ ID NO: 3)	MGHTRRQGTSPSKCPYLNFFQLLVL AGLSHFCSGV (SEQ ID NO: 4)
CD80 extracellular domain (ECD)	
Nucleotide	**Amino acid**
ATTCACGTTACTAAAGAGGTCAAG GAGGTCGCAACATTGAGTTGTGGC CATAACGTATCAGTTGAAGAACTC GCGCAGACACGGATTTACTGGCAA AAGGAAAAGAAGATGGTGTTGAC AATGATGAGCGGTGACATGAACAT TTGGCCAGAGTACAAAAATCGAAC GATATTCGATATAACCAATAACTT GTCCATAGTAATACTTGCCTTGCG ACCTTCTGACGAGGGAACGTATGA ATGTGTAGTGCTTAAGTATGAAAA AGATGCCTTTAAGCGGGAACACTT GGCTGAGGTTACACTCTCCGTTAA GGCGGACTTTCCTACGCCGTCTAT ATCCGACTTCGAGATACCCACTTC TAACATTCGACGCATCATTTGCTC AACCTCAGGTGGTTTCCCAGAGCC TCACTTGAGCTGGCTGGAGAATGG	IHVTKEVKEVATLSCGHNVSVEELA QTRIYWQKEKKMVLTMMSGDMNI WPEYKNRTIFDITNNLSIVILALRPSD EGTYECVVLKYEKDAFKREHLAEV TLSVKADFPTPSISDFEIPTSNIRRIICS TSGGFPEPHLSWLENGEELNAINTTV SQDPETELYAVSSKLDFNMTTNHSF MCLIKYGHLRVNQTFNWNTTKQEH FPDNLLPS (SEQ ID NO: 6)

CGAAGAACTTAACGCAATCAATAC CACGGTGTCCCAAGACCCGGAGAC AGAGCTGTACGCCGTGTCATCCAA ACTGGATTTTAACATGACGACAAA TCATAGTTTCATGTGTCTGATCAAA TATGGGCATCTCAGGGTGAATCAG ACTTTTAATTGGAACACTACCAAA CAAGAGCACTTCCCAGATAATCTG TTGCCAAGC (SEQ ID NO:5)	
CD80 transmembrane and intracellular domain	
Nucleotide	**Amino acid**
TGGGCGATAACTCTTATCTCCGTC AACGGTATCTTCGTAATTTGCTGCC TCACCTATTGTTTCGCGCCTCGATG CCGAGAA (SEQ ID NO: 7)	WAITLISVNGIFVICCLTYCFAPRCRE (SEQ ID NO: 8)

In some embodiments, lentiviral vectors comprising a CD80 targeting protein exhibit enhanced transduction CD4 T cells over CD8 T cells as a relative percentage of total CD4 and CD8 T cells compared to the transduction by standard LVV. As used herein, "standard lentiviral vector" or "standard LVV" refers to a lentiviral vector which does not contain a mutated VSV-G envelope protein and a lymphocyte targeting protein of the present disclosure. In some embodiments, a standard LVV is generated using a 3rd generation LVV packaging system. In some embodiments, lentiviral vectors comprising a CD80 targeting protein enhance transduction of CD4 T cells over CD8 T cells as a relative percentage of total CD4 and CD8 T cells at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more compared to standard LVV.

In some embodiments, the targeting protein is an anti-CD3 targeting protein. In some embodiments, an anti-CD3 targeting protein is an antibody or binding fragment thereof, including for example, a scFv. Where the targeting protein is an anti-CD3 targeting protein, it may include an anti-CD3 scFv having a light chain variable region according to SEQ ID NO: 14, a G_3S linker according to SEQ ID NO: 16, and a heavy chain variable region according to SEQ ID NO: 18. In some embodiments, an anti-CD3 targeting protein may comprise an anti-CD3 scFv having a light chain variable region according to SEQ ID NO: 115, a G_3S linker according to SEQ ID NO: 16, and a heavy chain variable region according to SEQ ID NO: 113. An anti-CD3 targeting protein as described may include a signal peptide according to SEQ ID NO: 12 and a hinge and

transmembrane domain according to SEQ ID NO: 20. In a specific embodiment, an anti-CD3 targeting protein included in a lentiviral vector as described herein includes an amino acid sequence according to SEQ ID NO: 10 or SEQ ID NO: 10 absent the signal peptide of SEQ ID NO: 12. In another specific embodiment, an anti-CD3 targeting protein included in a lentiviral vector as described herein includes an amino acid sequence according to SEQ ID NO: 117 or SEQ ID NO: 117 absent the signal peptide of SEQ ID NO: 12.

In some embodiments, where the targeting protein is an anti-CD3 targeting protein, it may include an anti-CD3 scFv having a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 14, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 18. An anti-CD3 targeting protein as described may include a signal peptide that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 12 and a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 20. In a specific embodiment, an anti-CD3 targeting protein included in a lentiviral vector as described herein includes an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 10 or SEQ ID NO: 10 absent the signal peptide of SEQ ID NO: 12.

In some embodiments, where the targeting protein is an anti-CD3 targeting protein, it may include an anti-CD3 scFv having a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 115, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 113. An anti-CD3 targeting protein as described may include a signal peptide that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 12 and a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to

SEQ ID NO: 20. In a specific embodiment, an anti-CD3 targeting protein included in a lentiviral vector as described herein includes an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 117 or SEQ ID NO: 117 absent the signal peptide of SEQ ID NO: 12.

TABLE 2: Anti-CD3 T cell targeting molecules

Anti-CD3 (UCTH1) Full molecule	
Nucleotide	**Amino acid (signal peptide underlined)**
ATGGCCTTACCAGTGACCGCCTTG	MALPVTALLLPLALLLHAARPDIQM
CTCCTGCCGCTGGCCTTGCTGCTCC	TQTTSSLSASLGDRVTISCRASQDIR
ACGCCGCCAGGCCGGACATCCAGA	NYLNWYQQKPDGTVKLLIYYTSRL
TGACCCAGACCACCTCCTCCCTGT	HSGVPSKFSGSGSGTDYSLTISNLEQ
CTGCCTCTCTGGGAGACAGAGTCA	EDIATYFCQQGNTLPWTFAGGTKLE
CCATCAGTTGCAGGGCAAGTCAGG	IKRAGGGSGGGSGGGSGGGSEVQLQ
ACATTAGAAATTATTTAAACTGGT	QSGPELVKPGASMKISCKASGYSFT
ATCAACAGAAACCAGATGGAACTG	GYTMNWVKQSHGKNLEWMGLINP
TTAAACTCCTGATCTACTACACATC	YKGVSTYNQKFKDKATLTVDKSSST
AAGATTACACTCAGGAGTCCCATC	AYMELLSLTSEDSAVYYCARSGYY
AAAGTTCAGTGGCAGTGGGTCTGG	GDSDWYFDVWGAGTTVTVSSTTTP
AACAGATTATTCTCTCACCATTAG	APRPPTPAPTIASQPLSLRPEACRPAA
CAACCTGGAGCAAGAGGATATTGC	GGAVHTRGLDFACDIYIWAPLAGTC
CACTTACTTTTGCCAACAGGGTAA	GVLLLSLVITLYC (SEQ ID NO: 10)
TACGCTTCCGTGGACGTTCGCTGG	
AGGCACCAAGCTGGAAATCAAAC	
GGGCTGGAGGCGGTAGTGGCGGTG	
GATCAGGTGGAGGCAGCGGTGGC	
GGATCTGAGGTGCAGCTCCAGCAG	
TCTGGACCTGAGCTGGTGAAGCCT	
GGAGCTTCAATGAAGATATCCTGC	
AAGGCTTCTGGTTACTCATTCACTG	
GCTACACCATGAACTGGGTGAAGC	
AGAGTCATGGAAAGAACCTTGAGT	
GGATGGGACTTATTAATCCTTACA	
AAGGTGTTAGTACCTACAACCAGA	
AGTTCAAGGACAAGGCCACATTAA	
CTGTAGACAAGTCATCCAGCACAG	
CCTACATGGAACTCCTCAGTCTGA	
CATCTGAGGACTCTGCAGTCTATT	
ACTGTGCAAGATCGGGGTACTACG	
GTGATAGTGACTGGTACTTCGATG	
TCTGGGGCGCAGGGACCACGGTCA	
CCGTCTCCTCAACCACtACaCCAGCa	
CCtaGACCACCAACACCtGCGCCaAC	
CATCGCaTCGCAGCCaCTGTCtCTGC	

GCCCAGAGGCaTGCCGGCCAGCaG CtGGGGGCGCAGTGCACACaAGGG GGCTGGACTTCGCaTGTGATATCTA CATCTGGGCaCCaTTGGCaGGGACT TGTGGGGTCCTTCTCCTGTCACTGG TTATCACCCTTTACTGC (SEQ ID NO: 9)	
CD8a signal peptide	
Nucleotide	**Amino acid**
ATGGCCTTACCAGTGACCGCCTTG CTCCTGCCGCTGGCCTTGCTGCTCC ACGCCGCCAGGCCG (SEQ ID NO: 11)	MALPVTALLLPLALLLHAARP (SEQ ID NO:12)
Anti-CD3 (UCTH1) Light chain variable region	
Nucleotide	**Amino acid**
GACATCCAGATGACCCAGACCACC TCCTCCCTGTCTGCCTCTCTGGGAG ACAGAGTCACCATCAGTTGCAGGG CAAGTCAGGACATTAGAAATTATT TAAACTGGTATCAACAGAAACCAG ATGGAACTGTTAAACTCCTGATCT ACTACACATCAAGATTACACTCAG GAGTCCCATCAAAGTTCAGTGGCA GTGGGTCTGGAACAGATTATTCTC TCACCATTAGCAACCTGGAGCAAG AGGATATTGCCACTTACTTTTGCCA ACAGGGTAATACGCTTCCGTGGAC GTTCGCTGGAGGCACCAAGCTGGA AATCAAACGGGCT (SEQ ID NO: 13)	DIQMTQTTSSLSASLGDRVTISCRAS QDIRNYLNWYQQKPDGTVKLLIYYT SRLHSGVPSKFSGSGSGTDYSLTISN LEQEDIATYFCQQGNTLPWTFAGGT KLEIKRA (SEQ ID NO:14)
G_3S linker	
Nucleotide	Amino acid
GGAGGCGGTAGTGGCGGTGGATCA GGTGGAGGCAGCGGTGGCGGATCT (SEQ ID NO: 15)	GGGSGGGSGGGSGGGS (SEQ ID NO:16)
Anti-CD3 (UCTH1) heavy chain variable region	
Nucleotide	**Amino acid**
GAGGTGCAGCTCCAGCAGTCTGGA CCTGAGCTGGTGAAGCCTGGAGCT TCAATGAAGATATCCTGCAAGGCT TCTGGTTACTCATTCACTGGCTACA CCATGAACTGGGTGAAGCAGAGTC ATGGAAAGAACCTTGAGTGGATGG GACTTATTAATCCTTACAAAGGTG TTAGTACCTACAACCAGAAGTTCA AGGACAAGGCCACATTAACTGTAG ACAAGTCATCCAGCACAGCCTACA TGGAACTCCTCAGTCTGACATCTG AGGACTCTGCAGTCTATTACTGTG	EVQLQQSGPELVKPGASMKISCKAS GYSFTGYTMNWVKQSHGKNLEWM GLINPYKGVSTYNQKFKDKATLTVD KSSSTAYMELLSLTSEDSAVYYCAR SGYYGDSDWYFDVWGAGTTVTVSS (SEQ ID NO: 18)

CAAGATCGGGGTACTACGGTGATA GTGACTGGTACTTCGATGTCTGGG GCGCAGGGACCACGGTCACCGTCT CCTCA (SEQ ID NO: 17)	
CD8a hinge and transmembrane	
Nucleotide	**Amino acid**
ACCACTACACCAGCACCTAGACCA CCAACACCTGCGCCAACCATCGCA TCGCAGCCACTGTCTCTGCGCCCA GAGGCATGCCGGCCAGCAGCTGGG GGCGCAGTGCACACAAGGGGGCT GGACTTCGCATGTGATATCTACAT CTGGGCACCATTGGCAGGGACTTG TGGGGTCCTTCTCCTGTCACTGGTT ATCACCCTTTACTGC (SEQ ID NO: 19)	TTTPAPRPPTPAPTIASQPLSLRPEAC RPAAGGAVHTRGLDFACDIYIWAPL AGTCGVLLLSLVITLYC (SEQ ID NO: 20)
Anti-CD3 (12F6) heavy chain variable region	
Nucleotide	**Amino Acid**
CAAGTGCAGCTCCAGCAGAGCGGC GCTGAGCTGGCCCGGCCCGGCGCC AGCGTGAAGATGAGCTGTAAAGCC AGCGGCTATACATTTACCAGCTAC ACCATGCACTGGGTCAAGCAGCGG CCTGGCCAGGGCCTGGAATGGATT GGATATATCAACCCCAGCAGCGGC TACACCAAGTACAACCAGAAATTC AAGGACAAGGCCACCCTGACCGCC GACAAGAGCTCCTCAACAGCCTAC ATGCAACTGAGCAGCCTGACCAGC GAGGATAGCGCCGTGTACTACTGC GCCAGATGGCAGGACTACGACGTG TACTTCGACTACTGGGGCCAAGGC ACAACACTGACCGTGTCCAGC (SEQ ID NO: 112)	QVQLQQSGAELARPGASVKMSCKA SGYTFTSYTMHWVKQRPGQGLEWI GYINPSSGYTKYNQKFKDKATLTAD KSSSTAYMQLSSLTSEDSAVYYCAR WQDYDVYFDYWGQGTTLTVSS (SEQ ID NO: 113)
Anti-CD3 (12F6) light chain variable region	
Nucleotide	**Amino Acid**
CAGATCGTGCTGAGCCAGTCCCCA GCCATCCTGTCTGCCAGCCCTGGC GAGAAGGTGACCATGACCTGCAGA GCCTCTTCTTCTGTTTCCTACATGC ACTGGTATCAGCAAAAGCCCGGCA GCTCTCCTAAGCCTTGGATCTACG CCACAAGCAACCTGGCTAGCGGCG TGCCTGCTCGCTTCAGCGGCAGCG GCAGCGGCACCAGCTACAGCCTGA CCATCAGCAGAGTGGAAGCCGAG GACGCCGCCACCTACTACTGCCAG CAGTGGTCCTCTAATCCTCCAACA	QIVLSQSPAILSASPGEKVTMTCRAS SSVSYMHWYQQKPGSSPKPWIYATS NLASGVPARFSGSGSGTSYSLTISRV EAEDAATYYCQQWSSNPPTFGGGT KLETKR (SEQ ID NO: 115)

TTCGGCGGCGGCACCAAGCTGGAA ACCAAAAGA (SEQ ID NO: 114)	
Anti-CD3 (12F6) Full molecule	
Nucleotide	**Amino Acid (signal peptide underlined)**
ATGGCCTTACCAGTGACCGCCTTG CTCCTGCCGCTGGCCTTGCTGCTCC ACGCCGCCAGGCCGCAAGTGCAGC TCCAGCAGAGCGGCGCTGAGCTGG CCCGGCCCGGCGCCAGCGTGAAGA TGAGCTGTAAAGCCAGCGGCTATA CATTTACCAGCTACACCATGCACT GGGTCAAGCAGCGGCCTGGCCAGG GCCTGGAATGGATTGGATATATCA ACCCCAGCAGCGGCTACACCAAGT ACAACCAGAAATTCAAGGACAAG GCCACCCTGACCGCCGACAAGAGC TCCTCAACAGCCTACATGCAACTG AGCAGCCTGACCAGCGAGGATAGC GCCGTGTACTACTGCGCCAGATGG CAGGACTACGACGTGTACTTCGAC TACTGGGGCCAAGGCACAACACTG ACCGTGTCCAGCGGAGGCGGTAGT GGCGGTGGATCAGGTGGAGGCAG CGGTGGCGGATCTCAGATCGTGCT GAGCCAGTCCCCAGCCATCCTGTC TGCCAGCCCTGGCGAGAAGGTGAC CATGACCTGCAGAGCCTCTTCTTCT GTTTCCTACATGCACTGGTATCAG CAAAAGCCCGGCAGCTCTCCTAAG CCTTGGATCTACGCCACAAGCAAC CTGGCTAGCGGCGTGCCTGCTCGC TTCAGCGGCAGCGGCAGCGGCACC AGCTACAGCCTGACCATCAGCAGA GTGGAAGCCGAGGACGCCGCCACC TACTACTGCCAGCAGTGGTCCTCT AATCCTCCAACATTCGGCGGCGGC ACCAAGCTGGAAACCAAAAGAAC CACTACACCAGCACCTAGACCACC AACACCTGCGCCAACCATCGCATC GCAGCCACTGTCTCTGCGCCCAGA GGCATGCCGGCCAGCAGCTGGGGG CGCAGTGCACACAAGGGGGCTGG ACTTCGCATGTGATATCTACATCTG GGCACCATTGGCAGGGACTTGTGG GGTCCTTCTCCTGTCACTGGTTATC ACCCTTTACTGC (SEQ ID NO: 116)	MALPVTALLLPLALLLHAARP QVQLQQSGAELARPGASVKMSCKA SGYTFTSYTMHWVKQRPGQGLEWI GYINPSSGYTKYNQKFKDKATLTAD KSSSTAYMQLSSLTSEDSAVYYCAR WQDYDVYFDYWGQGTTLTVSSGG GSGGGSGGGSGGGS QIVLSQSPAILSASPGEKVTMTCRAS SSVSYMHWYQQKPGSSPKPWIYATS NLASGVPARFSGSGSGTSYSLTISRV EAEDAATYYCQQWSSNPPTFGGGT KLETKRTTTPAPRPPTPAPTIASQPLS LRPEACRPAAGGAVHTRGLDFACDI YIWAPLAGTCGVLLLSLVITLYC (SEQ ID NO: 117)

In still further embodiments, a lentiviral vector according to the present description may include multiple targeting proteins or a single targeting protein having multiple different lymphocyte targeting domains. For example, the lentiviral vectors described herein can include at least two targeting proteins, with each of the targeting proteins having a NK cell targeting domain that specifically binds a different target. In another example, the lentiviral vectors described herein can include at least two targeting proteins, with each of the targeting proteins having a T cell targeting domain that specifically binds a different target. In another example, the lentiviral vectors described herein can include at least two targeting proteins, with each of the targeting proteins having a B cell targeting domain that specifically binds a different target. In yet another example, the lentiviral vectors described herein can include at least two targeting proteins, with a first targeting protein having a T cell targeting domain and a second targeting protein having a NK cell targeting domain. In yet another example, the lentiviral vectors described herein can include at least two targeting proteins, with a first targeting protein having a T cell targeting domain and a second targeting protein having a B cell targeting domain. In yet another example, the lentiviral vectors described herein can include at least two targeting proteins, with a first targeting protein having a B cell targeting domain and a second targeting protein having a NK cell targeting domain. In alternative embodiments, the lentiviral vectors described herein include a single targeting protein having two or more different lymphocyte targeting domains, two or more different T cell targeting domains, two or more different NK cell targeting domains, or two or more different B cell targeting domains (*e.g.*, a bi-specific or multi-specific targeting protein). In any such embodiments, the T lymphocyte targeting domains included in the targeting proteins can be selected from any of those described herein. In yet another example, the lentiviral vectors described herein can include at least two targeting proteins, with a first targeting protein having a lymphocyte targeting domain and a second targeting protein having a T cell targeting domain. In yet another example, the lentiviral vectors described herein can include at least two targeting proteins, with a first targeting protein having a lymphocyte targeting domain and a second targeting protein having a B cell targeting domain.

In an exemplary embodiment, a lentiviral vector having multiple targeting proteins includes a CD80 targeting protein and an anti-CD3 targeting protein as detailed

in Table 1 and Table 2, respectively. In some embodiments, a CD80 targeting protein includes an amino acid sequence according to SEQ ID NO: 2 or SEQ ID NO: 2 absent the signal peptide of SEQ ID NO: 4. In some embodiments, an anti-CD3 targeting protein includes an amino acid sequence according to SEQ ID NO: 10 or SEQ ID NO: 10 absent the signal peptide of SEQ ID NO: 12. In some embodiments, an anti-CD3 targeting protein includes an amino acid sequence according to SEQ ID NO: 117 or SEQ ID NO: 117 absent the signal peptide of SEQ ID NO: 12. Where the lentiviral vector includes a CD80 targeting protein and an anti-CD3 targeting protein, the targeting proteins may be encoded on separate expression vectors used in producing the lentiviral vector. Alternatively, both a CD80 targeting protein and an anti-CD3 targeting protein may be encoded on a tandem expression cassette, providing a single expression cassette in a single expression vector that provides expression of both targeting proteins for production of the lentiviral vector. Exemplary embodiments of nucleic acid sequences and the corresponding amino acid sequences for a CD80 targeting protein and an anti-CD3 targeting protein expressed from the same expression cassette are provided in Table 3. In such an embodiment, the two targeting proteins may be linked by a P2A self-cleaving peptide. Table 3 provides an exemplary nucleic acid that may be used in a tandem expression cassette that includes a CD80 binding protein linked to an anti-CD3 binding protein via a P2A sequence. In a particular embodiment, the P2A sequence is encoded by a nucleic acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 29 and the amino acid sequence of the P2A sequence has an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 30. In some embodiments, the tandem expression cassette that includes a CD80 binding protein linked to an anti-CD3 binding protein comprises the nucleic acid sequence of SEQ ID NO: 21 or has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID: 21. In some embodiments, the tandem expression cassette that includes a CD80 binding protein linked to an anti-CD3 binding protein encodes an amino acid sequence of SEQ ID NO: 22 or SEQ ID NO: 22 absent the signal peptide of SEQ ID NO: 24. In some embodiments, the tandem expression cassette that includes a CD80 binding protein linked to an anti-CD3 binding protein encodes an amino acid sequence having at least

about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 22 or SEQ ID NO: 22 absent the signal peptide of SEQ ID NO: 24.

TABLE 3: Co-expressed CD80 and anti-CD3 T cell targeting molecules

Full molecule	
Nucleotide	**Amino acid (Signal peptide underlined)**
ATGGGTCATACACGCCGCCAAGGA ACCTCACCATCTAAGTGCCCATAT CTGAATTTCTTTCAACTTCTCGTGC TGGCGGGGCTCAGTCATTTCTGCA GTGGGGTCATTCACGTTACTAAAG AGGTCAAGGAGGTCGCAACATTGA GTTGTGGCCATAACGTATCAGTTG AAGAACTCGCGCAGACACGGATTT ACTGGCAAAAGGAAAAGAAGATG GTGTTGACAATGATGAGCGGTGAC ATGAACATTTGGCCAGAGTACAAA AATCGAACGATATTCGATATAACC AATAACTTGTCCATAGTAATACTT GCCTTGCGACCTTCTGACGAGGGA ACGTATGAATGTGTAGTGCTTAAG TATGAAAAAGATGCCTTTAAGCGG GAACACTTGGCTGAGGTTACACTC TCCGTTAAGGCGGACTTTCCTACG CCGTCTATATCCGACTTCGAGATA CCCACTTCTAACATTCGACGCATC ATTTGCTCAACCTCAGGTGGTTTCC CAGAGCCTCACTTGAGCTGGCTGG AGAATGGCGAAGAACTTAACGCA ATCAATACCACGGTGTCCCAAGAC CCGGAGACAGAGCTGTACGCCGTG TCATCCAAACTGGATTTTAACATG ACGACAAATCATAGTTTCATGTGT CTGATCAAATATGGGCATCTCAGG GTGAATCAGACTTTTAATTGGAAC ACTACCAAACAAGAGCACTTCCCA GATAATCTGTTGCCAAGCTGGGCG ATAACTCTTATCTCCGTCAACGGT ATCTTCGTAATTTGCTGCCTCACCT ATTGTTTCGCGCCTCGATGCCGAG AAGGCAGCGGCGCCACCAACTTCT CCCTGCTGAAGCAGGCCGGCGACG TGGAAGAAAACCCTGGCCCCATGG CCTTACCAGTGACCGCCTTGCTCCT GCCGCTGGCCTTGCTGCTCCACGC	MGHTRRQGTSPSKCPYLNFFQLLVL AGLSHFCSGVIHVTKEVKEVATLSC GHNVSVEELAQTRIYWQKEKKMVL TMMSGDMNIWPEYKNRTIFDITNNL SIVILALRPSDEGTYECVVLKYEKDA FKREHLAEVTLSVKADFPTPSISDFEI PTSNIRRIICSTSGGFPEPHLSWLENG EELNAINTTVSQDPETELYAVSSKLD FNMTTNHSFMCLIKYGHLRVNQTFN WNTTKQEHFPDNLLPSWAITLISVN GIFVICCLTYCFAPRCREGSGATNFS LLKQAGDVEENPGPMALPVTALLLP LALLLHAARPDIQMTQTTSSLSASLG DRVTISCRASQDIRNYLNWYQQKPD GTVKLLIYYTSRLHSGVPSKFSGSGS GTDYSLTISNLEQEDIATYFCQQGNT LPWTFAGGTKLEIKRAGGGSGGGSG GGSGGGSEVQLQQSGPELVKPGAS MKISCKASGYSFTGYTMNWVKQSH GKNLEWMGLINPYKGVSTYNQKFK DKATLTVDKSSSTAYMELLSLTSED SAVYYCARSGYYGDSDWYFDVWG AGTTVTVSSTTTPAPRPPTPAPTIASQ PLSLRPEACRPAAGGAVHTRGLDFA CDIYIWAPLAGTCGVLLLSLVITLYC (SEQ ID NO: 22)

CGCCAGGCCGGACATCCAGATGAC CCAGACCACCTCCTCCCTGTCTGCC TCTCTGGGAGACAGAGTCACCATC AGTTGCAGGGCAAGTCAGGACATT AGAAATTATTTAAACTGGTATCAA CAGAAACCAGATGGAACTGTTAAA CTCCTGATCTACTACACATCAAGA TTACACTCAGGAGTCCCATCAAAG TTCAGTGGCAGTGGGTCTGGAACA GATTATTCTCTCACCATTAGCAACC TGGAGCAAGAGGATATTGCCACTT ACTTTTGCCAACAGGGTAATACGC TTCCGTGGACGTTCGCTGGAGGCA CCAAGCTGGAAATCAAACGGGCTG GAGGCGGTAGTGGCGGTGGATCAG GTGGAGGCAGCGGTGGCGGATCTG AGGTGCAGCTCCAGCAGTCTGGAC CTGAGCTGGTGAAGCCTGGAGCTT CAATGAAGATATCCTGCAAGGCTT CTGGTTACTCATTCACTGGCTACAC CATGAACTGGGTGAAGCAGAGTCA TGGAAAGAACCTTGAGTGGATGGG ACTTATTAATCCTTACAAAGGTGTT AGTACCTACAACCAGAAGTTCAAG GACAAGGCCACATTAACTGTAGAC AAGTCATCCAGCACAGCCTACATG GAACTCCTCAGTCTGACATCTGAG GACTCTGCAGTCTATTACTGTGCA AGATCGGGGTACTACGGTGATAGT GACTGGTACTTCGATGTCTGGGGC GCAGGGACCACGGTCACCGTCTCC TCAACCACtACaCCAGCaCCtaGACC ACCAACACCtGCGCCaACCATCGCa TCGCAGCCaCTGTCtCTGCGCCCAG AGGCaTGCCGGCCAGCaGCtGGGGG CGCAGTGCACACaAGGGGGCTGGA CTTCGCaTGTGATATCTACATCTGG GCaCCaTTGGCaGGGACTTGTGGGG TCCTTCTCCTGTCACTGGTTATCAC CCTTTACTGC (SEQ ID NO: 21)	
CD80 signal peptide	
Nucleotide	**Amino acid**
ATGGGTCATACACGCCGCCAAGGA ACCTCACCATCTAAGTGCCCATAT CTGAATTTCTTTCAACTTCTCGTGC TGGCGGGGCTCAGTCATTTCTGCA GTGGGGTC (SEQ ID NO: 23)	MGHTRRQGTSPSKCPYLNFFQLLVL AGLSHFCSGV (SEQ ID NO: 24)
CD80 extracellular domain (ECD)	

Nucleotide	Amino acid
ATTCACGTTACTAAAGAGGTCAAG GAGGTCGCAACATTGAGTTGTGGC CATAACGTATCAGTTGAAGAACTC GCGCAGACACGGATTTACTGGCAA AAGGAAAAGAAGATGGTGTTGAC AATGATGAGCGGTGACATGAACAT TTGGCCAGAGTACAAAAATCGAAC GATATTCGATATAACCAATAACTT GTCCATAGTAATACTTGCCTTGCG ACCTTCTGACGAGGGAACGTATGA ATGTGTAGTGCTTAAGTATGAAAA AGATGCCTTTAAGCGGGAACACTT GGCTGAGGTTACACTCTCCGTTAA GGCGGACTTTCCTACGCCGTCTAT ATCCGACTTCGAGATACCCACTTC TAACATTCGACGCATCATTTGCTC AACCTCAGGTGGTTTCCCAGAGCC TCACTTGAGCTGGCTGGAGAATGG CGAAGAACTTAACGCAATCAATAC CACGGTGTCCCAAGACCCGGAGAC AGAGCTGTACGCCGTGTCATCCAA ACTGGATTTTAACATGACGACAAA TCATAGTTTCATGTGTCTGATCAAA TATGGGCATCTCAGGGTGAATCAG ACTTTTAATTGGAACACTACCAAA CAAGAGCACTTCCCAGATAATCTG TTGCCAAGC (SEQ ID NO: 25)	IHVTKEVKEVATLSCGHNVSVEELA QTRIYWQKEKKMVLTMMSGDMNI WPEYKNRTIFDITNNLSIVILALRPSD EGTYECVVLKYEKDAFKREHLAEV TLSVKADFPTPSISDFEIPTSNIRRIICS TSGGFPEPHLSWLENGEELNAINTTV SQDPETELYAVSSKLDFNMTTNHSF MCLIKYGHLRVNQTFNWNTTKQEH FPDNLLPS (SEQ ID NO: 26)
CD80 transmembrane and intracellular domain	
Nucleotide	**Amino acid**
TGGGCGATAACTCTTATCTCCGTC AACGGTATCTTCGTAATTTGCTGCC TCACCTATTGTTTCGCGCCTCGATG CCGAGAA (SEQ ID NO: 27)	WAITLISVNGIFVICCLTYCFAPRCRE (SEQ ID NO: 28)
P2A self-cleaving peptide	
Nucleotide	**Amino acid**
GGCAGCGGCGCCACCAACTTCTCC CTGCTGAAGCAGGCCGGCGACGTG GAAGAAAACCCTGGCCCC (SEQ ID NO: 29)	GSGATNFSLLKQAGDVEENPGP (SEQ ID NO: 30)
CD8a signal peptide	
Nucleotide	**Amino acid**
ATGGCCTTACCAGTGACCGCCTTG CTCCTGCCGCTGGCCTTGCTGCTCC ACGCCGCCAGGCCG (SEQ ID NO: 31)	MALPVTALLLPLALLLHAARP (SEQ ID NO: 32)
Anti-CD3 Light chain variable region	
Nucleotide	**Amino acid**

GACATCCAGATGACCCAGACCACC TCCTCCCTGTCTGCCTCTCTGGGAG ACAGAGTCACCATCAGTTGCAGGG CAAGTCAGGACATTAGAAATTATT TAAACTGGTATCAACAGAAACCAG ATGGAACTGTTAAACTCCTGATCT ACTACACATCAAGATTACACTCAG GAGTCCCATCAAAGTTCAGTGGCA GTGGGTCTGGAACAGATTATTCTC TCACCATTAGCAACCTGGAGCAAG AGGATATTGCCACTTACTTTTGCCA ACAGGGTAATACGCTTCCGTGGAC GTTCGCTGGAGGCACCAAGCTGGA AATCAAACGGGCT (SEQ ID NO:33)	DIQMTQTTSSLSASLGDRVTISCRAS QDIRNYLNWYQQKPDGTVKLLIYYT SRLHSGVPSKFSGSGSGTDYSLTISN LEQEDIATYFCQQGNTLPWTFAGGT KLEIKRA (SEQ ID NO: 34)
G_3S linker	
Nucleotide	**Amino acid**
GGAGGCGGTAGTGGCGGTGGATCA GGTGGAGGCAGCGGTGGCGGATCT (SEQ ID NO: 35)	GGGSGGGSGGGSGGGS (SEQ ID NO: 36)
Anti-CD3 heavy chain variable region	
Nucleotide	**Amino acid**
GAGGTGCAGCTCCAGCAGTCTGGA CCTGAGCTGGTGAAGCCTGGAGCT TCAATGAAGATATCCTGCAAGGCT TCTGGTTACTCATTCACTGGCTACA CCATGAACTGGGTGAAGCAGAGTC ATGGAAAGAACCTTGAGTGGATGG GACTTATTAATCCTTACAAAGGTG TTAGTACCTACAACCAGAAGTTCA AGGACAAGGCCACATTAACTGTAG ACAAGTCATCCAGCACAGCCTACA TGGAACTCCTCAGTCTGACATCTG AGGACTCTGCAGTCTATTACTGTG CAAGATCGGGGTACTACGGTGATA GTGACTGGTACTTCGATGTCTGGG GCGCAGGGACCACGGTCACCGTCT CCTCA (SEQ ID NO: 37)	EVQLQQSGPELVKPGASMKISCKAS GYSFTGYTMNWVKQSHGKNLEWM GLINPYKGVSTYNQKFKDKATLTVD KSSSTAYMELLSLTSEDSAVYYCAR SGYYGDSDWYFDVWGAGTTVTVSS (SEQ ID NO: 38)
CD8a hinge and transmembrane	
Nucleotide	**Amino acid**
ACCACTACACCAGCACCTAGACCA CCAACACCTGCGCCAACCATCGCA TCGCAGCCACTGTCTCTGCGCCCA GAGGCATGCCGGCCAGCAGCTGGG GGCGCAGTGCACACAAGGGGGCT GGACTTCGCATGTGATATCTACAT CTGGGCACCATTGGCAGGGACTTG TGGGGTCCTTCTCCTGTCACTGGTT	TTTPAPRPPTPAPTIASQPLSLRPEAC RPAAGGAVHTRGLDFACDIYIWAPL AGTCGVLLLSLVITLYC (SEQ ID NO: 40)

ATCACCCTTTACTGC (SEQ ID NO: 39)	

Transgene

The lentiviral vectors described herein include a transgene encoding one or more proteins that is delivered to and expressed by a lymphocyte (e.g., T cell, B cell, or NK cell) targeted by the vector. In particular embodiments, the transgene encodes a chimeric antigen receptor (CAR) that includes: an extracellular domain comprising a binding domain that specifically binds to a target molecule; an intracellular signaling domain, wherein the intracellular signaling domain comprises an immunoreceptor tyrosine-based activation motif (ITAM); and a transmembrane domain connecting the extracellular domain and intracellular signaling domain.

Binding domains suitable for use in CARs of the present disclosure include any antigen-binding polypeptide. A binding domain may comprise an antibody or antigen binding fragment thereof, including for example, a full length heavy chain, Fab fragment, Fab', $F(ab')_2$, sFv, VH domain, VL domain, dAb, VHH, CDR, and scFv, specific for a target disease antigen. In certain embodiments, a CAR binding domain is murine, chimeric, human, or humanized. In further embodiments, CAR binding domain is an scFv having V_H and V_L regions that are human or humanized. In further embodiments, the CAR binding domain is an scFv having a $(GGGS)_N$ linker or $(GGGGS)_N$ linker, wherein N=1-10, joining the V_H and V_L regions. In other embodiments, the CAR binding domain is an scFv having a linker comprising SEQ ID NO: 64 joining the V_H and V_L regions.

A target molecule that is bound by an extracellular domain of a CAR of the present disclosure may be found on or in association with a target cell. Exemplary target cells include a cancer cell, a cell associated with an autoimmune disease or disorder, a neurodegenerative disease, or with an inflammatory disease or disorder, an infectious microbe (*e.g.*, bacteria, virus, or fungi), and an infected cell (*e.g.*, virus-infected cell). A cell of an infectious organism, such as a mammalian parasite, is also contemplated as a target cell.

The extracellular domain included in a CAR as described herein can include a binding domain that targets one or more of a variety of target molecules. In certain embodiments, the CAR includes a binding domain that specifically binds a tumor antigen. In some such embodiments, the CAR binding domain specifically binds one or more of CD19, BCMA, alpha folate receptor, 5T4, Ab integrin, B7-H3, B7-H6, CAIX, CD20, CD22, CD23, CD30, CD33, CD38, CD44, CD44v6, CD44v7/8, CD52, CD70, CD79a, CD79b, CD80, CD123, CD138, CD171, CEA, CSPG4, EGFR, ErbB2 (HER2), EGFRvIII, EGP2, EGP40, EpCAM, FAP, fetal AchR, FLT3, Fra, GD2, GD3, Glypican-3 (GPC3), HLA-A1 + MAGE1, HLA-A2 + MAGE1, HLA-A3 + MAGE1, HLA-A1 + NY-ESO-1, HLA-A2 + NY-ESO-1, HLA-A3 + NY-ESO-1, HLADR, IL-11Ralpha, IL-13 Ralpha2, Lambda, Lewis-Y, Kappa, mesothelin, Muc1, Muc16, NCAM, NKG2d ligands, NY-ESO-1, PRAME, PSCA, PSMA, ROR1, SSX, Survivin, TAG72, TEMs, VEGFR2, BAFF-R, Claudin18.2, CD86, FcRL5, GPRC5, and TACI.

In certain embodiments, the extracellular domain of a CAR encoded by the transgene of the lentiviral vectors described the present disclosure optionally comprises an extracellular, non-signaling spacer or linker domain between the binding domain and the transmembrane domain. Where included, such a spacer or linker domain may position the binding domain away from the host cell surface to further enable proper cell-to-cell contact, binding, and activation. An extracellular spacer domain is generally located between the extracellular binding domain and the transmembrane domain of the CAR. The length of the extracellular spacer may be varied to optimize target molecule binding based on the selected target molecule, selected binding epitope, binding domain size and affinity (*see*, *e.g.*, Guest *et al.*, *J. Immunother.* *28*:203-11, 2005; PCT Publication No. WO 2014/031687). In certain embodiments, an extracellular spacer domain is an immunoglobulin hinge region (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, IgD). An immunoglobulin hinge region may be a wild type immunoglobulin hinge region or an altered wild type immunoglobulin hinge region. An altered IgG_4 hinge region is described in PCT Publication No. WO 2014/031687, which hinge region is incorporated herein by reference in its entirety. In some embodiments, an extracellular spacer domain comprises a modified IgG_4 hinge region having an amino acid sequence of ESKYGPPCPPCP (SEQ ID NO: 118).

Other examples of hinge regions that may be used in the CARs described herein include the hinge region from the extracellular regions of type 1 membrane proteins, such as CD8a, CD4, CD28 and CD7, which may be wild-type or variants thereof. In some embodiments, an extracellular spacer domain comprises all or a portion of an immunoglobulin Fc domain selected from: a CH1 domain, a CH2 domain, a CH3 domain, or combinations thereof (*see, e.g.*, PCT Publication WO2014/031687, which spacers are incorporated herein by reference in their entirety). In yet further embodiments, an extracellular spacer domain may comprise a stalk region of a type II C-lectin (the extracellular domain located between the C-type lectin domain and the transmembrane domain). Type II C-lectins include CD23, CD69, CD72, CD94, NKG2A, and NKG2D.

CARs of the present disclosure comprise a transmembrane domain that connects and is positioned between the extracellular domain and the intracellular signaling domain. The transmembrane domain typically ranges in length from about 15 amino acids to about 30 amino acids. The transmembrane domain is a hydrophobic alpha helix that transverses the host cell membrane and anchors the CAR in the host cell membrane. The transmembrane domain may be directly fused to the binding domain or to the extracellular spacer domain if present. In certain embodiments, the transmembrane domain is derived from an integral membrane protein (*e.g.*, receptor, cluster of differentiation (CD) molecule, enzyme, transporter, cell adhesion molecule, or the like). The transmembrane domain can be selected from the same molecule as the extracellular domain or the intracellular signaling domain. In some embodiments, the transmembrane domain and the extracellular domain are each selected from different molecules. In some embodiments, the transmembrane domain and the intracellular signaling domain are each selected from different molecules. In yet other embodiments, the transmembrane domain, the extracellular domain, and the intracellular signaling domain are each selected from different molecules.

Exemplary transmembrane domains for use in CARs of the present disclosure include a CD28, CD2, CD4, CD8a, CD5, CD3ε, CD3δ, CD3ζ, CD9, CD16, CD22, CD25, CD27, CD33, CD37, CD40, CD45, CD64, CD79A, CD79B, CD80, CD86, CD95 (Fas), CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD154 (CD40L), CD200R, CD223 (LAG3), CD270 (HVEM), CD272 (BTLA), CD273

(PD-L2), CD274 (PD-L1), CD278 (ICOS), CD279 (PD-1), CD300, CD357 (GITR), A2aR, DAP10, FcRα, FcRβ, FcRγ, Fyn, GAL9, KIR, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPα, pTα, TCRα, TCRβ, TIM3, TRIM, LPA5, and Zap70 transmembrane domain. An exemplary CD8a transmembrane domain with CD8a hinge region comprises an amino acid sequence of SEQ ID NO: 52.

The intracellular signaling domain of a CAR is an intracellular effector domain and is capable of transmitting functional signals to a cell in response to binding of the extracellular domain of the CAR to a target molecule and activates at least one of the normal effector functions or responses of the immune cell, *e.g.*, a T cell engineered to express the CAR. In some embodiments, the CAR induces a function of a T cell such as cytolytic activity or T helper activity, such as secretion of cytokines or other factors. The intracellular signaling domain may be any portion of an intracellular signaling molecule that retains sufficient signaling activity. In some embodiments, the intracellular signaling domain is obtained from an antigen receptor component (*e.g.*, TCR) or costimulatory molecule. In some embodiments, a full-length intracellular signaling domain of an antigen receptor or costimulatory molecule is used. In some embodiments, a truncated portion of an intracellular signaling domain of an antigen receptor or costimulatory molecule is used, provided that the truncated portion retains sufficient signal transduction activity. In further embodiments, an intracellular signaling domain is a variant of a full length or truncated portion of an intracellular signaling domain of an antigen receptor co stimulatory molecule, provided that the variant retains sufficient signal transduction activity (*i.e.*, is a functional variant).

In certain embodiments, the intracellular signaling domain of a CAR comprises an immunoreceptor tyrosine-based activation motif (ITAM) containing signaling domain. An ITAM containing signaling domain generally contains at least one (one, two, three, four, or more) ITAMs, which refer to a conserved motif of YXXL/I-X_{6-8}-YXXL/I. An ITAM containing signaling domain may initiate T cell activation signaling following antigen binding or ligand engagement. ITAM-signaling domains include, for example, intracellular signaling domains of CD3γ, CD3δ, CD3ε, CD3ζ, CD5, CD22, CD79a, CD278 (ICOS), DAP10, DAP12, FcRγ, and CD66d. Exemplary

CD3ζ signaling domains that may be used in CARs of the present disclosure comprise an amino acid sequence of SEQ ID NO: 56 or SEQ ID NO: 122.

CAR intracellular signaling domains optionally comprise a costimulatory signaling domain, which, when activated in conjunction with a primary or classic (*e.g.*, ITAM-driven) activation signal, promotes or enhances T cell response, such as T cell activation, cytokine production, proliferation, differentiation, survival, effector function, or combinations thereof. Costimulatory signaling domains for use in CARs include, for example, CD27, CD28, CD40L, GITR, NKG2C, CARD1, CD2, CD7, CD27, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX-40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD226, CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, LFA-1, LIGHT, NKG2C, NKD2C, SLP76, TRIM, ZAP70, or any combination thereof. In a particular embodiment, the costimulatory signaling domain comprises a OX40, CD2, CD27, CD28, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), or 4-1BB (CD137) signaling domain. An exemplary 4-1BB costimulatory signaling domain comprises an amino acid sequence of SEQ ID NO: 54. In certain embodiments, a CAR comprises one, two, or more costimulatory signaling domains.

In some embodiments, a CAR of the present disclosure is a first generation CAR, a second generation CAR, or a third generation CAR. A first generation CAR generally has an intracellular signaling domain comprising an intracellular signaling domain of CD3ζ, FcγRI, or other ITAM-containing activating domain to provide a T cell activation signal. Second generation CARs further comprise a costimulatory signaling domain (*e.g.*, a costimulatory signaling domain from an endogenous T cell costimulatory receptor, such as CD28, 4-1BB, or ICOS). Third generation CARs comprise an ITAM-containing activating domain, a first costimulatory signaling domain and a second costimulatory signaling domain.

In some embodiments, one or more of the extracellular domain, the binding domain, the linker, the transmembrane domain, the intracellular signaling domain, or the costimulatory domain comprises junction amino acids. "Junction amino acids" or "junction amino acid residues" refer to one or more (*e.g.*, about 2-20) amino acid residues between two adjacent domains, motifs, regions, modules, or fragments of a protein, such as between a binding domain and an adjacent linker, between a

transmembrane domain and an adjacent extracellular or intracellular domain, or on one or both ends of a linker that links two domains, motifs, regions, modules, or fragments (e.g., between a linker and an adjacent binding domain or between a linker and an adjacent hinge). Junction amino acids may result from the construct design of a fusion protein (e.g., amino acid residues resulting from the use of a restriction enzyme site or self-cleaving peptide sequences during the construction of a polynucleotide encoding a fusion protein). For example, a transmembrane domain of a fusion protein may have one or more junction amino acids at the amino-terminal end, carboxy -terminal end, or both.

In specific embodiments, the transgene encodes an anti-CD19 CAR molecule. In such embodiments, the extracellular binding domain may include an scFv as detailed in Table 4, having an anti-CD19 light chain variable region of SEQ ID NO: 46, a G4S linker of SEQ ID NO: 48, and an anti-CD19 heavy chain variable region of SEQ ID NO: 50. An anti-CD19 CAR useful in the context of the present description may include a CD8a hinge and transmembrane domain of SEQ ID NO: 52. In certain embodiments, the anti-CD19 CAR includes a 4-1BB costimulatory domain according to SEQ ID NO: 54 and a CD3ζ effector domain according to SEQ ID NO: 56. In a specific embodiment, an anti-CD19 CAR encoded by the transgene has an amino acid sequence according to SEQ ID NO: 42 or SEQ ID NO: 42 absent the signal peptide of SEQ ID NO: 44.

In other embodiments, the transgene encodes an anti-CD19 CAR molecule and extracellular binding domain may include an scFv having an anti-CD19 light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 46, and an anti-CD19 heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 50. An anti-CD19 CAR useful in the context of the present description may include a CD8a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 52. In certain embodiments, the anti-CD19 CAR includes a 4-1BB costimulatory domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 54 and a CD3ζ effector

domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 56. In a specific embodiment, an anti-CD19 CAR encoded by the transgene has an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 42 or SEQ ID NO: 42 absent the signal peptide of SEQ ID NO: 44.

TABLE 4: Anti-CD19 CAR molecule

Full molecule	
Nucleotide	**Amino acid (signal peptide underlined)**
ATGGCCTTACCAGTGACCGCCTTG	MALPVTALLLPLALLLHAARPDIQM
CTCCTGCCGCTGGCCTTGCTGCTCC	TQTTSSLSASLGDRVTISCRASQDISK
ACGCCGCCAGGCCGGACATCCAGA	YLNWYQQKPDGTVKLLIYHTSRLHS
TGACACAGACTACATCCTCCCTGT	GVPSRFSGSGSGTDYSLTISNLEQEDI
CTGCCTCTCTGGGAGACAGAGTCA	ATYFCQQGNTLPYTFGGGTKLEITG
CCATCAGTTGCAGGGCAAGTCAGG	GGGSGGGGSGGGGSEVKLQESGPG
ACATTAGTAAATATTTAAATTGGT	LVAPSQSLSVTCTVSGVSLPDYGVS
ATCAGCAGAAACCAGATGGAACTG	WIRQPPRKGLEWLGVIWGSETTYYN
TTAAACTCCTGATCTACCATACATC	SALKSRLTIIKDNSKSQVFLKMNSLQ
AAGATTACACTCAGGAGTCCCATC	TDDTAIYYCAKHYYYGGSYAMDY
AAGGTTCAGTGGCAGTGGGTCTGG	WGQGTSVTVSSTTTPAPRPPTPAPTI
AACAGATTATTCTCTCACCATTAG	ASQPLSLRPEACRPAAGGAVHTRGL
CAACCTGGAGCAAGAAGATATTGC	DFACDIYIWAPLAGTCGVLLLSLVIT
CACTTACTTTTGCCAACAGGGTAA	LYCKRGRKKLLYIFKQPFMRPVQTT
TACGCTTCCGTACACGTTCGGAGG	QEEDGCSCRFPEEEEGGCELRVKFSR
GGGGACCAAGCTGGAGATCACAG	SADAPAYKQGQNQLYNELNLGRRE
GTGGaGGTGGaTCGGGCGGTGGTG	EYDVLDKRRGRDPEMGGKPRRKNP
GGTCGGGTGGCGGCGGATCTGAGG	QEGLYNELQKDKMAEAYSEIGMKG
TGAAACTGCAGGAGTCAGGACCTG	ERRRGKGHDGLYQGLSTATKDTYD
GCCTGGTGGCGCCCTCACAGAGCC	ALHMQALPPR (SEQ ID NO: 42)
TGTCCGTCACATGCACTGTCTCAG	
GGGTCTCATTACCCGACTATGGTG	
TAAGCTGGATTCGCCAGCCTCCAC	
GAAAGGGTCTGGAGTGGCTGGGA	
GTAATATGGGGTAGTGAAACCACA	
TACTATAATTCAGCTCTCAAATCC	
AGACTGACCATCATCAAGGACAAC	
TCCAAGAGCCAAGTTTTCTTAAAA	
ATGAACAGTCTGCAAACTGATGAC	
ACAGCCATTTACTACTGTGCCAAA	
CATTATTACTACGGTGGTAGCTAT	
GCTATGGACTACTGGGGCCAAGGA	
ACCTCAGTCACCGTCTCCTCAACC	
ACtACaCCAGCaCCtaGACCACCAAC	
ACCtGCGCCaACCATCGCaTCGCAG	
CCaCTGTCtCTGCGCCCAGAGGCaT	

GCCGGCCAGCaGCtGGGGGCGCAG TGCACACaAGGGGGCTGGACTTCG CaTGTGATATCTACATCTGGGCaCC aTTGGCaGGGACTTGTGGGGTCCTT CTCCTGTCACTGGTTATCACCCTTT ACTGCAAACGGGGCAGAAAGAAA CTCCTGTATATATTCAAACAACCA TTTATGAGACCAGTACAAACTACT CAAGAGGAAGATGGCTGTAGCTGC CGATTTCCAGAAGAAGAAGAAGG AGGATGTGAACTGAGAGTGAAGTT CAGCAGGAGCGCAGACGCCCCCGC GTACAAGCAGGGCCAGAACCAGCT CTATAACGAGCTCAATCTAGGACG AAGAGAGGAGTACGATGTTTTGGA CAAGAGACGTGGCCGGGACCCTGA GATGGGGGGAAAGCCGAGAAGGA AGAACCCTCAGGAAGGCCTGTACA ATGAACTGCAGAAAGATAAGATG GCGGAGGCCTACAGTGAGATTGGG ATGAAAGGCGAGCGCCGGAGGGG CAAGGGGCACGATGGCCTTTACCA GGGTCTCAGTACAGCCACCAAGGA CACCTACGACGCCCTTCACATGCA GGCCCTGCCCCCTCGC (SEQ ID NO: 41)	
CD8a signal peptide	
Nucleotide	**Amino acid**
ATGGCCTTACCAGTGACCGCCTTG CTCCTGCCGCTGGCCTTGCTGCTCC ACGCCGCCAGGCCG (SEQ ID NO: 43)	MALPVTALLLPLALLLHAARP (SEQ ID NO: 44)
Anti-CD19 light chain variable region	
Nucleotide	**Amino acid**
GACATCCAGATGACACAGACTACA TCCTCCCTGTCTGCCTCTCTGGGAG ACAGAGTCACCATCAGTTGCAGGG CAAGTCAGGACATTAGTAAATATT TAAATTGGTATCAGCAGAAACCAG ATGGAACTGTTAAACTCCTGATCT ACCATACATCAAGATTACACTCAG GAGTCCCATCAAGGTTCAGTGGCA GTGGGTCTGGAACAGATTATTCTC TCACCATTAGCAACCTGGAGCAAG AAGATATTGCCACTTACTTTTGCCA ACAGGGTAATACGCTTCCGTACAC	DIQMTQTTSSLSASLGDRVTISCRAS QDISKYLNWYQQKPDGTVKLLIYHT SRLHSGVPSRFSGSGSGTDYSLTISN LEQEDIATYFCQQGNTLPYTFGGGT KLEIT (SEQ ID NO: 46)

GTTCGGAGGGGGGACCAAGCTGG AGATCACA (SEQ ID NO: 45)	
G_4S Linker	
Nucleotide	**Amino acid**
GGTGGaGGTGGaTCGGGCGGTGGT GGGTCGGGTGGCGGCGGATCT (SEQ ID NO: 47)	GGGGSGGGGSGGGGS (SEQ ID NO: 48)
Anti-CD19 heavy chain variable region	
Nucleotide	**Amino acid**
GAGGTGAAACTGCAGGAGTCAGG ACCTGGCCTGGTGGCGCCCTCACA GAGCCTGTCCGTCACATGCACTGT CTCAGGGGTCTCATTACCCGACTA TGGTGTAAGCTGGATTCGCCAGCC TCCACGAAAGGGTCTGGAGTGGCT GGGAGTAATATGGGGTAGTGAAAC CACATACTATAATTCAGCTCTCAA ATCCAGACTGACCATCATCAAGGA CAACTCCAAGAGCCAAGTTTTCTT AAAAATGAACAGTCTGCAAACTGA TGACACAGCCATTTACTACTGTGC CAAACATTATTACTACGGTGGTAG CTATGCTATGGACTACTGGGGCCA AGGAACCTCAGTCACCGTCTCCTC A (SEQ ID NO: 49)	EVKLQESGPGLVAPSQSLSVTCTVS GVSLPDYGVSWIRQPPRKGLEWLGV IWGSETTYYNSALKSRLTIIKDNSKS QVFLKMNSLQTDDTAIYYCAKHYY YGGSYAMDYWGQGTSVTVSS (SEQ ID NO: 50)
CD8a Hinge and Transmembrane	
Nucleotide	**Amino acid**
ACCACTACACCAGCACCTAGACCA CCAACACCTGCGCCAACCATCGCA TCGCAGCCACTGTCTCTGCGCCCA GAGGCATGCCGGCCAGCAGCTGGG GGCGCAGTGCACACAAGGGGGCT GGACTTCGCATGTGATATCTACAT CTGGGCACCATTGGCAGGGACTTG TGGGGTCCTTCTCCTGTCACTGGTT ATCACCCTTTACTGC (SEQ ID NO: 51)	TTTPAPRPPTPAPTIASQPLSLRPEAC RPAAGGAVHTRGLDFACDIYIWAPL AGTCGVLLLSLVITLYC (SEQ ID NO: 52)
4-1BB costimulatory domain	
Nucleotide	**Amino acid**
AAACGGGGCAGAAAGAAACTCCT GTATATATTCAAACAACCATTTAT GAGACCAGTACAAACTACTCAAGA GGAAGATGGCTGTAGCTGCCGATT	KRGRKKLLYIFKQPFMRPVQTTQEE DGCSCRFPEEEEGGCEL (SEQ ID NO: 54)

TCCAGAAGAAGAAGAAGGAGGAT GTGAACTG (SEQ ID NO: 53)	
CD3 zeta effector domain #1	
Nucleotide	**Amino acid**
AGAGTGAAGTTCAGCAGGAGCGC AGACGCCCCCGCGTACAAGCAGGG CCAGAACCAGCTCTATAACGAGCT CAATCTAGGACGAAGAGAGGAGT ACGATGTTTTGGACAAGAGACGTG GCCGGGACCCTGAGATGGGGGGA AAGCCGAGAAGGAAGAACCCTCA GGAAGGCCTGTACAATGAACTGCA GAAAGATAAGATGGCGGAGGCCT ACAGTGAGATTGGGATGAAAGGC GAGCGCCGGAGGGGCAAGGGGCA CGATGGCCTTTACCAGGGTCTCAG TACAGCCACCAAGGACACCTACGA CGCCCTTCACATGCAGGCCCTGCC CCCTCGC (SEQ ID NO: 55)	RVKFSRSADAPAYKQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYS EIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR (SEQ ID NO: 56)

In other embodiments, the transgene encodes an anti-BCMA CAR molecule. In such embodiments, the extracellular binding domain may include an scFv as detailed in Table 5, having an anti-BCMA light chain variable region of SEQ ID NO: 62, a linker of SEQ ID NO: 64, and an anti-BCMA heavy chain variable region of SEQ ID NO: 66. An anti-BCMA CAR useful in the context of the present description may include a CD8a hinge and transmembrane domain of SEQ ID NO: 52. In certain embodiments, the anti-BCMA CAR includes a 4-1BB costimulatory domain according to SEQ ID NO: 54 and a CD3ζ effector domain according to SEQ ID NO: 122. In a specific embodiment, an anti-BCMA CAR encoded by the transgene has an amino acid sequence according to SEQ ID NO: 58 or SEQ ID NO: 58 absent the signal peptide of SEQ ID NO: 60.

In some embodiments, the transgene encodes an anti-BCMA CAR molecule. In such embodiments, the extracellular binding domain may include an scFv having an anti-BCMA light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 62, and an anti-BCMA heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 66. An anti-BCMA CAR useful in the context of the present

description may include a CD8a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 52. In certain embodiments, the anti-BCMA CAR includes a 4-1BB costimulatory domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 54 and a CD3ζ effector domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 122. In a specific embodiment, an anti-BCMA CAR encoded by the transgene has an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 58 or SEQ ID NO: 58 absent the signal peptide of SEQ ID NO: 60.

TABLE 5: Anti-BCMA CAR molecule

Full molecule	
Nucleotide	**Amino acid (signal peptide underlined)**
ATGGCACTCCCCGTCACCGCCCTT CTCTTGCCCCTCGCCCTGCTGCTGC ATGCTGCCAGGCCCGACATTGTGC TCACTCAGTCACCTCCCAGCCTGG CCATGAGCCTGGGAAAAAGGGCC ACCATCTCCTGTAGAGCCAGTGAG TCCGTCACAATCTTGGGGAGCCAT CTTATTCACTGGTATCAGCAGAAG CCCGGGCAGCCTCCAACCCTTCTT ATTCAGCTCGCGTCAAACGTCCAG ACGGGTGTACCTGCCAGATTTTCT GGTAGCGGGTCCCGCACTGATTTT ACACTGACCATAGATCCAGTGGAA GAAGACGATGTGGCCGTGTATTAT TGTCTGCAGAGCAGAACGATTCCT CGCACATTTGGTGGGGGTACTAAG CTGGAGATTAAGGGAAGCACGTCC GGCTCAGGGAAGCCGGGCTCCGGC GAGGGAAGCACGAAGGGGCAAAT TCAGCTGGTCCAGAGCGGACCTGA GCTGAAAAAACCCGGCGAGACTGT TAAGATCAGTTGTAAAGCATCTGG CTATACCTTCACCGACTACAGCAT AAATTGGGTGAAACGAGCCCCTGG AAAGGGCCTCAAATGGATGGGTTG GATCAATACCGAAACTAGGGAGCC TGCTTATGCATATGACTTCCGCGG	MALPVTALLLPLALLLHAARPDIVL TQSPPSLAMSLGKRATISCRASESVTI LGSHLIHWYQQKPGQPPTLLIQLASN VQTGVPARFSGSGSRTDFTLTIDPVE EDDVAVYYCLQSRTIPRTFGGGTKL EIKGSTSGSGKPGSGEGSTKGQIQLV QSGPELKKPGETVKISCKASGYTFTD YSINWVKRAPGKGLKWMGWINTET REPAYAYDFRGRFAFSLETSASTAY LQINNLKYEDTATYFCALDYSYAM DYWGQGTSVTVSSAAATTTPAPRPP TPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLL LSLVITLYCKRGRKKLLYIFKQPFMR PVQTTQEEDGCSCRFPEEEEGGCELR VKFSRSADAPAYQQGQNQLYNELN LGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR (SEQ ID NO: 58)

GAGATTCGCCTTTTCACTCGAGAC ATCTGCCTCTACTGCTTACCTCCAA ATAAACAACCTCAAGTATGAAGAT ACAGCCACTTACTTTTGCGCCCTCG ACTATAGTTACGCCATGGACTACT GGGGACAGGGAACCTCCGTTACCG TCAGTTCCGCGGCCGCAACCACAA CACCTGCTCCAAGGCCCCCCACAC CCGCTCCAACTATAGCCAGCCAAC CATTGAGCCTCAGACCTGAAGCTT GCAGGCCCGCAGCAGGAGGCGCC GTCCATACGCGAGGCCTGGACTTC GCGTGTGATATTTATATTTGGGCA CCTTTGGCCGGAACATGTGGGGTG TTGCTTCTCTCCCTTGTGATCACTC TGTATTGTAAGCGCGGGAGAAAGA AGCTCCTGTACATCTTCAAGCAGC CTTTTATGCGACCTGTGCAAACCA CTCAGGAAGAAGATGGGTGTTCAT GCCGCTTCCCCGAGGAGGAAGAAG GAGGGTGTGAACTGAGGGTGAAAT TTTCTAGAAGCGCCGATGCTCCCG CATATCAGCAGGGTCAGAATCAGC TCTACAATGAATTGAATCTCGGCA GGCGAGAAGAGTACGATGTTCTGG ACAAGAGACGGGGCAGGGATCCC GAGATGGGGGGAAAGCCCCGGAG AAAAAATCCTCAGGAGGGGTTGTA CAATGAGCTGCAGAAGGACAAGA TGGCTGAAGCCTATAGCGAGATCG GAATGAAAGGCGAAAGACGCAGA GGCAAGGGGCATGACGGTCTGTAC CAGGGTCTCTCTACAGCCACCAAG GACACTTATGATGCGTTGCATATG CAAGCCTTGCCACCCCGC (SEQ ID NO: 57)	
CD8a signal peptide	
Nucleotide	**Amino acid**
ATGGCACTCCCCGTCACCGCCCTT CTCTTGCCCCTCGCCCTGCTGCTGC ATGCTGCCAGGCCC (SEQ ID NO: 59)	MALPVTALLLPLALLLHAARP (SEQ ID NO: 60)
Anti-BCMA light chain variable region	
Nucleotide	**Amino acid**
GACATTGTGCTCACTCAGTCACCT CCCAGCCTGGCCATGAGCCTGGGA AAAAGGGCCACCATCTCCTGTAGA GCCAGTGAGTCCGTCACAATCTTG	DIVLTQSPPSLAMSLGKRATISCRAS ESVTILGSHLIHWYQQKPGQPPTLLI QLASNVQTGVPARFSGSGSRTDFTL

GGGAGCCATCTTATTCACTGGTAT CAGCAGAAGCCCGGGCAGCCTCCA ACCCTTCTTATTCAGCTCGCGTCAA ACGTCCAGACGGGTGTACCTGCCA GATTTTCTGGTAGCGGGTCCCGCA CTGATTTTACACTGACCATAGATC CAGTGGAAGAAGACGATGTGGCC GTGTATTATTGTCTGCAGAGCAGA ACGATTCCTCGCACATTTGGTGGG GGTACTAAGCTGGAGATTAAG (SEQ ID NO: 61)	TIDPVEEDDVAVYYCLQSRTIPRTFG GGTKLEIK (SEQ ID NO: 62)
218 Linker	
Nucleotide	**Amino acid**
GGAAGCACGTCCGGCTCAGGGAA GCCGGGCTCCGGCGAGGGAAGCA CGAAGGGG (SEQ ID NO: 63)	GSTSGSGKPGSGEGSTKG (SEQ ID NO: 64)
Anti-BCMA heavy chain variable region	
Nucleotide	**Amino acid**
CAAATTCAGCTGGTCCAGAGCGGA CCTGAGCTGAAAAAACCCGGCGAG ACTGTTAAGATCAGTTGTAAAGCA TCTGGCTATACCTTCACCGACTAC AGCATAAATTGGGTGAAACGAGCC CCTGGAAAGGGCCTCAAATGGATG GGTTGGATCAATACCGAAACTAGG GAGCCTGCTTATGCATATGACTTC CGCGGGAGATTCGCCTTTTCACTC GAGACATCTGCCTCTACTGCTTAC CTCCAAATAAACAACCTCAAGTAT GAAGATACAGCCACTTACTTTTGC GCCCTCGACTATAGTTACGCCATG GACTACTGGGGACAGGGAACCTCC GTTACCGTCAGTTCC (SEQ ID NO: 65)	QIQLVQSGPELKKPGETVKISCKASG YTFTDYSINWVKRAPGKGLKWMG WINTETREPAYAYDFRGRFAFSLETS ASTAYLQINNLKYEDTATYFCALDY SYAMDYWGQGTSVTVSS (SEQ ID NO: 66)
CD8a Hinge and Transmembrane	
Nucleotide	**Amino acid**
ACCACAACACCTGCTCCAAGGCCC CCCACACCCGCTCCAACTATAGCC AGCCAACCATTGAGCCTCAGACCT GAAGCTTGCAGGCCCGCAGCAGGA GGCGCCGTCCATACGCGAGGCCTG GACTTCGCGTGTGATATTTATATTT GGGCACCTTTGGCCGGAACATGTG GGGTGTTGCTTCTCTCCCTTGTGAT CACTCTGTATTGT (SEQ ID NO: 119)	TTTPAPRPPTPAPTIASQPLSLRPEAC RPAAGGAVHTRGLDFACDIYIWAPL AGTCGVLLLSLVITLYC (SEQ ID NO: 52)
4-1BB costimulatory domain	
Nucleotide	**Amino acid**

AAGCGCGGGAGAAAGAAGCTCCT GTACATCTTCAAGCAGCCTTTTATG CGACCTGTGCAAACCACTCAGGAA GAAGATGGGTGTTCATGCCGCTTC CCCGAGGAGGAAGAAGGAGGGTG TGAACTG (SEQ ID NO: 120)	KRGRKKLLYIFKQPFMRPVQTTQEE DGCSCRFPEEEEGGCEL (SEQ ID NO: 54)
CD3 zeta effector domain #2	
Nucleotide	**Amino acid**
AGGGTGAAATTTTCTAGAAGCGCC GATGCTCCCGCATATCAGCAGGGT CAGAATCAGCTCTACAATGAATTG AATCTCGGCAGGCGAGAAGAGTAC GATGTTCTGGACAAGAGACGGGGC AGGGATCCCGAGATGGGGGGAAA GCCCCGGAGAAAAAATCCTCAGGA GGGGTTGTACAATGAGCTGCAGAA GGACAAGATGGCTGAAGCCTATAG CGAGATCGGAATGAAAGGCGAAA GACGCAGAGGCAAGGGGCATGAC GGTCTGTACCAGGGTCTCTCTACA GCCACCAAGGACACTTATGATGCG TTGCATATGCAAGCCTTGCCACCC CGC (SEQ ID NO: 121)	RVKFSRSADAPAYQQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYS EIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR (SEQ ID NO: 122)

CARs of the present disclosure may comprise polynucleotide sequences derived from any mammalian species, including humans, primates, cows, horses, goats, sheep, dogs, cats, mice, rats, rabbits, guinea pigs, pigs, transgenic species thereof, or any combination thereof. In some embodiments, the chimeric antigen receptor is murine, chimeric, human, or humanized.

Methods for Producing Lentiviral Vectors

Methods for producing the LVV disclosed are provided herein. LVV have been developed and based on the retroviral genome by combining its components into recombinant plasmid DNA vectors. The plasmid DNA vectors can then be transfected into producer cell lines to transfer genes required for lentiviral particle production. LVV packaging systems generally comprise a transfer plasmid encoding the transgene of interest, an envelope plasmid (e.g., VSV-G), and packaging plasmid(s). Second generation LVV packaging systems contain a single packaging plasmid encoding Gag, Pol, Rev, and Tat genes and a separate Env plasmid. In some embodiments, the methods described herein utilize a third generation vector system for producing LVV.

Third generation vector production systems improve upon the safety of 2nd generation LVV packaging systems and are typically four plasmid systems that include a transgene plasmid combined with three packaging plasmids, VSV-G, GagPol, and Rev. Thus, Rev and GagPol are separated into two plasmids. Tat is also eliminated from 3rd generation LVV packaging systems by the addition of a chimeric 5' LTR fused to a heterologous promoter (e.g., CMV or RSV promoter) on the transfer plasmid. The *gag* gene encodes a Gag polyprotein precursor comprising structural proteins of the lentivirus, including matrix, capsid, and nucleocapsid. The *pol* gene encodes a Pol polyprotein precursor supplying enzymatic functions of the lentivirus essential for replication, including protease, reverse transcriptase, and integrase. The *rev* gene encodes the Rev protein which binds to the Rev Response Element (RRE) to allow nuclear export of unspliced and singly spliced HIV RNA during viral replication. The Gag and Pol polyprotein precursors are cleaved during production of viral particles.

Producer cells that may be used for making LVV of the present disclosure include human embryonic kidney (HEK) 293 cells and derivatives thereof. Producer cells may be an adherent cell line, such as HEK293T producer cells, or a suspension cell line, such as HEK293T/17 SF producer cells.

The transgene plasmid is combined with the three packaging plasmids, GagPol plasmid, VSV-G env plasmid, and Rev plasmid, and used to transfect producer cells, such as HEK293 producer cells. In some embodiments, the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid. In some embodiments, the ratios of the packaging plasmids are determined by mass. In some embodiments, the mass of each of the transgene plasmid and GagPol plasmid are higher than the the mass of each of the VSV-G env plasmid and Rev plasmid. In some embodiments, the defined ratio of transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid is at about 1:1:1:1 to about 5:4:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 2:1:1:1 to about 5:4:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 2:1:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid,

VSV-G env plasmid, and Rev plasmid at about 3:1:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 3.125:3.125:2.5:1.25. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 4:2:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 5:4:1:1. Productive lentiviral particles are harvested from the producer cell culture media. Exemplary materials and methods for producing LVV particles are described in *Production of Lentiviral Vectors*, Merten et al., Molecular Therapy – Methods & Clinical Development (2016), 3, 16017, the contents of which are incorporated herein by reference. Third generation production systems are used for research and development and clinical purposes. Schematic representations of helper plasmids suitable for use in a third generation LVV production system are provided in **FIG. 1**.

The transgene plasmid encodes the only genetic materials that is transferred to the lymphocytes (e.g., T cells or NK cells) targeted by the resulting LVV and comprises a lentiviral backbone containing the transgene expression cassette flanked by cis-acting elements for encapsidation, reverse transcription and integration. In specific embodiments, the transgene plasmid includes a deletion in the U3 element of the 3' LTR that causes loss of transcriptional capacity of the viral LTR once transferred into the target cells. The expression cassette included in the transgene plasmid may encode a single heterologous protein (*e.g.*, a single CAR as described herein) or multiple heterologous proteins (e.g., multiple CARs as described herein) for introduction into and expression by the target cell.

In some embodiments of a four plasmid LVV system, the VSV-G env plasmid includes a tandem expression cassette that encodes a mutated VSV-G envelope protein and a targeting protein as disclosed herein. In specific embodiments, the tandem expression cassette included in the VSV-G env plasmid includes a polynucleotide that encodes a first signal peptide, a polynucleotide that encodes a targeting protein, a polynucleotide that encodes one of an internal ribosomal entry site (IRES), a furin cleavage site, or a viral 2A peptide, a polynucleotide that encodes a second signal peptide, and a polynucleotide that encodes a mutated VSV-G envelope protein. In

certain embodiments, the polynucleotide encoding the mutant VSV-G envelope protein is positioned 5' to the polynucleotide encoding the targeting protein. In other embodiments, the polynucleotide encoding the mutant VSV-G envelope protein is positioned 3' to the polynucleotide encoding the targeting protein. The polynucleotide that encodes the targeting protein and the polynucleotide that encodes the mutated VSV-G are separated in the tandem cassette by the polynucleotide that encodes the IRES, furin cleavage site, or viral 2A peptide, which allows for co-expression of the two proteins from a single mRNA. In certain embodiments, a viral 2A peptide is a porcine teschovirus-1 (P2A), *Thosea asigna* virus (T2A), equine rhinitis A virus (E2A), foot-and-mouth disease virus (F2A), or a variant thereof. Specific examples of amino acid and nucleotide sequences for the viral 2A peptides are provided in Table 7.

Table 7: 2A sequences

T2A peptide from Thosea asigna virus capsid protein	EGRGSLLTCGD VEENPGP (SEQ ID NO: 79)	GAGGGCAGGGGAAGTCTTC TAACATGCGGGGACGTGGA GGAAAATCCCGGCCCC (SEQ ID NO: 80)
P2A peptide from porcine teschovirus-1 polyprotein	ATNFSLLKQAG DVEENPGP (SEQ ID NO: 81)	GCCACGAACTTCTCTCTGTT AAAGCAAGCAGGAGACGT GGAAGAAAACCCCGGTCCT (SEQ ID NO: 82)
F2A peptide from foot-and-mouth disease virus polyprotein	VKQTLNFDLLK LAGDVESNPGP (SEQ ID NO: 83)	GTGAAACAGACTTTGAATT TTGACCTTCTCAAGTTGGC GGGAGACGTCGAGTCCAAC CCTGGGCCC (SEQ ID NO: 84)
E2A peptide from equine rhinitis A virus polyprotein	QCTNYALLKLA GDVESNPGP (SEQ ID NO: 85)	CAGTGTACTAATTATGCTC TCTTGAAATTGGCTGGAGA TGTTGAGAGCAACCCAGGT CCC (SEQ ID NO: 86)

In certain embodiments, the present disclosure provides a method for producing a lymphocyte targeted lentiviral vector according to any one of the preceding claims, the method comprising: transfecting a producer cell with a GagPol plasmid, a Rev plasmid, a transgene plasmid, and a VSV-G env plasmid, wherein; the GagPol plasmid comprises a one or more polynucleotides encoding a lentiviral gag gene and a lentiviral pol gene and is capable of expressing a lentiviral gag polyprotein and a lentiviral pol polyprotein within the producer cell;

the Rev plasmid comprises a polynucleotide encoding a lentiviral rev gene and is capable expressing a lentiviral rev protein within the producer cell; the transgene plasmid comprises an expression cassette comprising a polynucleotide encoding a CAR; and the VSV-G env plasmid comprises a tandem expression cassette, wherein the tandem expression cassette comprises a polynucleotide encoding a mutated VSV-G envelope protein as disclosed herein and a polynucleotide encoding a lymphocyte targeting protein as disclosed herein and the VSV-G env plasmid is capable of expressing the mutated VSV-G envelope protein and the lymphocyte targeting protein within the producer cell; culturing the producer cell in a culture medium; and harvesting lentiviral vector from the culture medium.

In some embodiments, the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid. In some embodiments, the defined ratio of transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 1:1:1:1 to about 5:4:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 2:1:1:1 to about 5:4:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 2:1:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 3:1:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 3.125:3.125:2.5:1.25. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 4:2:1:1. In some embodiments, the the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, and Rev plasmid at about 5:4:1:1.

In one embodiment, the tandem expression cassette of the VSV-G env plasmid encodes an anti-CD3 targeting protein and a mutated VSV-G envelope protein. For example, in a specific embodiment, the tandem expression cassette may comprise a polynucleotide that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 67, which encodes an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 68. Such an embodiment is described in more detail in Table 8. In another embodiment, the tandem expression cassette may comprise a polynucleotide that has about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 67 absent the signal peptide of SEQ ID NO: 31, which encodes an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 68 absent the signal peptide of SEQ ID NO: 32. As illustrated in Table 8, the anti-CD3 targeting protein is separated from the mutated VSV-G envelope protein via a P2A self-cleaving peptide. In certain embodiments, that P2A peptide may be encoded by a nucleic acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 69, and the P2A peptide may have an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 70.

TABLE 8: Anti-CD3 targeting molecule co-expressed with mutant VSV-G

Full molecule	
Nucleotide	**Amino acid (signal peptide underlined)**
ATGGCCTTACCAGTGACCGCCTTG	MALPVTALLLPLALLLHAARPDIQM
CTCCTGCCGCTGGCCTTGCTGCTCC	TQTTSSLSASLGDRVTISCRASQDIR
ACGCCGCCAGGCCGGACATCCAGA	NYLNWYQQKPDGTVKLLIYYTSRL
TGACCCAGACCACCTCCTCCCTGT	HSGVPSKFSGSGSGTDYSLTISNLEQ
CTGCCTCTCTGGGAGACAGAGTCA	EDIATYFCQQGNTLPWTFAGGTKLE
CCATCAGTTGCAGGGCAAGTCAGG	IKRAGGGSGGGSGGGSGGGSEVQLQ
ACATTAGAAATTATTTAAACTGGT	QSGPELVKPGASMKISCKASGYSFT
ATCAACAGAAACCAGATGGAACTG	GYTMNWVKQSHGKNLEWMGLINP
TTAAACTCCTGATCTACTACACATC	YKGVSTYNQKFKDKATLTVDKSSST
AAGATTACACTCAGGAGTCCCATC	AYMELLSLTSEDSAVYYCARSGYY
AAAGTTCAGTGGCAGTGGGTCTGG	GDSDWYFDVWGAGTTVTVSSTTTP
AACAGATTATTCTCTCACCATTAG	APRPPTPAPTIASQPLSLRPEACRPAA
CAACCTGGAGCAAGAGGATATTGC	GGAVHTRGLDFACDIYIWAPLAGTC
CACTTACTTTTGCCAACAGGGTAA	GVLLLSLVITLYCGSGATNFSLLKQA
TACGCTTCCGTGGACGTTCGCTGG	GDVEENPGPMKCLLYLAFLFIGVNC
AGGCACCAAGCTGGAAATCAAAC	KFTIVFPHNQKGNWKNVPSNYHYCP
GGGCTGGAGGCGGTAGTGGCGGTG	SSSDLNWHNDLIGTALQVKMPQSH
GATCAGGTGGAGGCAGCGGTGGC	KAIQADGWMCHASKWVTTCDFRW
GGATCTGAGGTGCAGCTCCAGCAG	YGPKYITHSIRSFTPSVEQCKESIEQT
TCTGGACCTGAGCTGGTGAAGCCT	KQGTWLNPGFPPQSCGYATVTDAE
GGAGCTTCAATGAAGATATCCTGC	AVIVQVTPHHVLVDEYTGEWVDSQ
AAGGCTTCTGGTTACTCATTCACTG	FINGKCSNYICPTVHNSTTWHSDYK
GCTACACCATGAACTGGGTGAAGC	VKGLCDSNLISMDITFFSEDGELSSL

AGAGTCATGGAAAGAACCTTGAGT GGATGGGACTTATTAATCCTTACA AAGGTGTTAGTACCTACAACCAGA AGTTCAAGGACAAGGCCACATTAA CTGTAGACAAGTCATCCAGCACAG CCTACATGGAACTCCTCAGTCTGA CATCTGAGGACTCTGCAGTCTATT ACTGTGCAAGATCGGGGTACTACG GTGATAGTGACTGGTACTTCGATG TCTGGGGCGCAGGGACCACGGTCA CCGTCTCCTCAACCACTACACCAG CACCTAGACCACCAACACCTGCGC CAACCATCGCATCGCAGCCACTGT CTCTGCGCCCAGAGGCATGCCGGC CAGCAGCTGGGGGCGCAGTGCACA CAAGGGGGCTGGACTTCGCATGTG ATATCTACATCTGGGCACCATTGG CAGGGACTTGTGGGGTCCTTCTCC TGTCACTGGTTATCACCCTTTACTG CGGCAGCGGCGCCACCAACTTCTC CCTGCTGAAGCAGGCCGGCGACGT GGAAGAAAACCCTGGCCCCATGAA GTGTCTGCTGTACCTGGCGTTCCTG TTTATCGGGGTGAACTGCAAGTTC ACTATCGTGTTTCCGCACAACCAA AAGGGCAACTGGAAAAACGTGCCT TCAAATTACCATTATTGCCCCAGC AGCTCGGACCTGAACTGGCACAAT GACCTCATTGGAACCGCGCTGCAG GTGAAGATGCCACAGAGCCACAA GGCTATCCAGGCTGACGGATGGAT GTGCCACGCGTCAAAATGGGTGAC TACCTGCGATTTCCGCTGGTACGG ACCAAAATACATCACGCACAGCAT CAGATCATTCACCCCGTCAGTGGA ACAATGCAAAGAATCCATCGAACA GACTAAGCAGGGAACCTGGCTGAA CCCTGGATTTCCGCCGCAGTCGTG TGGGTACGCAACCGTGACCGATGC AGAGGCCGTGATCGTGCAAGTCAC GCCGCATCACGTGCTTGTGGACGA GTACACCGGAGAATGGGTCGATTC CCAGTTCATCAACGGCAAGTGCTC CAACTACATTTGCCCAACCGTGCA CAACAGCACTACTTGGCATAGCGA CTACAAAGTGAAGGGTCTGTGTGA TTCCAACCTGATCTCCATGGATATC ACTTTCTTCTCGGAAGACGGCGAA CTGTCCTCACTGGGCAAAGAAGGA	GKEGTGFRSNYFAYETGGKACKMQ YCKHWGVRLPSGVWFEMADKDLF AAARFPECPEGSSISAPSQTSVDVSLI QDVERILDYSLCQETWSKIRAGLPIS PVDLSYLAPKNPGTGPAFTIINGTLK YFETRYIRVDIAAPILSRMVGMISGT TTEAELWDDWAPYEDVEIGPNGVL RTSSGYKFPLYMIGHGMLDSDLHLS SKAQVFEHPHIQDAASQLPDDESLFF GDTGLSKNPIELVEGWFSSWKSSIAS FFFIIGLIIGLFLVLRVGIHLCIKLKHT KKRQIYTDIEMNRLGK (SEQ ID NO: 68)

ACTGGGTTTCGCTCAAATTACTTCG CCTACGAAACTGGAGGAAAAGCCT GCAAGATGCAGTACTGCAAGCACT GGGGCGTGAGACTACCCAGCGGTG TCTGGTTCGAGATGGCCGATAAGG ACCTGTTTGCAGCAGCGAGATTCC CGGAATGCCCTGAGGGATCGAGCA TCTCCGCTCCAAGCCAAACTTCAG TGGACGTGAGCCTGATCCAGGACG TGGAACGGATTCTCGACTACTCGC TGTGCCAGGAGACCTGGTCGAAGA TCAGAGCGGGACTGCCCATCTCAC CGGTGGACCTGTCCTACCTGGCGC CAAAGAATCCGGGCACTGGACCGG CGTTCACCATCATCAACGGCACCC TCAAATACTTCGAGACGCGGTACA TCCGGGTGGACATCGCAGCTCCGA TCCTCTCCCGGATGGTGGGAATGA TCTCGGGGACTACTACCGAAGCCG AGCTCTGGGACGACTGGGCACCTT ACGAGGATGTCGAGATCGGACCTA ACGGAGTGCTCCGGACCTCCTCCG GGTACAAGTTCCCTCTGTACATGA TCGGCCATGGCATGCTGGACTCGG ATCTGCATCTGTCGTCCAAAGCAC AGGTGTTTGAACACCCACACATTC AAGACGCCGCCAGCCAGCTGCCGG ACGATGAGTCGCTGTTCTTCGGAG ACACGGGCTTGTCAAAGAATCCCA TCGAGCTGGTGGAAGGATGGTTTT CATCCTGGAAAAGCAGCATCGCTT CATTCTTCTTCATCATTGGCCTGAT CATCGGCCTATTTCTAGTCCTGCGG GTGGGAATTCATCTGTGCATCAAG CTCAAGCACACTAAGAAGCGGCAA ATCTACACTGATATCGAGATGAAT CGCCTGGGCAAG (SEQ ID NO: 67)	
CD8a signal peptide	
Nucleotide	**Amino acid**
ATGGCCTTACCAGTGACCGCCTTG CTCCTGCCGCTGGCCTTGCTGCTCC ACGCCGCCAGGCCG (SEQ ID NO: 31)	MALPVTALLLPLALLLHAARP (SEQ ID NO: 32)
Anti-CD3 Light chain variable region	
Nucleotide	**Amino acid**
GACATCCAGATGACCCAGACCACC TCCTCCCTGTCTGCCTCTCTGGGAG ACAGAGTCACCATCAGTTGCAGGG	DIQMTQTTSSLSASLGDRVTISCRAS QDIRNYLNWYQQKPDGTVKLLIYYT SRLHSGVPSKFSGSGSGTDYSLTISN

CAAGTCAGGACATTAGAAATTATT TAAACTGGTATCAACAGAAACCAG ATGGAACTGTTAAACTCCTGATCT ACTACACATCAAGATTACACTCAG GAGTCCCATCAAAGTTCAGTGGCA GTGGGTCTGGAACAGATTATTCTC TCACCATTAGCAACCTGGAGCAAG AGGATATTGCCACTTACTTTTGCCA ACAGGGTAATACGCTTCCGTGGAC GTTCGCTGGAGGCACCAAGCTGGA AATCAAACGGGCT (SEQ ID NO: 33)	LEQEDIATYFCQQGNTLPWTFAGGT KLEIKRA (SEQ ID NO: 34)
G_3S linker	
Nucleotide	**Amino acid**
GGAGGCGGTAGTGGCGGTGGATCA GGTGGAGGCAGCGGTGGCGGATCT (SEQ ID NO: 35)	GGGSGGGSGGGSGGGS (SEQ ID NO: 36)
Anti-CD3 heavy chain variable region	
Nucleotide	**Amino acid**
GAGGTGCAGCTCCAGCAGTCTGGA CCTGAGCTGGTGAAGCCTGGAGCT TCAATGAAGATATCCTGCAAGGCT TCTGGTTACTCATTCACTGGCTACA CCATGAACTGGGTGAAGCAGAGTC ATGGAAAGAACCTTGAGTGGATGG GACTTATTAATCCTTACAAAGGTG TTAGTACCTACAACCAGAAGTTCA AGGACAAGGCCACATTAACTGTAG ACAAGTCATCCAGCACAGCCTACA TGGAACTCCTCAGTCTGACATCTG AGGACTCTGCAGTCTATTACTGTG CAAGATCGGGGTACTACGGTGATA GTGACTGGTACTTCGATGTCTGGG GCGCAGGGACCACGGTCACCGTCT CCTCA (SEQ ID NO: 37)	EVQLQQSGPELVKPGASMKISCKAS GYSFTGYTMNWVKQSHGKNLEWM GLINPYKGVSTYNQKFKDKATLTVD KSSSTAYMELLSLTSEDSAVYYCAR SGYYGDSDWYFDVWGAGTTVTVSS (SEQ ID NO: 38)
CD8a hinge and transmembrane	
Nucleotide	**Amino acid**
ACCACTACACCAGCACCTAGACCA CCAACACCTGCGCCAACCATCGCA TCGCAGCCACTGTCTCTGCGCCCA GAGGCATGCCGGCCAGCAGCTGGG GGCGCAGTGCACACAAGGGGGCT GGACTTCGCATGTGATATCTACAT CTGGGCACCATTGGCAGGGACTTG TGGGGTCCTTCTCCTGTCACTGGTT ATCACCCTTTACTGC (SEQ ID NO: 39)	TTTPAPRPPTPAPTIASQPLSLRPEAC RPAAGGAVHTRGLDFACDIYIWAPL AGTCGVLLLSLVITLYC (SEQ ID NO: 40)
P2A self-cleaving peptide	
Nucleotide	**Amino acid**

GGCAGCGGCGCCACCAACTTCTCC CTGCTGAAGCAGGCCGGCGACGTG GAAGAAAACCCTGGCCCC (SEQ ID NO: 69)	GSGATNFSLLKQAGDVEENPGP (SEQ ID NO: 70)
VSV-G signal sequence	
Nucleotide	**Amino acid**
ATGAAGTGTCTGCTGTACCTGGCG TTCCTGTTTATCGGGGTGAACTGC (SEQ ID NO: 71)	MKCLLYLAFLFIGVNC (SEQ ID NO: 72)
Mutant VSV-G (Trop-002)	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCAC AACCAAAAGGGCAACTGGAAAAA CGTGCCTTCAAATTACCATTATTGC CCCAGCAGCTCGGACCTGAACTGG CACAATGACCTCATTGGAACCGCG CTGCAGGTGAAGATGCCACAGAGC CACAAGGCTATCCAGGCTGACGGA TGGATGTGCCACGCGTCAAAATGG GTGACTACCTGCGATTTCCGCTGG TACGGACCAAAATACATCACGCAC AGCATCAGATCATTCACCCCGTCA GTGGAACAATGCAAAGAATCCATC GAACAGACTAAGCAGGGAACCTG GCTGAACCCTGGATTTCCGCCGCA GTCGTGTGGGTACGCAACCGTGAC CGATGCAGAGGCCGTGATCGTGCA AGTCACGCCGCATCACGTGCTTGT GGACGAGTACACCGGAGAATGGG TCGATTCCCAGTTCATCAACGGCA AGTGCTCCAACTACATTTGCCCAA CCGTGCACAACAGCACTACTTGGC ACAGCGACTACAAAGTGAAGGGTC TGTGTGATTCCAACCTGATCTCCAT GGATATCACTTTCTTCTCGGAAGA CGGCGAACTGTCCTCACTGGGCAA AGAAGGAACTGGGTTTCGCTCAAA TTACTTCGCCTACGAAACTGGAGG AAAAGCCTGCAAGATGCAGTACTG CAAGCACTGGGGCGTGAGACTACC CAGCGGTGTCTGGTTCGAGATGGC CGATAAGGACCTGTTTGCAGCAGC GAGATTCCCGGAATGCCCTGAGGG ATCGAGCATCTCCGCTCCAAGCCA AACTTCAGTGGACGTGAGCCTGAT CCAGGACGTGGAACGGATTCTCGA CTACTCGCTGTGCCAGGAGACCTG GTCGAAGATCAGAGCGGGACTGCC	KFTIVFPHNQKGNWKNVPSNYHYCP SSSDLNWHNDLIGTALQVKMPQSH KAIQADGWMCHASKWVTTCDFRW YGPKYITHSIRSFTPSVEQCKESIEQT KQGTWLNPGFPPQSCGYATVTDAE AVIVQVTPHHVLVDEYTGEWVDSQ FINGKCSNYICPTVHNSTTWHSDYK VKGLCDSNLISMDITFFSEDGELSSL GKEGTGFRSNYFAYETGGKACKMQ YCKHWGVRLPSGVWFEMADKDLF AAARFPECPEGSSISAPSQTSVDVSLI QDVERILDYSLCQETWSKIRAGLPIS PVDLSYLAPKNPGTGPAFTIINGTLK YFETRYIRVDIAAPILSRMVGMISGT TTEAELWDDWAPYEDVEIGPNGVL RTSSGYKFPLYMIGHGMLDSDLHLS SKAQVFEHPHIQDAASQLPDDESLFF GDTGLSKNPIELVEGWFSSWKSSIAS FFFIIGLIIGLFLVLRVGIHLCIKLKHT KKRQIYTDIEMNRLGK (SEQ ID NO: 74)

CATCTCACCGGTGGACCTGTCCTA CCTGGCGCCAAAGAATCCGGGCAC TGGACCGGCGTTCACCATCATCAA CGGCACCCTCAAATACTTCGAGAC GCGGTACATCCGGGTGGACATCGC AGCTCCGATCCTCTCCCGGATGGT GGGAATGATCTCGGGGACTACTAC CGAAGCCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTG GACTCGGATCTGCATCTGTCGTCC AAAGCACAGGTGTTTGAACACCCA CACATTCAAGACGCCGCCAGCCAG CTGCCGGACGATGAGTCGCTGTTC TTCGGAGACACGGGCTTGTCAAAG AATCCCATCGAGCTGGTGGAAGGA TGGTTTTCATCCTGGAAAAGCAGC ATCGCTTCATTCTTCTTCATCATTG GCCTGATCATCGGCCTATTTCTAGT CCTGCGGGTGGGAATTCATCTGTG CATCAAGCTCAAGCACACTAAGAA GCGGCAAATCTACACTGATATCGA GATGAATCGCCTGGGCAAG (SEQ ID NO: 73)	

In another embodiment, the tandem expression cassette of the VSV-G env plasmid encodes a CD80 targeting protein and a mutated VSV-G envelope protein. For example, in a specific embodiment, the tandem expression cassette may comprise a polynucleotide according to SEQ ID NO: 75, which encodes an amino acid sequence of SEQ ID NO: 76. Such an embodiment is described in more detail in Table 9. As illustrated in Table 9, the CD80 targeting protein is separated from the mutated VSV-G envelope protein via a P2A self-cleaving peptide encoded by SEQ ID NO: 69 and having an amino acid sequence according to SEQ ID NO: 70.

In some embodiments, where the tandem expression cassette of the VSV-G env plasmid encodes a CD80 targeting protein and a mutated VSV-G envelope protein, the tandem expression cassette may include a polynucleotide that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 75, which encodes an amino acid sequence that has at least

about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 76. In certain embodiments, the CD80 targeting protein is separated from the mutated VSV-G envelope protein via a P2A self-cleaving peptide encoded a nucleic acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 69, and the P2A self-cleaving peptide includes an amino acid that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 70.

TABLE 9: CD80 T cell targeting molecule co-expressed with mutant VSV-G

Full molecule	
Nucleotide	**Amino acid (signal sequence underlined)**
ATGGGTCATACACGCCGCCAAGGA	MGHTRRQGTSPSKCPYLNFFQLLVL
ACCTCACCATCTAAGTGCCCATAT	AGLSHFCSGVIHVTKEVKEVATLSC
CTGAATTTCTTTCAACTTCTCGTGC	GHNVSVEELAQTRIYWQKEKKMVL
TGGCGGGGCTCAGTCATTTCTGCA	TMMSGDMNIWPEYKNRTIFDITNNL
GTGGGGTCATTCACGTTACTAAAG	SIVILALRPSDEGTYECVVLKYEKDA
AGGTCAAGGAGGTCGCAACATTGA	FKREHLAEVTLSVKADFPTPSISDFEI
GTTGTGGCCATAACGTATCAGTTG	PTSNIRRIICSTSGGFPEPHLSWLENG
AAGAACTCGCGCAGACACGGATTT	EELNAINTTVSQDPETELYAVSSKLD
ACTGGCAAAAGGAAAAGAAGATG	FNMTTNHSFMCLIKYGHLRVNQTFN
GTGTTGACAATGATGAGCGGTGAC	WNTTKQEHFPDNLLPSWAITLISVN
ATGAACATTTGGCCAGAGTACAAA	GIFVICCLTYCFAPRCREGSGATNFS
AATCGAACGATATTCGATATAACC	LLKQAGDVEENPGPMKCLLYLAFLF
AATAACTTGTCCATAGTAATACTT	IGVNCKFTIVFPHNQKGNWKNVPSN
GCCTTGCGACCTTCTGACGAGGGA	YHYCPSSSDLNWHNDLIGTALQVK
ACGTATGAATGTGTAGTGCTTAAG	MPQSHKAIQADGWMCHASKWVTT
TATGAAAAAGATGCCTTTAAGCGG	CDFRWYGPKYITHSIRSFTPSVEQCK
GAACACTTGGCTGAGGTTACACTC	ESIEQTKQGTWLNPGFPPQSCGYAT
TCCGTTAAGGCGGACTTTCCTACG	VTDAEAVIVQVTPHHVLVDEYTGE
CCGTCTATATCCGACTTCGAGATA	WVDSQFINGKCSNYICPTVHNSTTW
CCCACTTCTAACATTCGACGCATC	HSDYKVKGLCDSNLISMDITFFSEDG
ATTTGCTCAACCTCAGGTGGTTTCC	ELSSLGKEGTGFRSNYFAYETGGKA
CAGAGCCTCACTTGAGCTGGCTGG	CKMQYCKHWGVRLPSGVWFEMAD
AGAATGGCGAAGAACTTAACGCA	KDLFAAARFPECPEGSSISAPSQTSV
ATCAATACCACGGTGTCCCAAGAC	DVSLIQDVERILDYSLCQETWSKIRA
CCGGAGACAGAGCTGTACGCCGTG	GLPISPVDLSYLAPKNPGTGPAFTIIN
TCATCCAAACTGGATTTTAACATG	GTLKYFETRYIRVDIAAPILSRMVGM
ACGACAAATCATAGTTTCATGTGT	ISGTTTEAELWDDWAPYEDVEIGPN
CTGATCAAATATGGGCATCTCAGG	GVLRTSSGYKFPLYMIGHGMLDSDL
GTGAATCAGACTTTTAATTGGAAC	HLSSKAQVFEHPHIQDAASQLPDDE

ACTACCAAACAAGAGCACTTCCCA GATAATCTGTTGCCAAGCTGGGCG ATAACTCTTATCTCCGTCAACGGT ATCTTCGTAATTTGCTGCCTCACCT ATTGTTTCGCGCCTCGATGCCGAG AAGGCAGCGGCGCCACCAACTTCT CCCTGCTGAAGCAGGCCGGCGACG TGGAAGAAAACCCTGGCCCCATGA AGTGTCTGCTGTACCTGGCGTTCCT GTTTATCGGGGTGAACTGCAAGTT CACTATCGTGTTTCCGCACAACCA AAAGGGCAACTGGAAAAACGTGC CTTCAAATTACCATTATTGCCCCAG CAGCTCGGACCTGAACTGGCACAA TGACCTCATTGGAACCGCGCTGCA GGTGAAGATGCCACAGAGCCACA AGGCTATCCAGGCTGACGGATGGA TGTGCCACGCGTCAAAATGGGTGA CTACCTGCGATTTCCGCTGGTACG GACCAAAATACATCACGCACAGCA TCAGATCATTCACCCCGTCAGTGG AACAATGCAAAGAATCCATCGAAC AGACTAAGCAGGGAACCTGGCTGA ACCCTGGATTTCCGCCGCAGTCGT GTGGGTACGCAACCGTGACCGATG CAGAGGCCGTGATCGTGCAAGTCA CGCCGCATCACGTGCTTGTGGACG AGTACACCGGAGAATGGGTCGATT CCCAGTTCATCAACGGCAAGTGCT CCAACTACATTTGCCCAACCGTGC ACAACAGCACTACTTGGCACAGCG ACTACAAAGTGAAGGGTCTGTGTG ATTCCAACCTGATCTCCATGGATA TCACTTTCTTCTCGGAAGACGGCG AACTGTCCTCACTGGGCAAAGAAG GAACTGGGTTTCGCTCAAATTACT TCGCCTACGAAACTGGAGGAAAAG CCTGCAAGATGCAGTACTGCAAGC ACTGGGGCGTGAGACTACCCAGCG GTGTCTGGTTCGAGATGGCCGATA AGGACCTGTTTGCAGCAGCGAGAT TCCCGGAATGCCCTGAGGGATCGA GCATCTCCGCTCCAAGCCAAACTT CAGTGGACGTGAGCCTGATCCAGG ACGTGGAACGGATTCTCGACTACT CGCTGTGCCAGGAGACCTGGTCGA AGATCAGAGCGGGACTGCCCATCT CACCGGTGGACCTGTCCTACCTGG CGCCAAAGAATCCGGGCACTGGAC	SLFFGDTGLSKNPIELVEGWFSSWKS SIASFFFIIGLIIGLFLVLRVGIHLCIKL KHTKKRQIYTDIEMNRLGK (SEQ ID NO: 76)

CGGCGTTCACCATCATCAACGGCA CCCTCAAATACTTCGAGACGCGGT ACATCCGGGTGGACATCGCAGCTC CGATCCTCTCCCGGATGGTGGGAA TGATCTCGGGGACTACTACCGAAG CCGAGCTCTGGGACGACTGGGCAC CTTACGAGGATGTCGAGATCGGAC CTAACGGAGTGCTCCGGACCTCCT CCGGGTACAAGTTCCCTCTGTACA TGATCGGCCATGGCATGCTGGACT CGGATCTGCATCTGTCGTCCAAAG CACAGGTGTTTGAACACCCACACA TTCAAGACGCCGCCAGCCAGCTGC CGGACGATGAGTCGCTGTTCTTCG GAGACACGGGCTTGTCAAAGAATC CCATCGAGCTGGTGGAAGGATGGT TTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCT GATCATCGGCCTATTTCTAGTCCTG CGGGTGGGAATTCATCTGTGCATC AAGCTCAAGCACACTAAGAAGCG GCAAATCTACACTGATATCGAGAT GAATCGCCTGGGCAAG (SEQ ID NO: 75)	
CD80 signal peptide	
Nucleotide	**Amino acid**
ATGGGTCATACACGCCGCCAAGGA ACCTCACCATCTAAGTGCCCATAT CTGAATTTCTTTCAACTTCTCGTGC TGGCGGGGCTCAGTCATTTCTGCA GTGGGGTC (SEQ ID NO: 3)	MGHTRRQGTSPSKCPYLNFFQLLVL AGLSHFCSGV (SEQ ID NO: 4)
CD80 extracellular domain (ECD)	
Nucleotide	**Amino acid**
ATTCACGTTACTAAAGAGGTCAAG GAGGTCGCAACATTGAGTTGTGGC CATAACGTATCAGTTGAAGAACTC GCGCAGACACGGATTTACTGGCAA AAGGAAAAGAAGATGGTGTTGAC AATGATGAGCGGTGACATGAACAT TTGGCCAGAGTACAAAAATCGAAC GATATTCGATATAACCAATAACTT GTCCATAGTAATACTTGCCTTGCG ACCTTCTGACGAGGGAACGTATGA ATGTGTAGTGCTTAAGTATGAAAA AGATGCCTTTAAGCGGGAACACTT GGCTGAGGTTACACTCTCCGTTAA GGCGGACTTTCCTACGCCGTCTAT ATCCGACTTCGAGATACCCACTTC	IHVTKEVKEVATLSCGHNVSVEELA QTRIYWQKEKKMVLTMMSGDMNI WPEYKNRTIFDITNNLSIVILALRPSD EGTYECVVLKYEKDAFKREHLAEV TLSVKADFPTPSISDFEIPTSNIRRIICS TSGGFPEPHLSWLENGEELNAINTTV SQDPETELYAVSSKLDFNMTTNHSF MCLIKYGHLRVNQTFNWNTTKQEH FPDNLLPS (SEQ ID NO: 6)

TAACATTCGACGCATCATTTGCTC AACCTCAGGTGGTTTCCCAGAGCC TCACTTGAGCTGGCTGGAGAATGG CGAAGAACTTAACGCAATCAATAC CACGGTGTCCCAAGACCCGGAGAC AGAGCTGTACGCCGTGTCATCCAA ACTGGATTTTAACATGACGACAAA TCATAGTTTCATGTGTCTGATCAAA TATGGGCATCTCAGGGTGAATCAG ACTTTTAATTGGAACACTACCAAA CAAGAGCACTTCCCAGATAATCTG TTGCCAAGC (SEQ ID NO: 5)	
CD80 transmembrane and intracellular domain	
Nucleotide	**Amino acid**
TGGGCGATAACTCTTATCTCCGTC AACGGTATCTTCGTAATTTGCTGCC TCACCTATTGTTTCGCGCCTCGATG CCGAGAA (SEQ ID NO: 7)	WAITLISVNGIFVICCLTYCFAPRCRE (SEQ ID NO: 8)
P2A self-cleaving	
Nucleotide	**Amino acid**
GGCAGCGGCGCCACCAACTTCTCC CTGCTGAAGCAGGCCGGCGACGTG GAAGAAAACCCTGGCCCC (SEQ ID NO: 69)	GSGATNFSLLKQAGDVEENPGP (SEQ ID NO: 70)
VSV-G signal sequence	
Nucleotide	**Amino acid**
ATGAAGTGTCTGCTGTACCTGGCG TTCCTGTTTATCGGGGTGAACTGC (SEQ ID NO: 71)	MKCLLYLAFLFIGVNC (SEQ ID NO: 72)
Mutant VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCAC AACCAAAAGGGCAACTGGAAAAA CGTGCCTTCAAATTACCATTATTGC CCCAGCAGCTCGGACCTGAACTGG CACAATGACCTCATTGGAACCGCG CTGCAGGTGAAGATGCCACAGAGC CACAAGGCTATCCAGGCTGACGGA TGGATGTGCCACGCGTCAAAATGG GTGACTACCTGCGATTTCCGCTGG TACGGACCAAAATACATCACGCAC AGCATCAGATCATTCACCCCGTCA GTGGAACAATGCAAAGAATCCATC GAACAGACTAAGCAGGGAACCTG GCTGAACCCTGGATTTCCGCCGCA GTCGTGTGGGTACGCAACCGTGAC CGATGCAGAGGCCGTGATCGTGCA AGTCACGCCGCATCACGTGCTTGT	KFTIVFPHNQKGNWKNVPSNYHYCP SSSDLNWHNDLIGTALQVKMPQSH KAIQADGWMCHASKWVTTCDFRW YGPKYITHSIRSFTPSVEQCKESIEQT KQGTWLNPGFPPQSCGYATVTDAE AVIVQVTPHHVLVDEYTGEWVDSQ FINGKCSNYICPTVHNSTTWHSDYK VKGLCDSNLISMDITFFSEDGELSSL GKEGTGFRSNYFAYETGGKACKMQ YCKHWGVRLPSGVWFEMADKDLF AAARFPECPEGSSISAPSQTSVDVSLI QDVERILDYSLCQETWSKIRAGLPIS PVDLSYLAPKNPGTGPAFTIINGTLK YFETRYIRVDIAAPILSRMVGMISGT TTEAELWDDWAPYEDVEIGPNGVL RTSSGYKFPLYMIGHGMLDSDLHLS SKAQVFEHPHIQDAASQLPDDESLFF

GGACGAGTACACCGGAGAATGGG TCGATTCCCAGTTCATCAACGGCA AGTGCTCCAACTACATTTGCCCAA CCGTGCACAACAGCACTACTTGGC ACAGCGACTACAAAGTGAAGGGTC TGTGTGATTCCAACCTGATCTCCAT GGATATCACTTTCTTCTCGGAAGA CGGCGAACTGTCCTCACTGGGCAA AGAAGGAACTGGGTTTCGCTCAAA TTACTTCGCCTACGAAACTGGAGG AAAAGCCTGCAAGATGCAGTACTG CAAGCACTGGGGCGTGAGACTACC CAGCGGTGTCTGGTTCGAGATGGC CGATAAGGACCTGTTTGCAGCAGC GAGATTCCCGGAATGCCCTGAGGG ATCGAGCATCTCCGCTCCAAGCCA AACTTCAGTGGACGTGAGCCTGAT CCAGGACGTGGAACGGATTCTCGA CTACTCGCTGTGCCAGGAGACCTG GTCGAAGATCAGAGCGGGACTGCC CATCTCACCGGTGGACCTGTCCTA CCTGGCGCCAAAGAATCCGGGCAC TGGACCGGCGTTCACCATCATCAA CGGCACCCTCAAATACTTCGAGAC GCGGTACATCCGGGTGGACATCGC AGCTCCGATCCTCTCCCGGATGGT GGGAATGATCTCGGGGACTACTAC CGAAGCCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTG GACTCGGATCTGCATCTGTCGTCC AAAGCACAGGTGTTTGAACACCCA CACATTCAAGACGCCGCCAGCCAG CTGCCGGACGATGAGTCGCTGTTC TTCGGAGACACGGGCTTGTCAAAG AATCCCATCGAGCTGGTGGAAGGA TGGTTTTCATCCTGGAAAAGCAGC ATCGCTTCATTCTTCTTCATCATTG GCCTGATCATCGGCCTATTTCTAGT CCTGCGGGTGGGAATTCATCTGTG CATCAAGCTCAAGCACACTAAGAA GCGGCAAATCTACACTGATATCGA GATGAATCGCCTGGGCAAG (SEQ ID NO: 73)	GDTGLSKNPIELVEGWFSSWKSSIAS FFFIIGLIIGLFLVLRVGIHLCIKLKHT KKRQIYTDIEMNRLGK (SEQ ID NO: 74)

In another embodiment, the tandem expression cassette of the VSV-G env plasmid encodes a CD80 targeting protein, an anti-CD3 targeting protein, and a mutated VSV-G envelope protein. For example, in a specific embodiment, the tandem expression cassette may comprise a CD80 targeting polynucleotide according to SEQ ID NO: 1, which encodes an amino acid sequence of SEQ ID NO: 2. In a specific embodiment, the tandem expression cassette may comprise an anti-CD3 targeting polynucleotide according to SEQ ID NO: 9, which encodes an amino acid sequence of SEQ ID NO: 10. Such an embodiment is described in more detail in Table 3. As illustrated in Table 3, the CD80 targeting protein is separated from the anti-CD3 targeting protein via a P2A self-cleaving peptide encoded by SEQ ID NO: 29 and having an amino acid sequence according to SEQ ID NO: 30.

In some embodiments, where the tandem expression cassette of the VSV-G env plasmid encodes a CD80 targeting protein, anti-CD3 targeting protein, and a mutated VSV-G envelope protein, the tandem expression cassette may include a polynucleotide encoding the CD80 targeting protein and anti-CD3 targeting protein that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 21, which encodes an amino acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 22. In certain embodiments, the CD80 targeting protein is separated from the anti-CD3 targeting protein via a P2A self-cleaving peptide encoded a nucleic acid sequence that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 29, and the P2A self-cleaving peptide includes an amino acid that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 30.

In some embodiments, wherein the tandem expression cassette of the VSV-G env plasmid encodes a CD80 targeting protein, anti-CD3 targeting protein, and a mutated VSV-G envelope protein, the CD80 targeting protein and anti-CD3 targeting protein are separated from the mutated VSV-G envelope protein via a 2A self-cleaving peptide.

In some embodiments of a four plasmid LVV system, the GagPol plasmid includes a tandem expression cassette that encodes the Gag polyprotein precursor and

Pol polyprotein precursor and a targeting protein as disclosed herein. In specific embodiments, the tandem expression cassette included in the GagPol plasmid includes a polynucleotide that encodes a first signal peptide, a polynucleotide that encodes a targeting protein, a polynucleotide that encodes one of an internal ribosomal entry site (IRES), a furin cleavage site, or a viral 2A peptide, a polynucleotide that encodes a second signal peptide, and a polynucleotide that encodes Gag polyprotein precursor and Pol polyprotein precursor. In certain embodiments, the polynucleotide encoding the Gag polyprotein precursor and Pol polyprotein precursor is positioned 5' to the polynucleotide encoding the targeting protein. In other embodiments, the polynucleotide encoding the Gag polyprotein precursor and Pol polyprotein precursor is positioned 3' to the polynucleotide encoding the targeting protein. The polynucleotide that encodes the targeting protein and the polynucleotide that encodes the Gag polyprotein precursor and Pol polyprotein precursor are separated in the tandem cassette by the polynucleotide that encodes the IRES, furin cleavage site, or viral 2A peptide, which allows for co-expression of Gag polyprotein precursor and Pol polyprotein precursor and the targeting protein from a single mRNA. In certain embodiments, a viral 2A peptide is a porcine teschovirus-1 (P2A), *Thosea asigna* virus (T2A), equine rhinitis A virus (E2A), foot-and-mouth disease virus (F2A), or a variant thereof. Specific examples of amino acid and nucleotide sequences for the viral 2A peptides are provided in Table 7.

In some embodiments, the GagPol plasmid containing the tandem expression cassette encoding Gag polyprotein precursor and Pol polyprotein precursor and the targeting protein is transfected into producer cells at a higher molar concentration than the VSV-G env plasmid. In some embodiments, the GagPol plasmid containing the tandem expression cassette encoding Gag polyprotein precursor and Pol polyprotein precursor and the targeting protein is transfected into producer cells at a lower molar concentration than the VSV-G env plasmid.

In one embodiment, the tandem expression cassette of the GagPol plasmid encodes an anti-CD3 targeting protein and Gag polyprotein precursor and Pol polyprotein precursor. In some embodiments, the anti-CD3 targeting protein is separated from the Gag polyprotein precursor and Pol polyprotein precursor via a P2A self-cleaving peptide.

In another embodiment, the tandem expression cassette of the GagPol plasmid encodes a CD80 targeting protein and Gag polyprotein precursor and Pol polyprotein precursor. In some embodiments, the CD80 targeting protein is separated from Gag polyprotein precursor and Pol polyprotein precursor via a P2A self-cleaving peptide.

In some embodiments of a four plasmid LVV system, the Rev plasmid includes a tandem expression cassette that encodes the Rev protein and a targeting protein as disclosed herein. In specific embodiments, the tandem expression cassette included in the Rev plasmid includes a polynucleotide that encodes a first signal peptide, a polynucleotide that encodes a targeting protein, a polynucleotide that encodes one of an internal ribosomal entry site (IRES), a furin cleavage site, or a viral 2A peptide, a polynucleotide that encodes a second signal peptide, and a polynucleotide that encodes a Rev protein. In certain embodiments, the polynucleotide encoding the Rev protein is positioned 5' to the polynucleotide encoding the targeting protein. In other embodiments, the polynucleotide encoding the Rev protein is positioned 3' to the polynucleotide encoding the targeting protein. The polynucleotide that encodes the targeting protein and the polynucleotide that encodes the Rev protein are separated in the tandem cassette by the polynucleotide that encodes the IRES, furin cleavage site, or viral 2A peptide, which allows for co-expression of the Rev protein and the targeting protein from a single mRNA. In certain embodiments, a viral 2A peptide is a porcine teschovirus-1 (P2A), *Thosea asigna* virus (T2A), equine rhinitis A virus (E2A), foot-and-mouth disease virus (F2A), or a variant thereof. Specific examples of amino acid and nucleotide sequences for the viral 2A peptides are provided in Table 7.

In some embodiments, the Rev plasmid containing the tandem expression cassette encoding the Rev protein and the targeting protein is transfected into producer cells at a higher molar concentration than the VSV-G env plasmid. In some embodiments, the Rev plasmid containing the tandem expression cassette encoding the Rev protein and the targeting protein is transfected into producer cells at a lower molar concentration than the VSV-G env plasmid.

In one embodiment, the tandem expression cassette of the Rev plasmid encodes an anti-CD3 targeting protein and a Rev protein. In some embodiments, the anti-CD3 targeting protein is separated from the Rev protein via a P2A self-cleaving peptide.

In another embodiment, the tandem expression cassette of the GagPol plasmid encodes a CD80 targeting protein and a Rev protein. In some embodiments, the CD80 targeting protein is separated from the Rev protein via a P2A self-cleaving peptide.

Examples of mutated VSV-G sequences that may be used in the four plasmid, 3rd generation lentiviral vector system of the present disclosure are provided in Table 10.

Table 10: WT VSV-G reference and mutant sequences

WT VSV-G	
Nucleotide	**Amino acid (signal sequence underlined)**
ATGAAGTGTCTGCTGTACCTGGCGT TCCTGTTTATCGGGGTGAACTGCAA GTTCACTATCGTGTTTCCGCACAAC CAAAAGGGCAACTGGAAAAACGTG CCTTCAAATTACCATTATTGCCCCA GCAGCTCGGACCTGAACTGGCACA ATGACCTCATTGGAACCGCGCTGCA GGTGAAGATGCCAAAGAGCCACAA GGCTATCCAGGCTGACGGATGGAT GTGCCACGCGTCAAAATGGGTGAC TACCTGCGATTTCCGCTGGTACGGA CCAAAATACATCACGCACAGCATC AGATCATTCACCCCGTCAGTGGAAC AATGCAAAGAATCCATCGAACAGA CTAAGCAGGGAACCTGGCTGAACC CTGGATTTCCGCCGCAGTCGTGTGG GTACGCAACCGTGACCGATGCAGA GGCCGTGATCGTGCAAGTCACGCC GCATCACGTGCTTGTGGACGAGTAC ACCGGAGAATGGGTCGATTCCCAG TTCATCAACGGCAAGTGCTCCAACT ACATTTGCCCAACCGTGCACAACAG CACTACTTGGCACAGCGACTACAA AGTGAAGGGTCTGTGTGATTCCAAC CTGATCTCCATGGATATCACTTTCT TCTCGGAAGACGGCGAACTGTCCTC ACTGGGCAAAGAAGGAACTGGGTT TCGCTCAAATTACTTCGCCTACGAA ACTGGAGGAAAAGCCTGCAAGATG CAGTACTGCAAGCACTGGGGCGTG AGACTACCCAGCGGTGTCTGGTTCG AGATGGCCGATAAGGACCTGTTTGC	MKCLLYLAFLFIGVNCKFTIVFPHN QKGNWKNVPSNYHYCPSSSDLNW HNDLIGTALQVKMPKSHKAIQADG WMCHASKWVTTCDFRWYGPKYIT HSIRSFTPSVEQCKESIEQTKQGTWL NPGFPPQSCGYATVTDAEAVIVQVT PHHVLVDEYTGEWVDSQFINGKCS NYICPTVHNSTTWHSDYKVKGLCD SNLISMDITFFSEDGELSSLGKEGTG FRSNYFAYETGGKACKMQYCKHW GVRLPSGVWFEMADKDLFAAARFP ECPEGSSISAPSQTSVDVSLIQDVERI LDYSLCQETWSKIRAGLPISPVDLS YLAPKNPGTGPAFTIINGTLKYFETR YIRVDIAAPILSRMVGMISGTTTERE LWDDWAPYEDVEIGPNGVLRTSSG YKFPLYMIGHGMLDSDLHLSSKAQ VFEHPHIQDAASQLPDDESLFFGDT GLSKNPIELVEGWFSSWKSSIASFFF IIGLIIGLFLVLRVGIHLCIKLKHTKK RQIYTDIEMNRLGK (SEQ ID NO: 78)

AGCAGCGAGATTCCCGGAATGCCC TGAGGGATCGAGCATCTCCGCTCCA AGCCAAACTTCAGTGGACGTGAGC CTGATCCAGGACGTGGAACGGATT CTCGACTACTCGCTGTGCCAGGAGA CCTGGTCGAAGATCAGAGCGGGAC TGCCCATCTCACCGGTGGACCTGTC CTACCTGGCGCCAAAGAATCCGGG CACTGGACCGGCGTTCACCATCATC AACGGCACCCTCAAATACTTCGAG ACGCGGTACATCCGGGTGGACATC GCAGCTCCGATCCTCTCCCGGATGG TGGGAATGATCTCGGGGACTACTAC CGAACGCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTGG ACTCGGATCTGCATCTGTCGTCCAA AGCACAGGTGTTTGAACACCCACA CATTCAAGACGCCGCCAGCCAGCT GCCGGACGATGAGTCGCTGTTCTTC GGAGACACGGGCTTGTCAAAGAAT CCCATCGAGCTGGTGGAAGGATGG TTTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCTG ATCATCGGCCTATTTCTAGTCCTGC GGGTGGGAATTCATCTGTGCATCAA GCTCAAGCACACTAAGAAGCGGCA AATCTACACTGATATCGAGATGAAT CGCCTGGGCAAG (SEQ ID NO: 77)	
WT VSV-G Signal Sequence	
Nucleotide	**Amino acid**
ATGAAGTGTCTGCTGTACCTGGCGT TCCTGTTTATCGGGGTGAACTGC (SEQ ID NO: 87)	MKCLLYLAFLFIGVNC (SEQ ID NO: 88)
WT VSV-G without signal sequence	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCACA ACCAAAAGGGCAACTGGAAAAACG TGCCTTCAAATTACCATTATTGCCC CAGCAGCTCGGACCTGAACTGGCA CAATGACCTCATTGGAACCGCGCTG CAGGTGAAGATGCCAAAGAGCCAC AAGGCTATCCAGGCTGACGGATGG ATGTGCCACGCGTCAAAATGGGTG	KFTIVFPHNQKGNWKNVPSNYHYC PSSSDLNWHNDLIGTALQVKMPKS HKAIQADGWMCHASKWVTTCDFR WYGPKYITHSIRSFTPSVEQCKESIE QTKQGTWLNPGFPPQSCGYATVTD AEAVIVQVTPHHVLVDEYTGEWVD SQFINGKCSNYICPTVHNSTTWHSD YKVKGLCDSNLISMDITFFSEDGEL

ACTACCTGCGATTTCCGCTGGTACG GACCAAAATACATCACGCACAGCA TCAGATCATTCACCCCGTCAGTGGA ACAATGCAAAGAATCCATCGAACA GACTAAGCAGGGAACCTGGCTGAA CCCTGGATTTCCGCCGCAGTCGTGT GGGTACGCAACCGTGACCGATGCA GAGGCCGTGATCGTGCAAGTCACG CCGCATCACGTGCTTGTGGACGAGT ACACCGGAGAATGGGTCGATTCCC AGTTCATCAACGGCAAGTGCTCCAA CTACATTTGCCCAACCGTGCACAAC AGCACTACTTGGCACAGCGACTAC AAAGTGAAGGGTCTGTGTGATTCCA ACCTGATCTCCATGGATATCACTTT CTTCTCGGAAGACGGCGAACTGTCC TCACTGGGCAAAGAAGGAACTGGG TTTCGCTCAAATTACTTCGCCTACG AAACTGGAGGAAAAGCCTGCAAGA TGCAGTACTGCAAGCACTGGGGCG TGAGACTACCCAGCGGTGTCTGGTT CGAGATGGCCGATAAGGACCTGTTT GCAGCAGCGAGATTCCCGGAATGC CCTGAGGGATCGAGCATCTCCGCTC CAAGCCAAACTTCAGTGGACGTGA GCCTGATCCAGGACGTGGAACGGA TTCTCGACTACTCGCTGTGCCAGGA GACCTGGTCGAAGATCAGAGCGGG ACTGCCCATCTCACCGGTGGACCTG TCCTACCTGGCGCCAAAGAATCCGG GCACTGGACCGGCGTTCACCATCAT CAACGGCACCCTCAAATACTTCGAG ACGCGGTACATCCGGGTGGACATC GCAGCTCCGATCCTCTCCCGGATGG TGGGAATGATCTCGGGGACTACTAC CGAACGCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTGG ACTCGGATCTGCATCTGTCGTCCAA AGCACAGGTGTTTGAACACCCACA CATTCAAGACGCCGCCAGCCAGCT GCCGGACGATGAGTCGCTGTTCTTC GGAGACACGGGCTTGTCAAAGAAT CCCATCGAGCTGGTGGAAGGATGG TTTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCTG ATCATCGGCCTATTTCTAGTCCTGC	SSLGKEGTGFRSNYFAYETGGKAC KMQYCKHWGVRLPSGVWFEMAD KDLFAAARFPECPEGSSISAPSQTSV DVSLIQDVERILDYSLCQETWSKIR AGLPISPVDLSYLAPKNPGTGPAFTI INGTLKYFETRYIRVDIAAPILSRMV GMISGTTTERELWDDWAPYEDVEI GPNGVLRTSSGYKFPLYMIGHGML DSDLHLSSKAQVFEHPHIQDAASQL PDDESLFFGDTGLSKNPIELVEGWF SSWKSSIASFFFIIGLIIGLFLVLRVGI HLCIKLKHTKKRQIYTDIEMNRLGK (SEQ ID NO: 90)

GGGTGGGAATTCATCTGTGCATCAA GCTCAAGCACACTAAGAAGCGGCA AATCTACACTGATATCGAGATGAAT CGCCTGGGCAAG (SEQ ID NO: 89)	
Trop-002 Mutated VSV-G	
Nucleotide	**Amino Acid**
AAGTTCACTATCGTGTTTCCGCACA ACCAAAAGGGCAACTGGAAAAACG TGCCTTCAAATTACCATTATTGCCC CAGCAGCTCGGACCTGAACTGGCA CAATGACCTCATTGGAACCGCGCTG CAGGTGAAGATGCCACAGAGCCAC AAGGCTATCCAGGCTGACGGATGG ATGTGCCACGCGTCAAAATGGGTG ACTACCTGCGATTTCCGCTGGTACG GACCAAAATACATCACGCACAGCA TCAGATCATTCACCCCGTCAGTGGA ACAATGCAAAGAATCCATCGAACA GACTAAGCAGGGAACCTGGCTGAA CCCTGGATTTCCGCCGCAGTCGTGT GGGTACGCAACCGTGACCGATGCA GAGGCCGTGATCGTGCAAGTCACG CCGCATCACGTGCTTGTGGACGAGT ACACCGGAGAATGGGTCGATTCCC AGTTCATCAACGGCAAGTGCTCCAA CTACATTTGCCCAACCGTGCACAAC AGCACTACTTGGCACAGCGACTAC AAAGTGAAGGGTCTGTGTGATTCCA ACCTGATCTCCATGGATATCACTTT CTTCTCGGAAGACGGCGAACTGTCC TCACTGGGCAAAGAAGGAACTGGG TTTCGCTCAAATTACTTCGCCTACG AAACTGGAGGAAAAGCCTGCAAGA TGCAGTACTGCAAGCACTGGGGCG TGAGACTACCCAGCGGTGTCTGGTT CGAGATGGCCGATAAGGACCTGTTT GCAGCAGCGAGATTCCCGGAATGC CCTGAGGGATCGAGCATCTCCGCTC CAAGCCAAACTTCAGTGGACGTGA GCCTGATCCAGGACGTGGAACGGA TTCTCGACTACTCGCTGTGCCAGGA GACCTGGTCGAAGATCAGAGCGGG ACTGCCCATCTCACCGGTGGACCTG TCCTACCTGGCGCCAAAGAATCCGG GCACTGGACCGGCGTTCACCATCAT CAACGGCACCCTCAAATACTTCGAG ACGCGGTACATCCGGGTGGACATC	KFTIVFPHNQKGNWKNVPSNYHYC PSSSDLNWHNDLIGTALQVKMPQS HKAIQADGWMCHASKWVTTCDFR WYGPKYITHSIRSFTPSVEQCKESIE QTKQGTWLNPGFPPQSCGYATVTD AEAVIVQVTPHHVLVDEYTGEWVD SQFINGKCSNYICPTVHNSTTWHSD YKVKGLCDSNLISMDITFFSEDGEL SSLGKEGTGFRSNYFAYETGGKAC KMQYCKHWGVRLPSGVWFEMAD KDLFAAARFPECPEGSSISAPSQTSV DVSLIQDVERILDYSLCQETWSKIR AGLPISPVDLSYLAPKNPGTGPAFTI INGTLKYFETRYIRVDIAAPILSRMV GMISGTTTEAELWDDWAPYEDVEI GPNGVLRTSSGYKFPLYMIGHGML DSDLHLSSKAQVFEHPHIQDAASQL PDDESLFFGDTGLSKNPIELVEGWF SSWKSSIASFFFIIGLIIGLFLVLRVGI HLCIKLKHTKKRQIYTDIEMNRLGK (SEQ ID NO: 74)

GCAGCTCCGATCCTCTCCCGGATGG TGGGAATGATCTCGGGGACTACTAC CGAAGCCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTGG ACTCGGATCTGCATCTGTCGTCCAA AGCACAGGTGTTTGAACACCCACA CATTCAAGACGCCGCCAGCCAGCT GCCGGACGATGAGTCGCTGTTCTTC GGAGACACGGGCTTGTCAAAGAAT CCCATCGAGCTGGTGGAAGGATGG TTTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCTG ATCATCGGCCTATTTCTAGTCCTGC GGGTGGGAATTCATCTGTGCATCAA GCTCAAGCACACTAAGAAGCGGCA AATCTACACTGATATCGAGATGAAT CGCCTGGGCAAG (SEQ ID NO: 73)	
Trop-002 Mutated VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCACA ACCAAAAGGGCAACTGGAAAAACG TGCCTTCAAATTACCATTATTGCCC CAGCAGCTCGGACCTGAACTGGCA CAATGACCTCATTGGAACCGCGCTG CAGGTGAAGATGCCACAGAGCCAC AAGGCTATCCAGGCTGACGGATGG ATGTGCCACGCGTCAAAATGGGTG ACTACCTGCGATTTCCGCTGGTACG GACCAAAATACATCACGCACAGCA TCAGATCATTCACCCCGTCAGTGGA ACAATGCAAAGAATCCATCGAACA GACTAAGCAGGGAACCTGGCTGAA CCCTGGATTTCCGCCGCAGTCGTGT GGGTACGCAACCGTGACCGATGCA GAGGCCGTGATCGTGCAAGTCACG CCGCATCACGTGCTTGTGGACGAGT ACACCGGAGAATGGGTCGATTCCC AGTTCATCAACGGCAAGTGCTCCAA CTACATTTGCCCAACCGTGCACAAC AGCACTACTTGGCATAGCGACTACA AAGTGAAGGGTCTGTGTGATTCCAA CCTGATCTCCATGGATATCACTTTC TTCTCGGAAGACGGCGAACTGTCCT CACTGGGCAAAGAAGGAACTGGGT TTCGCTCAAATTACTTCGCCTACGA	KFTIVFPHNQKGNWKNVPSNYHYC PSSSDLNWHNDLIGTALQVKMPQS HKAIQADGWMCHASKWVTTCDFR WYGPKYITHSIRSFTPSVEQCKESIE QTKQGTWLNPGFPPQSCGYATVTD AEAVIVQVTPHHVLVDEYTGEWVD SQFINGKCSNYICPTVHNSTTWHSD YKVKGLCDSNLISMDITFFSEDGEL SSLGKEGTGFRSNYFAYETGGKAC KMQYCKHWGVRLPSGVWFEMAD KDLFAAARFPECPEGSSISAPSQTSV DVSLIQDVERILDYSLCQETWSKIR AGLPISPVDLSYLAPKNPGTGPAFTI INGTLKYFETRYIRVDIAAPILSRMV GMISGTTTEAELWDDWAPYEDVEI GPNGVLRTSSGYKFPLYMIGHGML DSDLHLSSKAQVFEHPHIQDAASQL PDDESLFFGDTGLSKNPIELVEGWF SSWKSSIASFFFIIGLIIGLFLVLRVGI HLCIKLKHTKKRQIYTDIEMNRLGK (SEQ ID NO: 74)

AACTGGAGGAAAAGCCTGCAAGAT GCAGTACTGCAAGCACTGGGGCGT GAGACTACCCAGCGGTGTCTGGTTC GAGATGGCCGATAAGGACCTGTTT GCAGCAGCGAGATTCCCGGAATGC CCTGAGGGATCGAGCATCTCCGCTC CAAGCCAAACTTCAGTGGACGTGA GCCTGATCCAGGACGTGGAACGGA TTCTCGACTACTCGCTGTGCCAGGA GACCTGGTCGAAGATCAGAGCGGG ACTGCCCATCTCACCGGTGGACCTG TCCTACCTGGCGCCAAAGAATCCGG GCACTGGACCGGCGTTCACCATCAT CAACGGCACCCTCAAATACTTCGAG ACGCGGTACATCCGGGTGGACATC GCAGCTCCGATCCTCTCCCGGATGG TGGGAATGATCTCGGGGACTACTAC CGAAGCCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTGG ACTCGGATCTGCATCTGTCGTCCAA AGCACAGGTGTTTGAACACCCACA CATTCAAGACGCCGCCAGCCAGCT GCCGGACGATGAGTCGCTGTTCTTC GGAGACACGGGCTTGTCAAAGAAT CCCATCGAGCTGGTGGAAGGATGG TTTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCTG ATCATCGGCCTATTTCTAGTCCTGC GGGTGGGAATTCATCTGTGCATCAA GCTCAAGCACACTAAGAAGCGGCA AATCTACACTGATATCGAGATGAAT CGCCTGGGCAAGTAG (SEQ ID NO: 91)	
Trop-051 Mutated VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCACA ACCAAAAGGGCAACTGGAAAAACG TGCCTTCAAATTACCATTATTGCCC CAGCAGCTCGGACCTGAACTGGCA CAATGACCTCATTGGAACCGCGCTG CAGGTGAAGATGCCAAAGAGCCAC AAGGCTATCCAGGCTGACGGATGG ATGTGCCACGCGTCAAAATGGGTG ACTACCTGCGATTTCCGCTGGTACG	KFTIVFPHNQKGNWKNVPSNYHYC PSSSDLNWHNDLIGTALQVKMPKS HKAIQADGWMCHASKWVTTCDFR WYGPKYITHSIRSFTPSVEQCKESIE QTKQGTWLNPGFPPQSCGYATVTD AEAVIVQVTPHHVLVDEYTGEWVD SQFINGKCSNYICPTVHNSTTWHSD YKVKGLCDSALASMDITFFSEDGEL SSLGKEGTGFRSNYFAYETGGKAC

GACCAAAATACATCACGCACAGCA TCAGATCATTCACCCCGTCAGTGGA ACAATGCAAAGAATCCATCGAACA GACTAAGCAGGGAACCTGGCTGAA CCCTGGATTTCCGCCGCAGTCGTGT GGGTACGCAACCGTGACCGATGCA GAGGCCGTGATCGTGCAAGTCACG CCGCATCACGTGCTTGTGGACGAGT ACACCGGAGAATGGGTCGATTCCC AGTTCATCAACGGCAAGTGCTCCAA CTACATTTGCCCAACCGTGCACAAC AGCACTACTTGGCACAGCGACTAC AAAGTGAAGGGTCTGTGTGATTCCG CCCTGGCCTCCATGGATATCACTTT CTTCTCGGAAGACGGCGAACTGTCC TCACTGGGCAAAGAAGGAACTGGG TTTCGCTCAAATTACTTCGCCTACG AAACTGGAGGAAAAGCCTGCAAGA TGCAGTACTGCAAGCACTGGGGCG TGAGACTACCCAGCGGTGTCTGGTT CGAGATGGCCGATAAGGACCTGTTT GCAGCAGCGAGATTCCCGGAATGC CCTGAGGGATCGAGCATCTCCGCTC CAAGCCAAACTTCAGTGGACGTGA GCCTGATCCAGGACGTGGAACGGA TTCTCGACTACTCGCTGTGCCAGGA GACCTGGTCGAAGATCAGAGCGGG ACTGCCCATCTCACCGGTGGACCTG TCCTACCTGGCGCCAAAGAATCCGG GCACTGGACCGGCGTTCACCATCAT CAACGGCACCCTCAAATACTTCGAG ACGCGGTACATCCGGGTGGACATC GCAGCTCCGATCCTCTCCCGGATGG TGGGAATGATCTCGGGGACTACTGC CGCCCGCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTGG ACTCGGATCTGCATCTGTCGTCCAA AGCACAGGTGTTTGAACACCCACA CATTCAAGACGCCGCCAGCCAGCT GCCGGACGATGAGTCGCTGTTCTTC GGAGACACGGGCTTGTCAAAGAAT CCCATCGAGCTGGTGGAAGGATGG TTTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCTG ATCATCGGCCTATTTCTAGTCCTGC GGGTGGGAATTCATCTGTGCATCAA	KMQYCKHWGVRLPSGVWFEMAD KDLFAAARFPECPEGSSISAPSQTSV DVSLIQDVERILDYSLCQETWSKIR AGLPISPVDLSYLAPKNPGTGPAFTI INGTLKYFETRYIRVDIAAPILSRMV GMISGTTAARELWDDWAPYEDVEI GPNGVLRTSSGYKFPLYMIGHGML DSDLHLSSKAQVFEHPHIQDAASQL PDDESLFFGDTGLSKNPIELVEGWF SSWKSSIASFFFIIGLIIGLFLVLRVGI HLCIKLKHTKKRQIYTDIEMNRLGK (SEQ ID NO: 93)

GCTCAAGCACACTAAGAAGCGGCA AATCTACACTGATATCGAGATGAAT CGCCTGGGCAAGTAG (SEQ ID NO: 92)	
Trop-052 Mutated VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCACA ACCAAAAGGGCAACTGGAAAAACG TGCCTTCAAATTACCATTATTGCCC CAGCAGCTCGGACCTGAACTGGCA CAATGACCTCATTGGAACCGCGCTG CAGGTGAAGATGCCAAAGAGCCAC AAGGCTATCCAGGCTGACGGATGG ATGTGCCACGCGTCAAAATGGGTG ACTACCTGCGATTTCCGCTGGTACG GACCAAAATACATCACGCACAGCA TCAGATCATTCACCCCGTCAGTGGA ACAATGCAAAGAATCCATCGAACA GACTAAGCAGGGAACCTGGCTGAA CCCTGGATTTCCGCCGCAGTCGTGT GGGTACGCAACCGTGACCGATGCA GAGGCCGTGATCGTGCAAGTCACG CCGCATCACGTGCTTGTGGACGAGT ACACCGGAGAATGGGTCGATTCCC AGTTCATCAACGGCAAGTGCTCCAA CTACATTTGCCCAACCGTGCACAAC AGCACTACTTGGCACAGCGACTAC AAAGTGAAGGGTCTGTGTGATTCCA ACCTGATCTCCATGGATATCACTTT CTTCTCGGAAGACGGCGAACTGTCC TCACTGGGCAAAGAAGGAACTGGG TTTCGCTCAAATTACTTCGCCTACG AAACTGGAGGAAAAGCCTGCAAGA TGCAGTACTGCAAGCACTGGGGCG TGAGACTACCCAGCGGTGTCTGGTT CGAGATGGCCGATAAGGACCTGTTT GCAGCAGCGAGATTCCCGGAATGC CCTGAGGGATCGAGCATCTCCGCTC CAAGCCAAACTTCAGTGGACGTGA GCCTGATCCAGGACGTGGAACGGA TTCTCGACTACTCGCTGTGCCAGGA GACCTGGTCGAAGATCAGAGCGGG ACTGCCCATCTCACCGGTGGACCTG TCCTACCTGGCGCCAAAGAATCCGG GCACTGGACCGGCGTTCACCATCAT CAACGGCACCCTCAAATACTTCGAG ACGCGGTACATCCGGGTGGACATC	KFTIVFPHNQKGNWKNVPSNYHYC PSSSDLNWHNDLIGTALQVKMPKS HKAIQADGWMCHASKWVTTCDFR WYGPKYITHSIRSFTPSVEQCKESIE QTKQGTWLNPGFPPQSCGYATVTD AEAVIVQVTPHHVLVDEYTGEWVD SQFINGKCSNYICPTVHNSTTWHSD YKVKGLCDSNLISMDITFFSEDGEL SSLGKEGTGFRSNYFAYETGGKAC KMQYCKHWGVRLPSGVWFEMAD KDLFAAARFPECPEGSSISAPSQTSV DVSLIQDVERILDYSLCQETWSKIR AGLPISPVDLSYLAPKNPGTGPAFTI INGTLKYFETRYIRVDIAAPILSRMV GMISGTTWARELWDDWAPYEDVEI GPNGVLRTSSGYKFPLYMIGHGML DSDLHLSSKAQVFEHPHIQDAASQL PDDESLFFGDTGLSKNPIELVEGWF SSWKSSIASFFFIIGLIIGLFLVLRVGI HLCIKLKHTKKRQIYTDIEMNRLGK (SEQ ID NO: 95)

GCAGCTCCGATCCTCTCCCGGATGG TGGGAATGATCTCGGGGACTACTTG GGCCCGCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTGG ACTCGGATCTGCATCTGTCGTCCAA AGCACAGGTGTTTGAACACCCACA CATTCAAGACGCCGCCAGCCAGCT GCCGGACGATGAGTCGCTGTTCTTC GGAGACACGGGCTTGTCAAAGAAT CCCATCGAGCTGGTGGAAGGATGG TTTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCTG ATCATCGGCCTATTTCTAGTCCTGC GGGTGGGAATTCATCTGTGCATCAA GCTCAAGCACACTAAGAAGCGGCA AATCTACACTGATATCGAGATGAAT CGCCTGGGCAAGTAG (SEQ ID NO: 94)	
Trop-055 Mutated VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCACG CCGCCAAGGGCAACTGGAAAAACG TGCCTTCAAATTACCATTATTGCCC CAGCAGCTCGGACCTGAACTGGCA CAATGACCTCATTGGAACCGCGCTG CAGGTGAAGATGCCAAAGAGCCAC AAGGCTATCCAGGCTGACGGATGG ATGTGCCACGCGTCAAAATGGGTG ACTACCTGCGATTTCCGCTGGTACG GACCAAAATACATCACGCACAGCA TCAGATCATTCACCCCGTCAGTGGA ACAATGCAAAGAATCCATCGAACA GACTAAGCAGGGAACCTGGCTGAA CCCTGGATTTCCGCCGCAGTCGTGT GGGTACGCAACCGTGACCGATGCA GAGGCCGTGATCGTGCAAGTCACG CCGCATCACGTGCTTGTGGACGAGT ACACCGGAGAATGGGTCGATTCCC AGTTCATCAACGGCAAGTGCTCCAA CTACATTTGCCCAACCGTGCACAAC AGCACTACTTGGCACAGCGACTAC AAAGTGAAGGGTCTGTGTGATTCCG CCCTGATCTCCATGGATATCACTTT	KFTIVFPHAAKGNWKNVPSNYHYC PSSSDLNWHNDLIGTALQVKMPKS HKAIQADGWMCHASKWVTTCDFR WYGPKYITHSIRSFTPSVEQCKESIE QTKQGTWLNPGFPPQSCGYATVTD AEAVIVQVTPHHVLVDEYTGEWVD SQFINGKCSNYICPTVHNSTTWHSD YKVKGLCDSALISMDITFFSEDGEL SSLGKEGTGFRSNYFAYETGGKAC KMQYCKHWGVRLPSGVWFEMAD KDLFAAARFPECPEGSSISAPSQTSV DVSLIQDVERILDYSLCQETWSKIR AGLPISPVDLSYLAPKNPGTGPAFTI INGTLKYFETRYIRVDIAAPILSRMV GMISGTTTERELWDDWAPYEDVEI GPNGVLRTSSGYKFPLYMIGHGML DSDLHLSSKAQVFEHPHIQDAASQL PDDESLFFGDTGLSKNPIELVEGWF SSWKSSIASFFFIIGLIIGLFLVLRVGI HLCIKLKHTKKRQIYTDIEMNRLGK (SEQ ID NO: 97)

CTTCTCGGAAGACGGCGAACTGTCC TCACTGGGCAAAGAAGGAACTGGG TTTCGCTCAAATTACTTCGCCTACG AAACTGGAGGAAAAGCCTGCAAGA TGCAGTACTGCAAGCACTGGGGCG TGAGACTACCCAGCGGTGTCTGGTT CGAGATGGCCGATAAGGACCTGTTT GCAGCAGCGAGATTCCCGGAATGC CCTGAGGGATCGAGCATCTCCGCTC CAAGCCAAACTTCAGTGGACGTGA GCCTGATCCAGGACGTGGAACGGA TTCTCGACTACTCGCTGTGCCAGGA GACCTGGTCGAAGATCAGAGCGGG ACTGCCCATCTCACCGGTGGACCTG TCCTACCTGGCGCCAAAGAATCCGG GCACTGGACCGGCGTTCACCATCAT CAACGGCACCCTCAAATACTTCGAG ACGCGGTACATCCGGGTGGACATC GCAGCTCCGATCCTCTCCCGGATGG TGGGAATGATCTCGGGGACTACTAC CGAACGCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGAT CGGACCTAACGGAGTGCTCCGGAC CTCCTCCGGGTACAAGTTCCCTCTG TACATGATCGGCCATGGCATGCTGG ACTCGGATCTGCATCTGTCGTCCAA AGCACAGGTGTTTGAACACCCACA CATTCAAGACGCCGCCAGCCAGCT GCCGGACGATGAGTCGCTGTTCTTC GGAGACACGGGCTTGTCAAAGAAT CCCATCGAGCTGGTGGAAGGATGG TTTTCATCCTGGAAAAGCAGCATCG CTTCATTCTTCTTCATCATTGGCCTG ATCATCGGCCTATTTCTAGTCCTGC GGGTGGGAATTCATCTGTGCATCAA GCTCAAGCACACTAAGAAGCGGCA AATCTACACTGATATCGAGATGAAT CGCCTGGGCAAGTAG (SEQ ID NO: 96)	
Trop-056 Mutated VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCACA ACACCACACAAAAGGGCAACTGGA AAAACGTGCCTTCAAATTACCATTA TTGCCCCAGCAGCTCGGACCTGAAC TGGCACAATGACCTCATTGGAACCG CGCTGCAGGTGAAGATGCCAAAGA	KFTIVFPHNTTQKGNWKNVPSNYH YCPSSSDLNWHNDLIGTALQVKMP KSHKAIQADGWMCHASKWVTTCD FRWYGPKYITHSIRSFTPSVEQCKES IEQTKQGTWLNPGFPPQSCGYATVT DAEAVIVQVTPHHVLVDEYTGEWV

GCCACAAGGCTATCCAGGCTGACG GATGGATGTGCCACGCGTCAAAAT GGGTGACTACCTGCGATTTCCGCTG GTACGGACCAAAATACATCACGCA CAGCATCAGATCATTCACCCCGTCA GTGGAACAATGCAAAGAATCCATC GAACAGACTAAGCAGGGAACCTGG CTGAACCCTGGATTTCCGCCGCAGT CGTGTGGGTACGCAACCGTGACCG ATGCAGAGGCCGTGATCGTGCAAG TCACGCCGCATCACGTGCTTGTGGA CGAGTACACCGGAGAATGGGTCGA TTCCCAGTTCATCAACGGCAAGTGC TCCAACTACATTTGCCCAACCGTGC ACAACAGCACTACTTGGCACAGCG ACTACAAAGTGAAGGGTCTGTGTG ATTCCAACCTGATCTCCATGGATAT CACTTTCTTCTCGGAAGACGGCGAA CTGTCCTCACTGGGCAAAGAAGGA ACTGGGTTTCGCTCAAATTACTTCG CCTACGAAACTGGAGGAAAAGCCT GCAAGATGCAGTACTGCAAGCACT GGGGCGTGAGACTACCCAGCGGTG TCTGGTTCGAGATGGCCGATAAGG ACCTGTTTGCAGCAGCGAGATTCCC GGAATGCCCTGAGGGATCGAGCAT CTCCGCTCCAAGCCAAACTTCAGTG GACGTGAGCCTGATCCAGGACGTG GAACGGATTCTCGACTACTCGCTGT GCCAGGAGACCTGGTCGAAGATCA GAGCGGGACTGCCCATCTCACCGGT GGACCTGTCCTACCTGGCGCCAAAG AATCCGGGCACTGGACCGGCGTTC ACCATCATCAACGGCACCCTCAAAT ACTTCGAGACGCGGTACATCCGGGT GGACATCGCAGCTCCGATCCTCTCC CGGATGGTGGGAATGATCTCGGGG ACTACTACCGAACGCGAGCTCTGG GACGACTGGGCACCTTACGAGGAT GTCGAGATCGGACCTAACGGAGTG CTCCGGACCTCCTCCGGGTACAAGT TCCCTCTGTACATGATCGGCCATGG CATGCTGGACTCGGATCTGCATCTG TCGTCCAAAGCACAGGTGTTTGAAC ACCCACACATTCAAGACGCCGCCA GCCAGCTGCCGGACGATGAGTCGC TGTTCTTCGGAGACACGGGCTTGTC AAAGAATCCCATCGAGCTGGTGGA AGGATGGTTTTCATCCTGGAAAAGC	DSQFINGKCSNYICPTVHNSTTWHS DYKVKGLCDSNLISMDITFFSEDGE LSSLGKEGTGFRSNYFAYETGGKA CKMQYCKHWGVRLPSGVWFEMA DKDLFAAARFPECPEGSSISAPSQTS VDVSLIQDVERILDYSLCQETWSKI RAGLPISPVDLSYLAPKNPGTGPAF TIINGTLKYFETRYIRVDIAAPILSR MVGMISGTTTERELWDDWAPYED VEIGPNGVLRTSSGYKFPLYMIGHG MLDSDLHLSSKAQVFEHPHIQDAA SQLPDDESLFFGDTGLSKNPIELVEG WFSSWKSSIASFFFIIGLIIGLFLVLR VGIHLCIKLKHTKKRQIYTDIEMNR LGK (SEQ ID NO: 99)

AGCATCGCTTCATTCTTCTTCATCAT TGGCCTGATCATCGGCCTATTTCTA GTCCTGCGGGTGGGAATTCATCTGT GCATCAAGCTCAAGCACACTAAGA AGCGGCAAATCTACACTGATATCG AGATGAATCGCCTGGGCAAGTAG (SEQ ID NO: 98)	
Trop-058 Mutated VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCACA ACCAAAAGGGCAACTGGAAAAACG TGCCTTCAAATTACCATTATTGCCC CAGCAGCTCGGACCTGAACTGGCA CAATGACCTCATTGGAACCGCGCTG CAGGTGAAGATGCCAGGCGGCAGC AAGAGCCACAAGGCTATCCAGGCT GACGGATGGATGTGCCACGCGTCA AAATGGGTGACTACCTGCGATTTCC GCTGGTACGGACCAAAATACATCA CGCACAGCATCAGATCATTCACCCC GTCAGTGGAACAATGCAAAGAATC CATCGAACAGACTAAGCAGGGAAC CTGGCTGAACCCTGGATTTCCGCCG CAGTCGTGTGGGTACGCAACCGTG ACCGATGCAGAGGCCGTGATCGTG CAAGTCACGCCGCATCACGTGCTTG TGGACGAGTACACCGGAGAATGGG TCGATTCCCAGTTCATCAACGGCAA GTGCTCCAACTACATTTGCCCAACC GTGCACAACAGCACTACTTGGCAC AGCGACTACAAAGTGAAGGGTCTG TGTGATTCCAACCTGATCTCCATGG ATATCACTTTCTTCTCGGAAGACGG CGAACTGTCCTCACTGGGCAAAGA AGGAACTGGGTTTCGCTCAAATTAC TTCGCCTACGAAACTGGAGGAAAA GCCTGCAAGATGCAGTACTGCAAG CACTGGGGCGTGAGACTACCCAGC GGTGTCTGGTTCGAGATGGCCGATA AGGACCTGTTTGCAGCAGCGAGATT CCCGGAATGCCCTGAGGGATCGAG CATCTCCGCTCCAAGCCAAACTTCA GTGGACGTGAGCCTGATCCAGGAC GTGGAACGGATTCTCGACTACTCGC TGTGCCAGGAGACCTGGTCGAAGA TCAGAGCGGGACTGCCCATCTCACC GGTGGACCTGTCCTACCTGGCGCCA	KFTIVFPHNQKGNWKNVPSNYHYC PSSSDLNWHNDLIGTALQVKMPGG SKSHKAIQADGWMCHASKWVTTC DFRWYGPKYITHSIRSFTPSVEQCK ESIEQTKQGTWLNPGFPPQSCGYAT VTDAEAVIVQVTPHHVLVDEYTGE WVDSQFINGKCSNYICPTVHNSTT WHSDYKVKGLCDSNLISMDITFFSE DGELSSLGKEGTGFRSNYFAYETG GKACKMQYCKHWGVRLPSGVWFE MADKDLFAAARFPECPEGSSISAPS QTSVDVSLIQDVERILDYSLCQETW SKIRAGLPISPVDLSYLAPKNPGTGP AFTIINGTLKYFETRYIRVDIAAPILS RMVGMISGTTTERELWDDWAPYE DVEIGPNGVLRTSSGYKFPLYMIGH GMLDSDLHLSSKAQVFEHPHIQDA ASQLPDDESLFFGDTGLSKNPIELVE GWFSSWKSSIASFFFIIGLIIGLFLVL RVGIHLCIKLKHTKKRQIYTDIEMN RLGK (SEQ ID NO: 101)

AAGAATCCGGGCACTGGACCGGCG TTCACCATCATCAACGGCACCCTCA AATACTTCGAGACGCGGTACATCCG GGTGGACATCGCAGCTCCGATCCTC TCCCGGATGGTGGGAATGATCTCGG GGACTACTACCGAACGCGAGCTCT GGGACGACTGGGCACCTTACGAGG ATGTCGAGATCGGACCTAACGGAG TGCTCCGGACCTCCTCCGGGTACAA GTTCCCTCTGTACATGATCGGCCAT GGCATGCTGGACTCGGATCTGCATC TGTCGTCCAAAGCACAGGTGTTTGA ACACCCACACATTCAAGACGCCGC CAGCCAGCTGCCGGACGATGAGTC GCTGTTCTTCGGAGACACGGGCTTG TCAAAGAATCCCATCGAGCTGGTG GAAGGATGGTTTTCATCCTGGAAAA GCAGCATCGCTTCATTCTTCTTCAT CATTGGCCTGATCATCGGCCTATTT CTAGTCCTGCGGGTGGGAATTCATC TGTGCATCAAGCTCAAGCACACTAA GAAGCGGCAAATCTACACTGATAT CGAGATGAATCGCCTGGGCAAGTA G (SEQ ID NO: 100)	
Trop-061 Mutated VSV-G	
Nucleotide	**Amino acid**
AAGTTCACTATCGTGTTTCCGCAC GGCGGAAGCAACGGCGGGAGCCA AAAGGGCAACTGGAAAAACGTGC CTTCAAATTACCATTATTGCCCCA GCAGCTCGGACCTGAACTGGCAC AATGACCTCATTGGAACCGCGCT GCAGGTGAAGATGCCAAAGAGCC ACAAGGCTATCCAGGCTGACGGA TGGATGTGCCACGCGTCAAAATG GGTGACTACCTGCGATTTCCGCTG GTACGGACCAAAATACATCACGC ACAGCATCAGATCATTCACCCCGT CAGTGGAACAATGCAAAGAATCC ATCGAACAGACTAAGCAGGGAAC CTGGCTGAACCCTGGATTTCCGCC GCAGTCGTGTGGGTACGCAACCG TGACCGATGCAGAGGCCGTGATC GTGCAAGTCACGCCGCATCACGT GCTTGTGGACGAGTACACCGGAG AATGGGTCGATTCCCAGTTCATCA ACGGCAAGTGCTCCAACTACATTT GCCCAACCGTGCACAACAGCACT	KFTIVFPHGGSNGGSQKGNWKNVPS NYHYCPSSSDLNWHNDLIGTALQVK MPKSHKAIQADGWMCHASKWVTTC DFRWYGPKYITHSIRSFTPSVEQCKES IEQTKQGTWLNPGFPPQSCGYATVTD AEAVIVQVTPHHVLVDEYTGEWVDS QFINGKCSNYICPTVHNSTTWHSDYK VKGLCDSNLISMDITFFSEDGELSSLG KEGTGFRSNYFAYETGGKACKMQYC KHWGVRLPSGVWFEMADKDLFAAA RFPECPEGSSISAPSQTSVDVSLIQDVE RILDYSLCQETWSKIRAGLPISPVDLS YLAPKNPGTGPAFTIINGTLKYFETRY IRVDIAAPILSRMVGMISGTTTERELW DDWAPYEDVEIGPNGVLRTSSGYKFP LYMIGHGMLDSDLHLSSKAQVFEHP HIQDAASQLPDDESLFFGDTGLSKNPI ELVEGWFSSWKSSIASFFFIIGLIIGLFL VLRVGIHLCIKLKHTKKRQIYTDIEM NRLGK (SEQ ID NO: 103)

ACTTGGCACAGCGACTACAAAGT GAAGGGTCTGTGTGATTCCAACCT GATCTCCATGGATATCACTTTCTT CTCGGAAGACGGCGAACTGTCCT CACTGGGCAAAGAAGGAACTGGG TTTCGCTCAAATTACTTCGCCTAC GAAACTGGAGGAAAAGCCTGCAA GATGCAGTACTGCAAGCACTGGG GCGTGAGACTACCCAGCGGTGTC TGGTTCGAGATGGCCGATAAGGA CCTGTTTGCAGCAGCGAGATTCCC GGAATGCCCTGAGGGATCGAGCA TCTCCGCTCCAAGCCAAACTTCAG TGGACGTGAGCCTGATCCAGGAC GTGGAACGGATTCTCGACTACTCG CTGTGCCAGGAGACCTGGTCGAA GATCAGAGCGGGACTGCCCATCT CACCGGTGGACCTGTCCTACCTGG CGCCAAAGAATCCGGGCACTGGA CCGGCGTTCACCATCATCAACGGC ACCCTCAAATACTTCGAGACGCG GTACATCCGGGTGGACATCGCAG CTCCGATCCTCTCCCGGATGGTGG GAATGATCTCGGGGACTACTACC GAACGCGAGCTCTGGGACGACTG GGCACCTTACGAGGATGTCGAGA TCGGACCTAACGGAGTGCTCCGG ACCTCCTCCGGGTACAAGTTCCCT CTGTACATGATCGGCCATGGCATG CTGGACTCGGATCTGCATCTGTCG TCCAAAGCACAGGTGTTTGAACA CCCACACATTCAAGACGCCGCCA GCCAGCTGCCGGACGATGAGTCG CTGTTCTTCGGAGACACGGGCTTG TCAAAGAATCCCATCGAGCTGGT GGAAGGATGGTTTTCATCCTGGA AAAGCAGCATCGCTTCATTCTTCT TCATCATTGGCCTGATCATCGGCC TATTTCTAGTCCTGCGGGTGGGAA TTCATCTGTGCATCAAGCTCAAGC ACACTAAGAAGCGGCAAATCTAC ACTGATATCGAGATGAATCGCCT GGGCAAGTAG (SEQ ID NO: 102)	

In other embodiments, the methods for producing LVV according to the present disclosure may utilize a five plasmid system. Where a five plasmid system is used, the LVV production system utilizes four plasmids from a third generation vector system

(i.e., a transgene plasmid, a GagPol plasmid, an Env plasmid, and a VSV-G env plasmid). In addition, a five plasmid system as described herein includes a fifth plasmid that encodes a targeting protein (the "targeting protein plasmid"). The GagPol and Env packaging plasmids included in a five plasmid system may be standard packaging plasmids as described in *Production of Lentiviral Vectors, Merten et al., Molecular Therapy – Methods & Clinical Development* (2016), 3, 16017. The transgene plasmid includes an expression cassette that encodes one or more CAR transgenes according to the present disclosure, and the VSV-G env plasmid includes an expression cassette that encodes a mutant VSV-G as described herein.

In some embodiment, the fifth plasmid comprises an expression cassette encoding a CD80 targeting protein In some embodiments, the fifth plasmid comprises an expression cassette encoding an anti-CD3 targeting protein. In some embodiments, the fifth plasmid comprises an expression cassette encoding a CD80 targeting protein and an anti-CD3 targeting protein. Examples of CD80 targeting protein and anti-CD3 targeting proteins that may be used in the fifth plasmid are provided in Tables 1-3.

In certain embodiments, the present disclosure provides a method for producing a lymphocyte targeted lentiviral vector according to any one of the preceding claims, the method comprising: transfecting a producer cell with a GagPol plasmid, a Rev plasmid, a transgene plasmid, a VSV-G env plasmid, and a lymphocyte targeting protein plasmid; the GagPol plasmid comprises a one or more polynucleotides encoding a lentiviral gag gene and a lentiviral pol gene and is capable of expressing a lentiviral gag protein and a lentiviral pol protein within the producer cell; the Rev plasmid comprises a polynucleotide encoding a lentiviral rev gene and is capable expressing a lentiviral rev protein within the producer cell; the transgene plasmid comprises an expression cassette comprising a polynucleotide encoding a CAR; and the VSV-G env plasmid comprises a polynucleotide encoding a mutated VSV-G envelope protein as described herein and is capable of expressing the mutated VSV-G envelope protein within the producer cell; and the lymphocyte targeting protein plasmid comprises a polynucleotide encoding a lymphocyte targeting protein as described herein and is capable of expressing the lymphocyte targeting protein within the producer cell; culturing the producer cell in a culture medium; and harvesting lentiviral vector from the culture medium.

In some embodiments, the producer cells are transfected with a defined ratio of the transgene plasmid, GagPol plasmid, VSV-G env plasmid, Rev plasmid, and lymphocyte targeting plasmid. The lymphocyte targeting plasmid may be at a ratio of about 0.25 to about 5 relative to the VSV-G env plasmid (by mass). For example, using the defined ratios for the four packaging plasmid system (transgene plasmid, GagPol plasmid, VSV-G env plasmid, Rev plasmid) described herein, the ratio of the lymphocyte targeting protein plasmid can be adjusted relative to the VSV-G env plasmid. For example, the lymphocyte targeting plasmid may be at a ratio of about 0.25:1, 0.5:1, 1:1, 1.25:1, 1.5:1, 1.75:1, 2:1, 2.25:1, 2.5:1, 2.75:1, 3:1, 3.25:1, 3.5:1, 3.75:1, 4:1, 4.25:1, 4.5:1, 4.75:1 or 5:1 relative to the VSV-G env plasmid.

In embodiments of LVV production methods that utilize a combination of five plasmids, the fifth plasmid is a targeting protein plasmid and includes an expression cassette that encodes one or more targeting proteins. For example the targeting protein plasmid may include an expression cassette that includes a polynucleotide that encodes a CD80 targeting protein, an anti-CD3 targeting protein, or both a CD80 targeting protein and an anti-CD3 targeting protein. In certain embodiments, a targeting protein vector plasmid an expression cassette comprising a polynucleotide according to SEQ ID NO: 1, which encodes a CD80 targeting protein according to SEQ ID NO: 2. In other embodiments, a targeting protein plasmid includes an expression cassette comprising a polynucleotide according to SEQ ID NO: 9, which encodes an anti-CD3 targeting protein according to SEQ ID NO: 10. In other embodiments, a targeting protein plasmid includes an expression cassette comprising a polynucleotide according to SEQ ID NO: 116, which encodes an anti-CD3 targeting protein according to SEQ ID NO: 117. In other embodiments, a targeting protein plasmid includes a tandem expression cassette that comprises a polynucleotide according to SEQ ID NO: 21, which encodes a polypeptide according to SEQ ID NO: 22.Examples of mutated VSV-G sequences that may be used in the five plasmid, 3rd generation lentiviral vector system of the present disclosure are provided in Table 10.

In the four plasmid packaging system, the concentration of the lymphocyte targeting protein on the surface of the transduced cells may be associated with the

concentration of the plasmid containing the targeting protein. For example if the lymphocyte targeting protein plasmid plasmid is contained in the VSV-G env plasmid, the concentration of the lymphocyte targeting protein on the surface of the transduced cells may be associated with the concentration of the VSV-G env plasmid. In another example, if the lymphocyte targeting protein plasmid plasmid is contained in the GagPol plasmid, the concentration of the lymphocyte targeting protein on the surface of the transduced cells may be associated with the concentration of the GagPol plasmid. In another example, if the lymphocyte targeting protein plasmid plasmid is contained in the Rev plasmid, the concentration of the lymphocyte targeting protein on the surface of the transduced cells may be associated with the concentration of the Rev plasmid. In the five plasmid packaging system, the concentration of the lymphocyte targeting protein on the surface of the transduced cells may be associated with the concentration of the plasmid containing the targeting protein.

In the four plasmid packaging system, the transduction efficiency may be associated with the concentration of the plasmid containing the targeting protein. For example if the lymphocyte targeting protein plasmid plasmid is contained in the VSV-G env plasmid, the transduction efficiency may be associated with the concentration of the VSV-G env plasmid. In another example, if the lymphocyte targeting protein plasmid plasmid is contained in the GagPol plasmid, the transduction efficiency may be associated with the concentration of the GagPol plasmid. In another example, if the lymphocyte targeting protein plasmid plasmid is contained in the Rev plasmid, the transduction efficiency may be associated with the concentration of the Rev plasmid. In the five plasmid packaging system, the transduction efficiency of may be associated with the concentration of the lymphocyte targeting protein plasmid.

Following transfection of producer cells, the lentiviral particles product may be harvested from the cell supernatant or culture media. Downstream processes of LVV to maximize LVV recovery while minimizing components which may negatively impact efficacy or safety are known in the art. A typical process, involves sequential purification steps comprising removing cells and their debris followed by enrichment of LVV and the removal of host cell or serum proteins, nucleic acids and lipids. The LVV product may be further concentrated prior to exchanging into a suitable formulation buffer for stability and then finally undergoing sterile filtration prior to storage or

application. A clarification step may be initially performed on the harvested LVV supernatants to remove large impurities such as aggregates and cell debris. In some embodiments, the clarification step comprises centrifugation and/or conventional flow filtration. In some embodiments, the clarification step includes a nuclease digestion step. In some embodiments, the LVV product undergoes further purification steps, including for example, ion exchange chromatography (e.g., anion exchange chromatography). The LVV may also be concentrated, e.g., by tangential flow filtration or ultrafiltration/diafiltration. In some embodiments, harvesting LVV from culture medium comprises centrifugation. In some embodiments, harvesting LVV from culture medium comprises anion exchange chromatography, In some embodiments, harvesting LVV from culture medium comprises anion exchange chromatography and tangential flow filtration.

In some embodiments, the lentiviral vectors described herein, which incorporate a mutated VSV-G env and one or more lymphocyte targeting proteins, are capable of producing a high titer LVV product, as compared to standard LVV incorporating another fusogenic env protein (e.g., cocal env, paramyxovirus env, truncated VSV-G env). As used herein viral titer refers to infectious virus particle titer as measured by transducing units (TU) per mL. In some embodiments, the titer of LVV described herein is at least 1e7 TU/mL, at least 1e8 TU/mL, at least 1e9 TU/mL, at least 1e10 TU/mL, at least 1e11 TU/mL, or at least 1e12 TU/mL as measured in concentrated LVV product. In some embodiments, the titer of LVV described herein is about 1e7 TU/mL to about 1e12 TU/mL in concentrated LVV product.

Engineered Lymphocytes

The LVV described herein can be used to modify targeted lymphocytes (e.g., T cells, B cells, or NK cells) to express a CAR encoded by a transgene carried by the LVV. In certain embodiments, the engineered lymphocytes have been transduced by an LVV according to the present disclosure by contacting the lymphocytes with the LVV of the present disclosure. In such embodiments, the engineered lymphocytes (e.g., T cells, B cells, or NK cells) express a CAR encoded by the transgene carried by the LVV. These lymphocytes are also referred to herein as "CAR modified lymphocytes." In specific embodiments, the engineered T cells are referred to herein as "CAR

modified T cells." In other specific embodiments, the engineered NK cells are referred to herein as "CAR modified NK cells." In specific embodiments, the engineered T cells are referred to herein as "CAR modified B cells." In specific embodiments the CAR modified T cell expresses a CAR encoded by a transgene carried by an LVV described herein and is selected from naïve T cells (CD45RA+, CCR7+, CD62L+, CD27+, CD45RO-), central memory T cells (CD45RA-, $CD45RO^+$, $CD62L^+$, CCR7+, CD27+), effector memory T cells (CD45RA-, CD45RO+, CCR7-, CD62L-, CD27-), γδ T cells, mucosal-associated invariant T (MAIT) cells, Tregs, natural killer T cells, and tissue resident T cells.

Transduction of targeted lymphocytes (e.g., T cells, B cells, or NK cells) by LVV described herein may be performed *ex vivo* or *in vivo*.

In certain embodiments, the lymphocytes (e.g., T cells, B cells, or NK cells) can be primary cells or cell lines derived from human, mouse, rat, or other mammals. If obtained from a mammal, a lymphocyte can be obtained from numerous sources, including blood, bone marrow, lymph node, thymus, or other tissues or fluids. A lymphocyte composition (e.g., T cell composition, B cell composition, or NK cell composition) may be enriched or purified. T cell lines are well known in the art, some of which are described in Sandberg *et al.*, *Leukemia 21*:230, 2000. In certain embodiments, the T cells lack endogenous expression of a TCRα gene, TCRβ gene, or both. Such T cells may naturally lack endogenous expression of TCRα and β chains, or may have been modified to block expression (*e.g.*, T cells from a transgenic mouse that does not express TCR α and β chains or cells that have been manipulated to inhibit expression of TCR α and β chains) or to knockout a TCRα chain, a TCRβ chain, or both genes.

In some embodiments, prior to genetic modification of the lymphocytes (e.g., T cells, B cells, or NK cells) with a polynucleotide encoding a CAR molecule, a source of lymphocytes (e.g., T cells or NK cells) may be obtained from a subject (*e.g.*, whole blood, peripheral blood mononuclear cells (PBMCs), bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue). The lymphocytes, T cells, B cells, or NK cells can be enriched or isolated from a sample taken from the subject using methods known in the art. For example, T cells can be enriched in a whole blood sample taken from a subject using

known methods for hypotonic lysis of red blood cells or T cells can be isolated from a sample taken from a subject using known gradient sedimentation, e.g., Ficoll® reduction) techniques. Alternatively, T cells can be isolated from whole blood using magnetic antibody-labeled beads followed by column separation. Specific host cell subsets can be collected in accordance with known techniques and enriched or depleted by known techniques, such as affinity binding to antibodies, flow cytometry and/or immunomagnetic selection. In some embodiments, after enrichment and/or depletion steps, the T cells are placed in contact with LVV as disclosed such that T cells obtained from the subject are transduced by the LVV and express the CAR encoded by the transgene carried by the LVV. In some embodiments, after enrichment and/or depletion steps, the NK cells are placed in contact with LVV as disclosed such that NK cells obtained from the subject are transduced by the LVV and express the CAR encoded by the transgene carried by the LVV. In some embodiments, after enrichment and/or depletion steps, the B cells are placed in contact with LVV as disclosed such that B cells obtained from the subject are transduced by the LVV and express the CAR encoded by the transgene carried by the LVV.

Generally, resting T cells, such as resting CD4 and CD8 lymphocytes, resting B cells, and resting NK cells are refractory to genetic transduction by lentiviral vectors. Resting T cells, also known as quiescent T cells or naïve T cells, refer to T cells that are not mitotically active or have not been exposed to a cognate antigen presented on an antigen presenting cell, such as a macrophage or dendritic cell. An example of a marker for resting T cells is CD28. Alternatively, markers that are expressed on activated T cells but not resting T cells include, for example, 4-1BB, PD-1, and HLA-DR. Similarly, resting B cells and resting NK cells, refer to B cells and NK cells that are not mitotically active or have not been exposed to a cognate antigen, respectively. Examples of markers for resting B cells include CD21 and CD23, and the absence of CD80, CD86, CD95, or CD25. CD137 and GITR, which are expressed on activated NK cells, are absent on resting NK cells. As a result, in order to facilitate transduction of T cells, B cells, or NK cells using a lentiviral vector, the T cells, B cells, or NK cells are typically activated in vitro using stimulation reagents before genetic modification via a lentiviral vector can occur. Following stimulation and transduction, the genetically modified cells are then typically expanded in vitro and subsequently reintroduced into a

patient. However, LVV described herein are capable of transducing resting T cells, B cells, and/or resting NK cells. In certain embodiments, methods of transducing T cells with an LVV according to the present description include, placing the LVV in contact with a population of T cells, wherein in the T cells are not activated and the T cells are not exposed to an exogenous stimulating agent during the transduction process. In some such embodiments, the methods of transducing T cells wherein the T cells are not activated and the T cells are not exposed to an exogenous stimulating agent during the transduction process comprises transducing T cells with a LVV comprising a CD80 targeting protein, an anti-CD3 targeting protein, or both a CD80 targeting protein and an anti-CD3 targeting protein. Examples of exogenous T cell stimulating agents include an anti-CD3 antibody or antigen binding fragment thereof (i.e., anti-CD3 antibody or antigen binding fragment thereof that is not part of the LVV), an anti-CD28 antibody or antigen binding fragment thereof (i.e., anti-CD28 antibody or antigen binding fragment thereof that is not part of the LVV), anti-CD2 antibody or antigen binding fragment thereof, IL-2, IL-7, IL-15, PHA, or any combination thereof.. Exogenous stimulating agents may be contacted to the T cell in soluble form or immobilized on a solid substrate, such as a bead or cell culture plate. In some embodiments, an anti-CD3 antibody or antigen binding fragment thereof, an anti-CD28 antibody or antigen binding fragment thereof, or both the anti-CD3 antibody and anti-CD28 antibody or antigen binding fragments thereof are contacted to the T cells in soluble form or immobilized on a solid substrate. Examples of anti-CD3 antibodies include OKT3, UCTH1, and BW264/56. An example of an anti-CD2 antibody is LT2. An example of an anti-CD28 antibody is 15E8. In certain embodiments, methods of transducing NK cells with an LVV according to the present description include, placing the LVV in contact with a population of NK cells, wherein in the NK cells are not activated and the NK cells are not exposed to an exogenous stimulating agent during the transduction process. Examples of exogenous NK cell stimulating agents include anti-CD2 antibody or antigen binding fragment thereof, anti-CD335 antibody or antigen binding fragment thereof, IL-2, IL-15, IL-12, IL-18, IL-21, or any combination thereof. In certain embodiments, methods of transducing B cells with an LVV according to the present description include, placing the LVV in contact with a population of B cells, wherein in the B cells are not activated and the B cells are not exposed to an exogenous

stimulating agent during the transduction process. Examples of exogenous B cell stimulating agents include CD154 and mixed Ig $F(ab)_2$.

In some embodiments, a lentiviral vector having a CD80 targeting protein and an anti-CD3 targeting protein is capable of efficiently transducing both CD4 and CD8 T cells as compared to standard LVV.

In some embodiments, a lentiviral vector having a CD80 targeting protein and an anti-CD3 targeting protein is capable of activating T cells without exogenous stimulation (e.g., T cell stimulating agent) to a comparable level as T cells transduced with standard LVV that are treated with exogenous stimulation. Examples of exogenous T cell stimulating agents include an anti-CD3 antibody or antigen binding fragment thereof (i.e., anti-CD3 antibody or antigen binding fragment thereof that is not part of the LVV), an anti-CD28 antibody or antigen binding fragment thereof (i.e., anti-CD28 antibody or antigen binding fragment thereof that is not part of the LVV), anti-CD2 antibody or antigen binding fragment thereof, IL-2, IL-7, IL-15, PHA, or any combination thereof..

In certain embodiments, methods of transducing T cells with an LVV according to the present description exhibit enhanced transduction of CD4 T cells compared to standard LVV. In some such embodiments, the methods of enhancing transduction of CD4 T cells in a mixed population of T cells (e.g., composed of CD4 and CD8 T cells) comprises transducing the mixed population of T cells with a LVV according to the present description comprising a CD80 targeting protein.

In certain embodiments, methods of transducing T cells with an LVV according to the present description efficiently transduce both CD4 and CD8 T cells. In some such embodiments, the methods of transducing T cells wherein both CD4 and CD8 T cells are efficiently transduced comprises transducing T cells with a LVV comprising a CD80 targeting protein and an anti-CD3 targeting protein.

In certain embodiments, an LVV according to the present description is capable of transducing T cells under "stressed" conditions, e.g., at low multiplicity of infection (MOI) or without treatment with exogenous IL-2 during transduction. In some embodiments, the LVV comprises a CD80 targeting protein and an anti-CD3 targeting protein. In some embodiments, the LVV according to the present description is capable

of transducing T cells at a LVV concentration about 5 to about 25X lower than a non-lymphocyte targeting LVV.

The expression of a CAR molecule on host cells may be assessed by methods known in the art, such as quantitative PCR or flow cytometry following staining with a fluorescently labeled antigen for the CAR binding domain.

The expression of a CAR molecule on host cells may be functionally characterized according to any of a large number of art-accepted methodologies for assaying host T cell activity, including determination of T cell binding, activation or induction and also including determination of T cell responses that are antigen-specific. Examples include determination of T cell proliferation, T cell cytokine release, antigen-specific T cell stimulation, CTL activity (*e.g.*, by detecting ^{51}Cr or Europium release from pre-loaded target cells, induction of caspase activity in target cells, extracellular release of lactate dehydrogenase by target cells), changes in T cell phenotypic marker expression, and other measures of T cell functions. Assaying host NK cell activity may also be assayed using similar methodologies. Procedures for performing these and similar assays are may be found, for example, in Lefkovits (*Immunology Methods Manual: The Comprehensive Sourcebook of Techniques*, 1998). *See, also, Current Protocols in Immunology*; Weir, *Handbook of Experimental Immunology*, Blackwell Scientific, Boston, MA (1986); Mishell and Shigii (eds.) *Selected Methods in Cellular Immunology*, Freeman Publishing, San Francisco, CA (1979); Green and Reed, *Science* 281:1309 (1998) and references cited therein. Cytokine levels may be determined according to methods known in the art, including for example, ELISA, ELISPOT, intracellular cytokine staining, flow cytometry, and any combination thereof (*e.g.*, intracellular cytokine staining and flow cytometry). Immune cell proliferation and clonal expansion resulting from an antigen-specific elicitation or stimulation of an immune response may be determined by isolating lymphocytes, such as circulating lymphocytes in samples of peripheral blood cells or cells from lymph nodes, stimulating the cells with antigen, and measuring cytokine production, cell proliferation and/or cell viability, such as by incorporation of tritiated thymidine or non-radioactive assays, such as MTT assays and the like.

Methods of & Compositions for Treatment

In one aspect, the present disclosure provides methods of treating a disease in a subject comprising administering to the subject an effective amount of an LVV as described herein, a CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) as described herein, or pharmaceutical compositions thereof. In another aspect, the methods of treating a disease in a subject according to the present description include administering to the subject an effective amount of an LVV as described herein, a CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) as described herein, or pharmaceutical compositions thereof, in combination with one or more additional therapeutic agents.

Diseases that may be treated with the LVV or CAR modified lymphocytes (e.g., T cells, B cells, or NK cells) according to the present description include cancers. Adoptive immune and gene therapies are promising treatments for various types of cancer (Morgan *et al.*, *Science 314*:126, 2006; Schmitt *et al.*, *Hum. Gene Ther. 20*:1240, 2009; June, *J. Clin. Invest. 117*:1466, 2007) and infectious disease (Kitchen *et al.*, *PLoS One 4*:38208, 2009; Rossi *et al.*, *Nat. Biotechnol. 25*:1444, 2007; Zhang *et al.*, *PLoS Pathog. 6*:e1001018, 2010; Luo *et al.*, *J. Mol. Med. 89*:903, 2011).

A wide variety of cancers, including solid tumors and leukemias are amenable to the compositions and methods disclosed herein. Exemplary cancers that may be treated using the receptors, modified host cells, and composition described herein include adenocarcinoma of the breast, prostate, and colon; all forms of bronchogenic carcinoma of the lung; myeloid leukemia; melanoma; hepatoma; neuroblastoma; papilloma; apudoma; choristoma; branchioma; malignant carcinoid syndrome; carcinoid heart disease; and carcinoma (*e.g.*, Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell). Additional types of cancers that may be treated using the receptors, modified host cells, and composition described herein include histiocytic disorders; malignant histiocytosis; leukemia; Hodgkin's disease; immunoproliferative small; non-Hodgkin's lymphoma; plasmacytoma; multiple myeloma; chronic myeloid leukemia (CML); acute myeloid leukemia (AML); plasmacytoma; reticuloendotheliosis; melanoma; chondroblastoma; chondroma; chondrosarcoma; fibroma; fibrosarcoma; giant cell

tumors; histiocytoma; lipoma; liposarcoma; mesothelioma; myxoma; myxosarcoma; osteoma; osteosarcoma; chordoma; craniopharyngioma; dysgerminoma; hamartoma; mesenchymoma; mesonephroma; myosarcoma; ameloblastoma; cementoma; odontoma; teratoma; thymoma; trophoblastic tumor. Further, the following types of cancers are also contemplated as amenable to treatment using the receptors, modified host cells, and composition described herein: adenoma; cholangioma; cholesteatoma; cyclindroma; cystadenocarcinoma; cystadenoma; granulosa cell tumor; gynandroblastoma; hepatoma; hidradenoma; islet cell tumor; Leydig cell tumor; papilloma; sertoli cell tumor; theca cell tumor; leimyoma; leiomyosarcoma; myoblastoma; myomma; myosarcoma; rhabdomyoma; rhabdomyosarcoma; ependymoma; ganglioneuroma; glioma; medulloblastoma; meningioma; neurilemmoma; neuroblastoma; neuroepithelioma; neurofibroma; neuroma; paraganglioma; paraganglioma nonchromaffin. The types of cancers that may be treated also include angiokeratoma; angiolymphoid hyperplasia with eosinophilia; angioma sclerosing; angiomatosis; glomangioma; hemangioendothelioma; hemangioma; hemangiopericytoma; hemangiosarcoma; lymphangioma; lymphangiomyoma; lymphangiosarcoma; pinealoma; carcinosarcoma; chondrosarcoma; cystosarcoma phyllodes; fibrosarcoma; hemangiosarcoma; leiomyosarcoma; leukosarcoma; liposarcoma; lymphangiosarcoma; myosarcoma; myxosarcoma; ovarian carcinoma; rhabdomyosarcoma; sarcoma; neoplasms; nerofibromatosis; cervical dysplasia, and peritoneal cancer.

Examples of hyperproliferative disorders amenable to therapy using the receptors, modified host cells, and composition described herein include B-cell cancers (B-cell malignancies), including B-cell lymphomas (such as various forms of Hodgkin's disease, non-Hodgkin's lymphoma (NHL) or central nervous system lymphomas), leukemias (such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia, B cell blast transformation of chronic myeloid leukemia, acute myeloid leukemia (AML), chronic myeloid leukemia, and myelomas (such as multiple myeloma). Additional B cell cancers that may be treated using the receptors, modified host cells, and composition described herein include small lymphocytic lymphoma, B-cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, splenic marginal zone lymphoma, plasma cell myeloma, solitary plasmacytoma of bone, extraosseous plasmacytoma, extra-nodal marginal zone B-cell lymphoma of mucosa-

associated (MALT) lymphoid tissue, nodal marginal zone B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, diffuse large B-cell lymphoma, mediastinal (thymic) large B-cell lymphoma, intravascular large B-cell lymphoma, primary effusion lymphoma, Burkitt's lymphoma/leukemia, B-cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, and post-transplant lymphoproliferative disorder.

The CAR modified lymphocytes included in the compositions disclosed herein and administered to a subject may include, CAR modified T cells, *e.g.*, CD4+ T cells, CD8+ T cells, Natural Killer T cells, gamma delta T cells, or MAIT cells; CAR modified B cells, or CAR modified NK cells. In certain embodiments, methods of treating a subject comprise administering an effective amount of an LVV as described herein or CAR modified lymphocytes (*i.e.*, recombinant cells that express one or more CARs) according to the present disclosure. In some embodiments, CAR modified T cells are administered to a subject. In some embodiments, CAR modified NK cells are administered to a subject. In some embodiments, CAR modified B cells are administered to a subject. The CAR modified lymphocytes (e.g., T cells, B cells, or NK cells) may be xenogeneic, syngeneic, allogeneic, or autologous to the subject.

Pharmaceutical compositions including the LVV or CAR engineered lymphocytes may be administered in a manner appropriate to the disease or condition to be treated (or prevented) as determined by persons skilled in the medical art. An appropriate dose, suitable duration, and frequency of administration of the compositions will be determined by such factors as the condition of the patient, size, weight, body surface area, age, sex, type and severity of the disease, particular therapy to be administered, particular form of the active ingredient, time and the method of administration, and other drugs being administered concurrently. The present disclosure provides pharmaceutical compositions comprising LVV or CAR modified lymphocytes and a pharmaceutically acceptable carrier, diluent, or excipient. Suitable excipients include water, saline, dextrose, glycerol, or the like and combinations thereof. Other suitable infusion medium can be any isotonic medium formulation, including saline, Normosol R (Abbott), Plasma-Lyte A (Baxter), 5% dextrose in water, or Ringer's lactate.

A treatment effective amount of engineered lymphocytes (e.g., T cells, B cells, or NK cells) in a pharmaceutical composition is at least one cell (for example, one CAR modified T cell) and is more typically greater than 10^2 cells, for example, up to 10^6, up to 10^7, up to 10^8 cells, up to 10^9 cells, up to 10^{10} cells, or up to 10^{11} cells or more. In certain embodiments, the cells are administered in a range from about 10^6 to about 10^{10} cells/m^2, preferably in a range of about 10^7 to about 10^9 cells/m^2. The number of cells will depend upon the ultimate use for which the composition is intended as well the type of cells included therein. For example, a composition comprising T cells modified to contain a CAR will comprise a T cell population containing from about 5% to about 95% or more of such cells. In certain embodiments, a composition comprising CAR modified T cells comprises a cell population comprising at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of such cells. In certain embodiments, a composition comprising CAR modified NK cells comprises a cell population comprising at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of such cells. In certain embodiments, a composition comprising CAR modified B cells comprises a cell population comprising at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of such cells. For uses provided herein, the lymphocytes (e.g., T cells, B cells, or NK cells) are generally in a volume of a liter or less, 500 mls or less, 250 mls or less, or 100 mls or less. Hence the density of the desired cells is typically greater than 10^4 cells/ml and generally is greater than 10^7 cells/ml, generally 10^8 cells/ml or greater. The lymphocytes (e.g., T cells, B cells, or NK cells) may be administered as a single infusion or in multiple infusions over a range of time. Repeated infusions of CAR modified lymphocytes (e.g., T cells, B cells, or NK cells) may be separated by days, weeks, months, or even years if relapses of disease or disease activity are present. A clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} cells. A preferred dose for administration of a host cell comprising a recombinant expression vector as described herein is about 10^7 cells/m^2, about 5 x 10^7 cells/m^2, about 10^8 cells/m^2, about 5 x 10^8 cells/m^2, about 10^9 cells/m^2, about 5 x 10^9 cells/m^2, about 10^{10} cells/m^2, about 5 x 10^{10} cells/m^2, or about 10^{11} cells/m^2.

The LVV and/or CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) compositions as described herein may be administered to a subject intravenously, intraperitoneally, intratumorly, into the bone marrow (*e.g.*, intraosseous administration), into the lymph node (intranodally), and /or into cerebrospinal fluid.

The LVV vectors and/or CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) compositions may be administered to a subject in combination with one or more additional therapeutic agents. Examples of therapeutic agents that may be administered in combination with the LVV vectors or CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) according to the present description include radiation therapy, adoptive cellular immunotherapy agent (*e.g.*, recombinant TCR, enhanced affinity TCR, CAR, TCR-CAR, scTCR fusion protein, dendritic cell vaccine), antibody therapy, immune checkpoint molecule inhibitor therapy, UV light therapy, electric pulse therapy, high intensity focused ultrasound therapy, oncolytic virus therapy, or a pharmaceutical therapy, such as a chemotherapeutic agent, a therapeutic peptide, a hormone, an aptamer, antibiotic, anti-viral agent, anti-fungal agent, anti-inflammatory agent, a small molecule therapy, or any combination thereof.

Radiation therapy includes external beam radiation therapy (e.g., conventional external beam radiation therapy, stereotactic radiation, 3-dimensional conformal radiation therapy, intensity-modulated radiation therapy, volumetric modulated arc therapy, particle therapy, proton therapy, and auger therapy), brachytherapy, systemic radioisotope therapy, intraoperative radiotherapy, or any combination thereof.

Exemplary antibodies that may be used in conjunction with the LVV or CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) compositions described herein include rituxmab, pertuzumab, trastuzumab, alemtuzumab, Ibritumomab tiuxetan, Brentuximab vedotin, cetuximab, bevacizumab, abciximab, adalimumab, alefacept, basilizimab, belimumab, bezlotoxumab, canakinumab, certolizumab pegol, daclizumab, denosumab, efalizumab, golimumab, olaratumab, palivizumab, panitumumab, and tocilizumab.

Exemplary inhibitors of immune checkpoint molecules that that may be used in conjunction with the LVV or CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) compositions described herein include checkpoint inhibitors targeting PD-L1, PD-L2, CD80, CD86, B7-H3, B7-H4, HVEM, adenosine, GAL9, VISTA, CEACAM-1,

CEACAM-3, CEACAM-5, PVRL2, PD-1, CTLA-4, BTLA, KIR, LAG3, TIM3, A2aR, CD244/2B4, CD160, TIGIT, LAIR-1, PVRIG/CD112R, or any combination thereof. In certain embodiments, an immune checkpoint inhibitor may be an antibody, a peptide, an RNAi agent, or a small molecule. An antibody specific for CTLA-4 may be ipilimumab or tremelimumab. An antibody specific for PD-1 may be pidilizumab, nivolumab, or pembrolizumab. An antibody specific for PD-L1 may be durvalumab, atezolizumab, or avelumab.

Exemplary chemotherapeutics that may be used in conjunction with the LVV or CAR modified lymphocyte (e.g., T cell, B cell, or NK cell) compositions described herein may include an alkylating agent, a platinum based agent, a cytotoxic agent, an inhibitor of chromatin function, a topoisomerase inhibitor, a microtubule inhibiting drug, a DNA damaging agent, an antimetabolite (such as folate antagonists, pyrimidine analogs, purine analogs, and sugar-modified analogs), a DNA synthesis inhibitor, a DNA interactive agent (such as an intercalating agent), and a DNA repair inhibitor.

As referred to herein, a chemotherapeutic includes non-specific cytotoxic agents that inhibit mitosis or cell division, as well as molecularly targeted therapy that blocks the growth and spread of cancer cells by targeting specific molecules that are involved in tumor growth, progression, and metastasis (*e.g.*, oncogenes). Exemplary non-specific chemotherapeutics for use in conjunction with the expression cassette compositions described herein may include an alkylating agent, a platinum based agent, a cytotoxic agent, an inhibitor of chromatin function, a topoisomerase inhibitor, a microtubule inhibiting drug, a DNA damaging agent, an antimetabolite (such as folate antagonists, pyrimidine analogs, purine analogs, and sugar-modified analogs), a DNA synthesis inhibitor, a DNA interactive agent (such as an intercalating agent), hypomethylating agent, and a DNA repair inhibitor.

Examples of chemotherapeutic agents considered for use in combination therapies contemplated herein include vemurafenib, dabrafenib, trametinib, cobimetinib, anastrozole (Arimidex®), bicalutamide (Casodex®), bleomycin sulfate (Blenoxane®), busulfan (Myleran®), busulfan injection (Busulfex®), capecitabine (Xeloda®), N4-pentoxycarbonyl-5-deoxy-5-fluorocytidine, carboplatin (Paraplatin®), carmustine (BiCNU®), chlorambucil (Leukeran®), cisplatin (Platinol®), cladribine (Leustatin®), cyclophosphamide (Cytoxan® or Neosar®), cytarabine, cytosine

arabinoside (Cytosar-U®), cytarabine liposome injection (DepoCyt®), dacarbazine (DTIC-Dome®), dactinomycin (Actinomycin D, Cosmegan), daunorubicin hydrochloride (Cerubidine®), daunorubicin citrate liposome injection (DaunoXome®), dexamethasone, docetaxel (Taxotere®), doxorubicin hydrochloride (Adriamycin®, Rubex®), etoposide (Vepesid®), fludarabine phosphate (Fludara®), 5-fluorouracil (Adrucil®, Efudex®), flutamide (Eulexin®), tezacitibine, Gemcitabine (difluorodeoxycitidine), hydroxyurea (Hydrea®), Idarubicin (Idamycin®), ifosfamide (IFEX®), irinotecan (Camptosar®), L-asparaginase (ELSPAR®), leucovorin calcium, melphalan (Alkeran®), 6-mercaptopurine (Purinethol®), methotrexate (Folex®), mitoxantrone (Novantrone®), mylotarg, paclitaxel (Taxol®), phoenix (Yttrium90/MX-DTPA), pentostatin, polifeprosan 20 with carmustine implant (Gliadel®),fdabra tamoxifen citrate (Nolvadex®), teniposide (Vumon®), 6-thioguanine, thiotepa, tirapazamine (Tirazone®), topotecan hydrochloride for injection (Hycamptin®), vinblastine (Velban®), vincristine (Oncovin®), ibrutinib, venetoclax, crizotinib, alectinib, brigatinib, ceritinib, and vinorelbine (Navelbine®).

Exemplary alkylating agents for use in combination therapies contemplated herein include nitrogen mustards, ethylenimine derivatives, alkyl sulfonates, nitrosoureas and triazenes): uracil mustard (Aminouracil Mustard®, Chlorethaminacil®, Demethyldopan®, Desmethyldopan®, Haemanthamine®, Nordopan®, Uracil nitrogen Mustard®, Uracillost®, Uracilmostaza®, Uramustin®, Uramustine®), chlormethine (Mustargen®), cyclophosphamide (Cytoxan®, Neosar®, Clafen®, Endoxan®, Procytox®, Revimmune™), ifosfamide (Mitoxana®), melphalan (Alkeran®), Chlorambucil (Leukeran®), pipobroman (Amedel®, Vercyte®), triethylenemelamine (Hemel®, Hexalen®, Hexastat®), triethylenethiophosphoramine, Temozolomide (Temodar®), thiotepa (Thioplex®), busulfan (Busilvex®, Myleran®), carmustine (BiCNU®), lomustine (CeeNU®), streptozocin (Zanosar®), and Dacarbazine (DTIC-Dome®). Additional exemplary alkylating agents for use in combination therapies contemplated herein include, without limitation, Oxaliplatin (Eloxatin®); Temozolomide (Temodar® and Temodal®); Dactinomycin (also known as actinomycin-D, Cosmegen®); Melphalan (also known as L-PAM, L-sarcolysin, and phenylalanine mustard, Alkeran®); Altretamine (also known as hexamethylmelamine (HMM), Hexalen®); Carmustine (BiCNU®); Bendamustine (Treanda®); Busulfan

(Busulfex® and Myleran®); Carboplatin (Paraplatin®); Lomustine (also known as CCNU, CeeNU®); Cisplatin (also known as CDDP, Platinol® and Platinol®-AQ); Chlorambucil (Leukeran®); Cyclophosphamide (Cytoxan® and Neosar®); Dacarbazine (also known as DTIC, DIC and imidazole carboxamide, DTIC-Dome®); Altretamine (also known as hexamethylmelamine (HMM), Hexalen®); Ifosfamide (Ifex®); Prednumustine; Procarbazine (Matulane®); Mechlorethamine (also known as nitrogen mustard, mustine and mechloroethamine hydrochloride, Mustargen®); Streptozocin (Zanosar®); Thiotepa (also known as thiophosphoamide, TESPA and TSPA, Thioplex®); Cyclophosphamide (Endoxan®, Cytoxan®, Neosar®, Procytox®, Revimmune®); and Bendamustine HCl (Treanda®).

Exemplary platinum based agents for use in combination therapies contemplated herein include carboplatin, cisplatin, oxaliplatin, nedaplatin, picoplatin, satraplatin, phenanthriplatin, and triplatin tetranitrate.

Exemplary hypomethylating agents for use in combination therapies include azacitidine and decitabine.

Exemplary molecularly targeted inhibitors for use in combination therapies contemplated herein include small molecules that target molecules involved in cancer cell growth and survival, including for example, receptor tyrosine kinase inhibitors, RAF inhibitors, BCL-2 inhibitors, ABL inhibitors, TRK inhibitors, c-KIT inhibitors, c-MET inhibitors, CDK4/6 inhibitors, FAK inhibitors, FGFR inhibitors, FLT3 inhibitors, IDH1 inhibitors, IDH2 inhibitors, PDGFRA inhibitors, and RET inhibitors

Exemplary molecularly targeted therapy includes hormone antagonists, signal transduction inhibitors, gene expression inhibitors (*e.g.*, translation inhibitors), apoptosis inducers, angiogenesis inhibitors (*e.g.*, a VEGF pathway inhibitor), tyrosine kinase inhibitors (*e.g.*, an EGF/EGFR pathway inhibitor), growth factor inhibitors, GTPase inhibitors, serine/threonine kinase inhibitors, transcription factor inhibitors, inhibitors of driver mutations associated with cancer, B-Raf inhibitors, RAF inhibitors, a MEK inhibitors, mTOR inhibitors, adenosine pathway inhibitors, EGFR inhibitors, PI3K inhibitors, BCL2 inhibitors, VEGFR inhibitors, MET inhibitors, MYC inhibitors, BCR-ABL inhibitors, ABL inhibitors, HER2 inhibitors, H-RAS inhibitors, K-RAS inhibitors, PDGFR inhibitors, ALK inhibitors, ROS1 inhibitors, BTK inhibitors, TRK inhibitors, c-KIT inhibitors, c-MET inhibitors, CDK4/6 inhibitors, FAK inhibitors,

FGFR inhibitors, FLT3 inhibitors, IDH1 inhibitors, IDH2 inhibitors, PARP inhibitors, PARP inhibitors, PDGFRA inhibitors, and RET inhibitors.

Exemplary angiogenesis inhibitors include, without limitation A6 (Angstrom Pharmaceuticals), ABT-510 (Abbott Laboratories), ABT-627 (Atrasentan) (Abbott Laboratories/Xinlay), ABT-869 (Abbott Laboratories), Actimid (CC4047, Pomalidomide) (Celgene Corporation), AdGVPEDF.11D (GenVec), ADH-1 (Exherin) (Adherex Technologies), AEE788 (Novartis), AG-013736 (Axitinib) (Pfizer), AG3340 (Prinomastat) (Agouron Pharmaceuticals), AGX1053 (AngioGenex), AGX51 (AngioGenex), ALN-VSP (ALN-VSP O2) (Alnylam Pharmaceuticals), AMG 386 (Amgen), AMG706 (Amgen), Apatinib (YN968D1) (Jiangsu Hengrui Medicine), AP23573 (Ridaforolimus/MK8669) (Ariad Pharmaceuticals), AQ4N (Novavea), ARQ 197 (ArQule), ASA404 (Novartis/Antisoma), Atiprimod (Callisto Pharmaceuticals), ATN-161 (Attenuon), AV-412 (Aveo Pharmaceuticals), AV-951 (Aveo Pharmaceuticals), Avastin (Bevacizumab) (Genentech), AZD2171 (Cediranib/Recentin) (AstraZeneca), BAY 57-9352 (Telatinib) (Bayer), BEZ235 (Novartis), BIBF1120 (Boehringer Ingelheim Pharmaceuticals), BIBW 2992 (Boehringer Ingelheim Pharmaceuticals), BMS-275291 (Bristol-Myers Squibb), BMS-582664 (Brivanib) (Bristol-Myers Squibb), BMS-690514 (Bristol-Myers Squibb), Calcitriol, CCI-779 (Torisel) (Wyeth), CDP-791 (ImClone Systems), Ceflatonin (Homoharringtonine/HHT) (ChemGenex Therapeutics), Celebrex (Celecoxib) (Pfizer), CEP-7055 (Cephalon/Sanofi), CHIR-265 (Chiron Corporation), NGR-TNF, COL-3 (Metastat) (Collagenex Pharmaceuticals), Combretastatin (Oxigene), CP-751,871(Figitumumab) (Pfizer), CP-547,632 (Pfizer), CS-7017 (Daiichi Sankyo Pharma), CT-322 (Angiocept) (Adnexus), Curcumin, Dalteparin (Fragmin) (Pfizer), Disulfiram (Antabuse), E7820 (Eisai Limited), E7080 (Eisai Limited), EMD 121974 (Cilengitide) (EMD Pharmaceuticals), ENMD-1198 (EntreMed), ENMD-2076 (EntreMed), Endostar (Simcere), Erbitux (ImClone/Bristol-Myers Squibb), EZN-2208 (Enzon Pharmaceuticals), EZN-2968 (Enzon Pharmaceuticals), GC1008 (Genzyme), Genistein, GSK1363089 (Foretinib) (GlaxoSmithKline), GW786034 (Pazopanib) (GlaxoSmithKline), GT-111 (Vascular Biogenics Ltd.), IMC-1121B (Ramucirumab) (ImClone Systems), IMC-18F1 (ImClone Systems), IMC-3G3 (ImClone LLC), INCB007839 (Incyte Corporation), INGN 241 (Introgen Therapeutics), Iressa

(ZD1839/Gefitinib), LBH589 (Faridak/Panobinostst) (Novartis), Lucentis (Ranibizumab) (Genentech/Novartis), LY317615 (Enzastaurin) (Eli Lilly and Company), Macugen (Pegaptanib) (Pfizer), MEDI522 (Abegrin) (MedImmune), MLN518 (Tandutinib) (Millennium), Neovastat (AE941/Benefin) (Aeterna Zentaris), Nexavar (Bayer/Onyx), NM-3 (Genzyme Corporation), Noscapine (Cougar Biotechnology), NPI-2358 (Nereus Pharmaceuticals), OSI-930 (OSI), Palomid 529 (Paloma Pharmaceuticals, Inc.), Panzem Capsules (2ME2) (EntreMed), Panzem NCD (2ME2) (EntreMed), PF-02341066 (Pfizer), PF-04554878 (Pfizer), PI-88 (Progen Industries/Medigen Biotechnology), PKC412 (Novartis), Polyphenon E (Green Tea Extract) (Polypheno E International, Inc.), PPI-2458 (Praecis Pharmaceuticals), PTC299 (PTC Therapeutics), PTK787 (Vatalanib) (Novartis), PXD101 (Belinostat) (CuraGen Corporation), RAD001 (Everolimus) (Novartis), RAF265 (Novartis), Regorafenib (BAY73-4506) (Bayer), Revlimid (Celgene), Retaane (Alcon Research), SN38 (Liposomal) (Neopharm), SNS-032 (BMS-387032) (Sunesis), SOM230 (Pasireotide) (Novartis), Squalamine (Genaera), Suramin, Sutent (Pfizer), Tarceva (Genentech), TB-403 (Thrombogenics), Tempostatin (Collard Biopharmaceuticals), Tetrathiomolybdate (Sigma-Aldrich), TG100801 (TargeGen), Thalidomide (Celgene Corporation), Tinzaparin Sodium, TKI258 (Novartis), TRC093 (Tracon Pharmaceuticals Inc.), VEGF Trap (Aflibercept) (Regeneron Pharmaceuticals), VEGF Trap-Eye (Regeneron Pharmaceuticals), Veglin (VasGene Therapeutics), Bortezomib (Millennium), XL184 (Exelixis), XL647 (Exelixis), XL784 (Exelixis), XL820 (Exelixis), XL999 (Exelixis), ZD6474 (AstraZeneca), Vorinostat (Merck), and ZSTK474.

Exemplary B-Raf inhibitors include vemurafenib, dabrafenib, and encorafenib.

Exemplary MEK inhibitors include binimetinib, cobimetinib, refametinib, selumetinib, and trametinib.

Exemplary BTK inhibitors include ibrutinib, Loxo-305, tirabrutinib, GDC-0853, acalabrutinib, ONO-4059, spebrutinib, BGB-3111, HM71224, and M7583.

Exemplary TRK inhibitors include entrectinib, larotrectinib, CH7057288, ONO-7579, LOXO-101, lestaurtinib, and LOXO-195.

Exemplary c-KIT inhibitors include imatinb, sunitinb, and ponatinib.

Exemplary c-MET inhibitors include capmatinib, crizotinib, tivantinib, onartuzumab, INCB28060, AMG-458, savolitinib, and tepotinib.

Exemplary CDK4/6 inhibitors include palbociclib, ribociclib, abemaciclib, and trilaciclib.

Exemplary FAK inhibitors include defactinib, GSK2256098, BI853520, and PF-00562271.

Exemplary FGFR inhibitors include erdafitinib, pemigatinib, infigratinib, rogaratinib, AZD4547, BGJ398, FP-1039, and ARQ 087.

Exemplary FLT-3 inhibitors include quizartinib, crenolanib, gilteritinib, midostaurin, and lestaurtinib.

Exemplary IDH1 inhibitors include ivosidenib, BAY-1436032, and AGI-5198.

An exemplary IDH2 inhibitor includes enasidenib.

Exemplary PARP inhibitors include talazoparib, niraparib, rucaparib, olaparib, veliparib, CEP 9722, E7016, AG014699, MK4827, BMN-673, and Pamiparib (BGB-290).

Exemplary PDGFRA inhibitors include imatinib, regorafenib, crenolanib, and olaratumab.

Exemplary pan-RAF inhibitors include belvarafenib, LXH254, LY3009120, INU-152, and HM95573.

Exemplary RET inhibitors include lenvatinib, alectinib, vandetanib, cabozantinib, BLU-667, and LOXO-292.

Exemplary ROS1 inhibitors include ceritinib, lorlatinib, entrectinib, crizotinib, TPX-0005, and DS-6051b.

Exemplary Vascular Endothelial Growth Factor (VEGF) receptor inhibitors include, but are not limited to, Bevacizumab (Avastin®), axitinib (Inlyta®); Brivanib alaninate (BMS-582664, (S)—((R)-1-(4-(4-Fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-yl)2-aminopropanoate); Sorafenib (Nexavar®); Pazopanib (Votrient®); Sunitinib malate (Sutent®); Cediranib (AZD2171, CAS 288383-20-1); Vargatef (BIBF1120, CAS 928326-83-4); Foretinib

(GSK1363089); Telatinib (BAY57-9352, CAS 332012-40-5); Apatinib (YN968D1, CAS 811803-05-1); Imatinib (Gleevec®); Ponatinib (AP24534, CAS 943319-70-8); Tivozanib (AV951, CAS 475108-18-0); Regorafenib (BAY73-4506, CAS 755037-03-7); Vatalanib dihydrochloride (PTK787, CAS 212141-51-0); Brivanib (BMS-540215, CAS 649735-46-6); Vandetanib (Caprelsa® or AZD6474); Motesanib diphosphate (AMG706, CAS 857876-30-3, N-(2,3-dihydro-3,3-dimethyl-1H-indol-6-yl)-2-[(4-pyridinylmethyl)amino]-3-pyridinecarboxamide, described in PCT Publication No. WO 02/066470); Dovitinib dilactic acid (TKI258, CAS 852433-84-2); Linfanib (ABT869, CAS 796967-16-3); Cabozantinib (XL184, CAS 849217-68-1); Lestaurtinib (CAS 111358-88-4); N-[5-[[[5-(1,1-Dimethylethyl)-2-oxazolyl]methyl]thio]-2-thiazolyl]-4-piperidinecarboxamide (BMS38703, CAS 345627-80-7); (3R,4R)-4-Amino-1-((4-((3-methoxyphenyl)amino)pyrrolo[2,1-f][1,2,4]triazin-5-yl)methyl)piperidin-3-ol (BMS690514); N-(3,4-Dichloro-2-fluorophenyl)-6-methoxy-7-[[(3aα,5β,6aα)-octahydro-2-methylcyclopenta[c]pyrrol-5-yl]methoxy]-4-quinazolinamine (XL647, CAS 781613-23-8); 4-Methyl-3-[[1-methyl-6-(3-pyridinyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl]amino]-N-[3-(trifluoromethyl)phenyl]-benzamide (BHG712, CAS 940310-85-0); and Aflibercept (Eylea®).

Exemplary EGF pathway inhibitors include, without limitation tyrphostin 46, EKB-569, erlotinib (Tarceva®), gefitinib (Iressa®), erbitux, nimotuzumab, lapatinib (Tykerb®), cetuximab (anti-EGFR mAb), ^{188}Re-labeled nimotuzumab (anti-EGFR mAb), and those compounds that are generically and specifically disclosed in WO 97/02266, EP 0 564 409, WO 99/03854, EP 0 520 722, EP 0 566 226, EP 0 787 722, EP 0 837 063, U.S. Pat. No. 5,747,498, WO 98/10767, WO 97/30034, WO 97/49688, WO 97/38983 and WO 96/33980. Exemplary EGFR antibodies include, but are not limited to, Cetuximab (Erbitux®); Panitumumab (Vectibix®); Matuzumab (EMD-72000); Trastuzumab (Herceptin®); Nimotuzumab (hR3); Zalutumumab; TheraCIM h-R3; MDX0447 (CAS 339151-96-1); and ch806 (mAb-806, CAS 946414-09-1). Exemplary Epidermal growth factor receptor (EGFR) inhibitors include, but not limited to, Erlotinib hydrochloride (Tarceva®); ceritinib; brigatinib; osimeritinib; icotinib; Gefitnib (Iressa®); N-[4-[(3-Chloro-4-fluorophenyl)amino]-7-[[(3″S″)-tetrahydro-3-furanyl]oxy]-6-quinazolinyl]-4(dimethylamino)-2-butenamide, Tovok®); Vandetanib (Caprelsa®); Lapatinib (Tykerb®); (3R,4R)-4-Amino-1-((4-((3-

methoxyphenyl)amino)pyrrolo[2,1-f][1,2,4]triazin-5-yl)methyl)piperidin-3-ol (BMS690514); Canertinib dihydrochloride (CI-1033); 6-[4-[(4-Ethyl-1-piperazinyl)methyl]phenyl]-N-[(1R)-1-phenylethyl]-7H-Pyrrolo[2,3-d]pyrimidin-4-amine (AEE788, CAS 497839-62-0); Mubritinib (TAK165); Pelitinib (EKB569); Afatinib (BIBW2992); Neratinib (HKI-272); N-[4-[[1-[(3-Fluorophenyl)methyl]-1H-indazol-5-yl]amino]-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yl]-carbamic acid, (3S)-3-morpholinylmethyl ester (BMS599626); N-(3,4-Dichloro-2-fluorophenyl)-6-methoxy-7-[[(3aα,5β,6aα)-octahydro-2-methylcyclopenta[c]pyrrol-5-yl]methoxy]-4-quinazolinamine (XL647, CAS 781613-23-8); 4-[4-[[(1R)-1-Phenylethyl]amino]-7H-pyrrolo[2,3-d]pyrimidin-6-yl]-phenol (PKI166, CAS 187724-61-4); rocelitinib.

Exemplary mTOR inhibitors include, without limitation, rapamycin (Rapamune®), and analogs and derivatives thereof; SDZ-RAD; Temsirolimus (Torisel®; also known as CCI-779); Ridaforolimus (formally known as deferolimus, (1R,2R,4S)-4-[(2R)-2[(1R,9S,12S,15R,16E,18R,19R,21R,23S,24E,26E,28Z,30S,32S,35R)-1,18-dihydroxy-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-2,3,10,14,20-pentaoxo-11,36-dioxa-4-azatricyclo[30.3.1.$0^{4,9}$]hexatriaconta-16,24,26,28-tetraen-12-yl]propyl]-2-methoxycyclohexyl dimethylphosphinate, also known as AP23573 and MK8669, and described in PCT Publication No. WO 03/064383); Everolimus (Afinitor® or RAD001); Rapamycin (AY22989, Sirolimus®); Simapimod (CAS 164301-51-3); (5-{2,4-Bis[(3S)-3-methylmorpholin-4-yl]pyrido[2,3-d]pyrimidin-7-yl}-2-methoxyphenyl)methanol (AZD8055); 2-Amino-8-[trans-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxy-3-pyridinyl)-4-methyl-pyrido[2,3-d]pyrimidin-7(8H)-one (PF04691502, CAS 1013101-36-4); and N^2-[1,4-dioxo-[[4-(4-oxo-8-phenyl-4H-1-benzopyran-2-yl)morpholinium-4-yl]methoxy]butyl]-L-arginylglycyl-L-α-aspartylL-serine-, inner salt (SF1126, CAS 936487-67-1).

Exemplary Phosphoinositide 3-kinase (PI3K) inhibitors include, but are not limited to, duvelisib, idelalisib, 4-[2-(1H-Indazol-4-yl)-6-[[4-(methylsulfonyl)piperazin-1-yl]methyl]thieno[3,2-d]pyrimidin-4-yl]morpholine (also known as GDC 0941 and described in PCT Publication Nos. WO 09/036082 and WO 09/055730); 2-Methyl-2-[4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydroimidazo[4,5-c]quinolin-1-yl]phenyl]propionitrile (also known as BEZ 235 or NVP-BEZ 235, and described in

PCT Publication No. WO 06/122806); 4-(trifluoromethyl)-5-(2,6-dimorpholinopyrimidin-4-yl)pyridin-2-amine (also known as BKM120 or NVP-BKM120, and described in PCT Publication No. WO2007/084786); Tozasertib (VX680 or MK-0457, CAS 639089-54-6); (5Z)-5-[[4-(4-Pyridinyl)-6-quinolinyl]methylene]-2,4-thiazolidinedione (GSK1059615, CAS 958852-01-2); (1E,4S,4aR,5R,6aS,9aR)-5-(Acetyloxy)-1-[(di-2-propenylamino)methylene]-4,4a,5,6,6a,8,9,9a-octahydro-11-hydroxy-4-(methoxymethyl)-4a,6a-dimethyl-cyclopenta[5,6]naphtho[1,2-c]pyran-2,7,10(1H)-trione (PX866, CAS 502632-66-8); and 8-Phenyl-2-(morpholin-4-yl)-chromen-4-one (LY294002, CAS 154447-36-6).

Exemplary Protein Kinase B (PKB) or AKT inhibitors include, but are not limited to. 8-[4-(1-Aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one (MK-2206, CAS 1032349-93-1); Perifosine (KRX0401); 4-Dodecyl-N-1,3,4-thiadiazol-2-yl -benzenesulfonamide (PHT-427, CAS 1191951-57-1); 4-[2-(4-Amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-[(3S)-3-piperidinylmethoxy]-1H-imidazo[4,5-c]pyridin-4-yl]-2-methyl-3-butyn-2-ol (GSK690693, CAS 937174-76-0); 8-(1-Hydroxyethyl)-2-methoxy-3-[(4-methoxyphenyl)methoxy]-6H-dibenzo[b,d]pyran-6-one (palomid 529, P529, or SG-00529); Tricirbine (6-Amino-4-methyl-8-(β-D-ribofuranosyl)-4H,8H-pyrrolo[4,3,2-de]pyrimido[4,5-c]pyridazine); (αS)-α-[[[5-(3-Methyl-1H-indazol-5-yl)-3-pyridinyl]oxy]methyl]-benzeneethanamine (A674563, CAS 552325-73-2); 4-[(4-Chlorophenyl)methyl]-1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-4-piperidinamine (CCT128930, CAS 885499-61-6); 4-(4-Chlorophenyl)-4-[4-(1H pyrazol-4-yl)phenyl]-piperidine (AT7867, CAS 857531-00-1); and Archexin (RX-0201, CAS 663232-27-7).

In certain embodiments, a tyrosine kinase inhibitor used in combination with the LVV or CAR modified T cells is an anaplastic lymphoma kinase (ALK) inhibitor. Exemplary ALK inhibitors include crizotinib, ceritinib, alectinib, brigatinib, dalantercept, entrectinib, and lorlatinib.

In certain embodiments where the LVV or CAR modified lymphocytes (e.g., T cells or NK cells) are administered in combination with one or more additional therapies, the LVV, CAR modified lymphocytes (e.g., T cells or NK cells), or one or more additional therapies may be administered at a dose that might otherwise be considered sub-therapeutic if administered as a monotherapy. Combination therapy

includes administration of an LVV or CAR modified lymphocyte (e.g., T cell or NK cell) composition as described herein before an additional therapy (*e.g.*, 1 day to 30 days or more before the additional therapy), concurrently with an additional therapy (on the same day), or after an additional therapy (*e.g.*, 1 day – 30 days or more after the additional therapy). In certain embodiments, the LVV or CAR modified lymphocytes (e.g., T cells or NK cells) are administered concurrently with the one or more additional therapies. In further embodiments, the LVV or CAR modified lymphocytes (e.g., T cells or NK cells) are administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days before or after administration of the one or more additional therapies. In still further embodiments, the LVV or CAR modified lymphocytes (e.g., T cells or NK cells) are administered within 4 weeks, within 3 weeks, within 2 weeks, or within 1 week before or after administration of the one or more additional therapies. Where the one or more additional therapies involves multiple doses, the LVV or CAR modified lymphocytes (e.g., T cells or NK cells) may be administered before or after the initial dose of the one or more additional therapies, after the final dose of the one or more additional therapies, or in between multiple doses of the one or more additional therapies.

Subjects that can be treated by the compositions and methods of the present disclosure include animals, such as humans, primates, cows, horses, sheep, dogs, cats, mice, rats, rabbits, guinea pigs, or pigs. The subject may be male or female, and can be any suitable age, including infant, juvenile, adolescent, adult, and geriatric subjects.

EXAMPLES

Example 1: Methods for generating lentiviral vectors with defined tropism

Self-inactivating lentiviral vectors (LVV) as described herein were produced using a third generation production system. An expression plasmid that contains the gene sequences desired for delivery by the LVV was combined at defined ratios with three packaging plasmids, VSV-G, GagPol, and Rev, and used to transfect HEK293 producer cells. Productive viral particles were harvested from the HEK293 culture media 2-3 days later.

The tropism of the resulting LVV was tested to determine whether it can be redirected using a non-viral targeting protein as described herein expressed from one of

the three packaging plasmids or by addition of a fifth plasmid to the transfection mix. The LVV vector includes a mutated VSV-G envelope protein, where the mutated VSV-G includes mutations that abolish binding of the mutated VSV-G to its native receptor such that viral entry occurs via the non-viral, tropism defining targeting protein. Mutations in the LDL-receptor binding site of VSV-G were found to abolish binding to its natural receptor. VSV-G bearing a variety of mutations (Trop-002 (SEQ ID NO: 74), Trop-051 (SEQ ID NO: 93), Trop-052 (SEQ ID NO: 95), Trop-055 (SEQ ID NO: 97), or Trop-061 (SEQ ID NO: 103)) was expressed together with CD80 (SEQ ID NO: 2) on the LVV. CD80 is the natural ligand to T cell surface receptors, CD28 and CTLA4, and provides tropism to T cells and not B cells. Binding to the natural receptor for VSV-G is assessed by transduction of Raji B cells. Several mutations in VSV-G prohibit transduction of Raji B cells yet transduction of Jurkat T cells is maintained (**FIGS. 2A-2B**).

In a first method, a tropism-defining targeting protein derived from CD80 (SEQ ID NO: 76) was expressed from the VSV-G packaging plasmid. CD80 is the natural ligand to T cell surface receptors, CD28 and CTLA4, and provides tropism to T cells. A codon optimized CD80 molecule (encoding SEQ ID NO: 2) was cloned downstream of the mutated VSV-G (Trop-002, encoding SEQ ID NO: 74), which contains mutations to abolish LDL receptor binding and was used to package a green fluorescent protein (GFP)-expressing LVV. CD80 and VSV-G were expressed at relatively equivalent levels on the surface of HEK293 producer cells, suggesting comparable decoration of the LVV particles (**FIG. 3A**). LVV generated with this approach were capable of transducing T cells as assessed by GFP expression (**FIG. 3B**).

In a second method, the tropism-defining CD80 targeting protein (encoding SEQ ID NO: 2) was cloned into the Rev packaging plasmid and transfected at a lower molar concentration to the VSV-G env (encoding Trop-002 (SEQ ID NO: 74)) plasmid. The modified plasmids was used to package a GFP-expressing LVV. Assessment of VSV-G and CD80 expression on the HEK-293 producer cells showed relative expression levels trended with the molar concentrations of the packaging plasmids. LVV generated with these packaging approaches were capable of transducing T cells as assessed by GFP expression (**FIG. 4**). Reduced expression of CD80 was observed in

LVVs packaged with CD80 targeting protein contained in the Rev packaging plasmid due to the lower ratio of the Rev plasmid.

In a third method, the tropism defining CD80 targeting protein (encoding SEQ ID NO: 2) was added as an additional plasmid during transfection of HEK293 cells intended to produce LVV (Trop-002 VSV-G env). The amount of the CD80 plasmid used during LVV production was associated with the expression on HEK293 producer cells and is likely representative of decoration of the LVV particles. LVV generated with this packaging approach were capable of transducing T cells as assessed by GFP expression and transduction efficiency was associated with CD80 levels on the LVV particles (**FIG. 5**).

Regardless of the method selected, high titer, tropism-redirected LVV can be generated with adherent or suspension HEK293 producer cells and concentrated by centrifugation or anion exchange chromatography (AEX) followed by tangential flow filtration (TFF) (**FIGS. 6A-6C**). Concentration by AEX/TFF resulted in LVV preparations with a high level of purity and recovery (**FIG. 6D**).

Example 2: CD4 T Cells Enriched by CD80 LVV

The tropism-defining targeting molecule precisely defines the specific cell type transduced with this LVV. The ligand for CD80, CD28, is naturally expressed on T cells. However, a higher frequency of CD4 T cells express CD28 in human peripheral blood compared to CD8 T cells (80% vs 50%). When a LVV was packaged to decorate the Trop-002 VSV-G envelope with a CD80 targeting protein (SEQ ID NO: 76) was used to transduce human PBMC, a higher composition of transduced CD4 T cells was observed compared to transduced CD8 T cells and was maintained for up to a week in culture (**FIG. 13**).

Example 3: Methods for ex vitro generation of tumor-reactive T cells engineered to express a chimeric antigen receptor (CAR) using a lentiviral vector containing T cell-specific tropism

Compared to standard lentiviral vectors (LVV) that use common third generation packaging systems, LVV packaged to redirect tropism as provided herein may have improved transduction efficiencies and specificity. The levels of available viral particles are likely increased when transducing a specific cell type as part of a heterogeneous mixture, as the LVV particles are not taken up by other, non-desired cell types. Whether improved transduction of T cells with a CAR molecule is possible in a heterogenous peripheral blood mononuclear cell (PBMC) mixture was evaluated. A representative anti-BCMA CAR (SEQ ID NO: 58) used to treat Multiple Myeloma or a representative anti-CD19 CAR (SEQ ID NO: 42) used to treat non-Hodgkin Lymphoma was used. GFP reporter was used as control. The LVV was packaged to decorate the envelope with anti-CD3 targeting protein and CD80 targeting protein and redirect tropism toward the T cell surface molecules, CD3, CD28 and CTLA4. The CD80 targeting protein was cloned into the Trop-002 VSV-G env packaging plasmid (SEQ ID NO: 76) and the anti-CD3 targeting protein (encoding SEQ ID NO: 10) was expressed from a fifth packaging plasmid.

The efficiency of T cell transduction within PBMC obtained from healthy donors leukapheresis after informed consent was tested. The PBMC were enriched using red cell lysis and matched donors were transduced with standard LVV or LVV with T cell redirected tropism (anti-CD3 and CD80) at LVV concentrations 5 to 25 times lower than typically used for CAR T cell transduction. Expression of an anti-BCMA CAR was assessed by flow cytometry after staining with a fluorescently labeled BCMA molecule to specifically detect anti-BCMA CAR T cells. Even with extremely low viral levels, T cells transduced using the T cell tropic LVV showed higher levels of transduction compared to standard LVV (**FIG.** 7) .

In the same study whether anti-BCMA CAR T cells can be generated without antibodies to activate T cells was evaluated. T cell activating antibodies (e.g., anti-CD28 and anti-CD3) are typically required for transduction using standard LVV during CAR T cell generation. It was confirmed that standard LVV indeed requires activation using antibodies to transduce normal human primary T cells (**FIG.** 7). By contrast, a T

cell redirected LVV (anti-CD3 and CD80) can cause transduction even in the absence of prior T cell activation (without the presence of IL-2 and exogenous activating antibodies anti-CD3 and anti-CD28) (**FIG. 7**). A consequence of T cell activation is the subsequent cellular expansion when cultured in media containing IL-2. PBMCs transduced in the presence or absence of exogenous activating anti-CD3 and anti-CD28 antibodies after standard LVV or T cell redirected LVV (anti-CD3 and CD80) showed that T cell redirected LVV was uniquely able to expand T cells even in the absence of antibody activation (**FIG. 8**). In another example, transduction of an anti-CD19 CAR also was achieved without prior T cell activation when using the T cell redirected LVV (anti-CD3 and CD80) (**FIG. 9**).

T cell activation and transduction efficiency can be dictated by the selection of anti-CD3 antibodies used to generate the T cell redirected LVV. Examples of envelope-bound anti-CD3 binding proteins having 12F6 binding domains in VH (SEQ ID NO:113)-VL (SEQ ID NO:115) orientation and VL (SEQ ID NO:115)-VH (SEQ ID NO:113) orientation are shown to cause a variable amount of T cell transduction as well as T cell activation (**FIG. 10**).

Example 4: T Cell Specificity of T cel targeted lentiviral vector

The T cell specificity of an LVV packaged to decorate the envelope (Trop-002 VSV-G env) with an anti-CD3 targeting protein (SEQ ID NO: 10) and CD80 targeting protein (SEQ ID NO: 2) was confirmed by transduction of a T cell line and a panel of B cell lines (Raji, Ramos, Jeko-1, NALM-6). Transduction of these cell lines assessed by flow cytometry for a GFP transgene was compared to standard LVV. Both standard LVV and a T cell-targeted LVV effectively transduced the T cell line (Jurkat). The standard LVV effectively transduced all the B cell lines, confirming these lines are sensitive to LVV transduction, but no substantial transduction by the T cell-targeting LVV was detected in any of the B cell lines (**FIG. 14**).

Example 5: Tumor-specific T cells generated using a T cell targeted lentiviral vector

Using methods described herein, T cells expressing an anti-BCMA CAR (SEQ ID NO: 58) were generated by transducing PBMC from healthy donors with LVVs having

Trop-002 VSV-G env and CD80 targeting protein and anti-CD3 targeting protein. The CD80 targeting protein was cloned into the Trop-002 VSV-G env packaging plasmid (SEQ ID NO: 76) and the anti-CD3 targeting protein (encoding SEQ ID NO: 10) was expressed from a fifth packaging plasmid to produce the T cell targeting LVVs. The T cell transductions were performed in the absence of any T cell stimulation typically required for efficient LVV-mediated gene transfer (exogenous anti-CD3 and anti-CD28).

First, the activity of anti-BCMA CAR T cells generated by T cell-redirected LVV was examined compared to standard LVV. Anti-tumor activity was assessed by intracellular cytokine staining for interferon-gamma and tumor necrosis factor-alpha after co-culture with a BCMA-negative cell line (Nalm-6) or BCMA-positive cell line (RPMI-8826). Anti-BCMA CAR T cells exhibited increased expression of T cell effector cytokines after culture with BCMA-positive cell lines that is not observed with BCMA-negative cell lines whether generated by standard LVV or T cell-redirected LVV (**FIG. 11**).

Example 6: Methods of T cell-specific in vivo delivery of genetic material using a T cell tropic lentiviral vector

The efficient and specific transduction of T cells with a T cell targeted LVV could permit safe and effective delivery of genetic material to T cells in vivo. A humanized mouse model infused intravenously with a GFP-expressing, T cell targeted LVV was used to test this hypothesis. LVVs having Trop-002 mutated VSV-G and coated with anti-CD3 targeting protein and CD80 targeting protein were produced. The CD80 targeting protein was cloned into the Trop-002 VSV-G env packaging plasmid (SEQ ID NO: 76) and the anti-CD3 targeting protein (encoding SEQ ID NO: 10) was expressed from a fifth packaging plasmid to produce the T cell targeting LVVs. Immunocompromised NCG mice were humanized via intravenous injection of human PBMC and supported by daily intraperitoneal administratin of recombinant IL-2 for 4 days. One day after PBMC administration, the T cell targeted lentiviral vector is administered intravenously to the mice Peripheral blood and splenic cells were harvested on day 7, and T cell specificity was assessed by flow cytometry. GFP positive cells were only detected in human T cells (CD3+) and not human B cells

(CD20+), confirming T cell specificity of LVV transduction (**FIG. 12A**). GFP was detected in both CD8+ and CD8- (CD4) T cells, indicating that the T cell specific LVV transduces both CD4 and CD8 T cells in vivo (**FIG. 12B**).

Specific T cell transduction has a key safety advantages to other in vivo CAR delivery approaches by minimizing risk of transducing tumor cells. Transduction of T cells without transduction of tumor cells is evaluated using PBMCs from chronic lymphocytic leukemia patients with detectable CD19 tumor cells. The PBMC are transduced with T cell targeted LVV and transduced cells are assessed using flow cytometry.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, including but not limited to U.S. Provisional Patent Application No. 63/154,639, filed on Feburary 26, 2021, are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

1. A lentiviral vector, comprising:

(a) a viral envelope comprising:

a heterologous lymphocyte targeting protein, the lymphocyte targeting protein comprising:

an extracellular domain comprising a lymphocyte targeting domain; and

a transmembrane domain; and

(b) a mutated VSV-G envelope protein that exhibits inhibited binding to an LDL-R but mediates membrane fusion; and

(c) an expression cassette that comprises a heterologous transgene.

2. The lentiviral vector according to claim 1, wherein the heterologous lymphocyte targeting protein is a heterologous T cell targeting protein comprising an extracellular domain comprising a T cell targeting domain.

3. The lentiviral vector according to claim 1, wherein the heterologous lymphocyte targeting protein is a heterologous natural killer (NK) cell targeting protein comprising an extracellular domain comprising a NK cell targeting domain.

4. The lentiviral vector according to claim 1, wherein the heterologous lymphocyte targeting protein is a heterologous B cell targeting protein comprising an extracellular domain comprising a B cell targeting domain.

5. A lentiviral vector according to claim 1 or 2, wherein the heterologous lymphocyte targeting protein is a heterologous T cell targeting protein that specifically binds to:
(a) a T cell marker selected from CD3, CD28, CD80, 4-1BB, AhR, CD3, CD2, CD7, CD4, CD8, CD25, CD44, CD45RA, CD47, CD62L, CD69, CD94, CD95, CD127, CD161, CD183 (CXCR3), CD184 (CXCR4), CD185 (CXCR5), CD193 (CCR3), CD194 (CCR4), CD195 (CCR5), CD196 (CCR6), CD197 (CCR7), CCR10, PD-1, TCRa/b, CD5, CD27, CD45RO, CD45RB, CD57, CD103, CD122, P2RX7, TIGIT, LAG-3, TIM-3, and IL6ST, or any combination thereof;
(b) a γδ T cell marker selected from γδ TCR, Vdelta1, Vdelta2, and NKG2D (KLRK1, CD314);

(c) an NK T cell marker selected from Invariant TCR (Va24-Ja18), CD185 (CXCR5), CXCR6, and IL-21R, or any combination thereof; or
(d) a MAIT cell marker selected from Va7.2, Ja33, CXCR6, IL-18R, KLRB1 (CD161), and VLA4 (alpha4beta1 integrin), or any combination thereof.

6. A lentiviral vector according to claim 1, 2, or 5, wherein the heterologous lymphocyte targeting protein is a heterologous T cell targeting protein comprising an extracellular domain comprising a T cell targeting domain that specifically binds to a CD3 protein or a CD28 protein.

7. A lentiviral vector according to claim 1 or 3, wherein the heterologous lymphocyte targeting protein is a heterologous NK cell targeting protein that specifically binds to a NK cell marker selected from CD56, NKp46, CD16, KIR(s), NKG2 proteins (e.g., NKG2D (KLRK1, CD314)), KLRB1 (CD161), KLRD1 (cd94), IL2Rb (CD122), IL-21R, SLAMF6 (CD352), SLAMF7 (CD319), and IL-18R, or any combination thereof.

8. A lentiviral vector according to claim 1 or 4, wherein the heterologous lymphocyte targeting protein is a B cell targeting protein that specifically binds to a B cell marker selected from CD19, CD20, CD21, CD22, CD24, CD38, CD40, CD72, CD32b, CD268, CD269, CD267, CD86, CD80, CD52, CD138, CD27, CD28, CD23, CD84, CD257, CD270, CD37, CD74, and CD269, or any combination thereof.

9. A lentiviral vector according to any one of claims 1, 2, 5, and 6, wherein the targeting protein comprises a CD80 protein comprising an extracellular domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 6.

10. A lentiviral vector according to any one of claims 1, 2, 5, 6, and 9, wherein the targeting protein comprises a CD80 protein comprising a transmembrane and intracellular domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 8

11. A lentiviral vector according to claim 1, 2, 5, 6, 9, or 10, wherein the targeting protein comprises a CD80 protein comprising SEQ ID NO: 2 or SEQ ID NO: 2 absent the signal peptide of SEQ ID NO: 4.

12. A lentiviral vector according to any one of claims 1, 2, 5, and 6, wherein the targeting protein comprises an anti-CD3 scFv having a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 14, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 18.

13. A lentiviral vector according to claim 12 wherein the targeting protein comprises an anti-CD3 targeting protein comprising: (i) SEQ ID NO: 10 or SEQ ID NO: 10 absent the signal peptide of SEQ ID NO: 12; or (ii) SEQ ID NO: 117 or SEQ ID NO: 117 absent the signal peptide of SEQ ID NO: 12.

14. A lentiviral vector according to any preceding claim, wherein the targeting protein as expressed in a host cell comprises a signal sequence.

15. A lentiviral vector according to any preceding claim, wherein the mature targeting protein lacks a signal sequence.

16. A lentiviral vector according to any of claims 1-14, wherein the mature targeting protein includes at least one amino acid from a signal sequence.

17. A lentiviral vector according to any preceding claim, wherein the mutated VSV-G envelope protein comprises a mutation selected from one or more of:

a mutation at H8, N9, Q10, K47, K50, A51, S183, S179, N180, I182, M184, Y209, I347, T350, T352, E353, and R354;

an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, and an insertion of GGS between N208 and Y209; and

a deletion of residues 1-8.

18. A lentiviral vector according to any preceding claim, wherein the mutated VSV-G envelope protein comprises a mutation selected from two or more of:

a mutation at H8, N9, Q10, K47, K50, A51, S183, S179, N180, I182, M184, Y209, I347, T350, T352, E353, and R354;

an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, and an insertion of GGS between N208 and Y209; and

a deletion of residues 1-8.

19. A lentiviral vector according to any preceding claim, wherein the mutated VSV-G envelope protein comprises a mutation selected from one or more of H8A, N9A, Q10A, K47A, K47Q, N180A, I182A, Y209A, T352A, T352W, E353A, R354A, and R354Q.

20. A lentiviral vector according to any preceding claim, wherein the mutated VSV-G envelope protein comprises a mutation selected from one or more of H8A, K47A, K47Q, Y209A, R354A, and R354Q.

21. A lentiviral vector according to any of the preceding claims, wherein the mutated VSV-G envelope protein comprises:

(a) a K47 mutation and a R354 mutation;

(b) a N180 mutation, a I182 mutation, a T352 mutation, and a E353 mutation;

(c) a T352 mutation and E353 mutation;

(d) a N9 mutation, a Q10 mutation, and a N180 mutation; or

(e) an insertion of GGS between H8 and N9 and an insertion of GGS between N9 and Q10;

(f) an insertion of TT between N9 and Q10; or

(g) an insertion of GGS between P46 and K47.

22. The lentiviral vector according to claim 21, wherein the mutated VSV-G envelope protein comprises:

(a) a K47Q mutation and a R354A mutation;

(b) a N180A mutation, a I182A mutation, a T352A mutation and a E353A mutation;
(c) a T352W mutation and a E353A mutation; or
(d) a N9A mutation, a Q10A mutation, and a N180A mutation.

23. The lentiviral vector according to any one of the preceding claims, wherein the mutated VSV-G envelope protein comprises the amino acid sequence of any one of SEQ ID NOS: 74, 93, 95, 97, 99 and 103.

24. A lentiviral vector according to any preceding claim, wherein the heterologous transgene encodes a chimeric antigen receptor (CAR) comprising an extracellular domain comprising a binding domain that specifically binds to a target molecule; an intracellular signaling domain, wherein the intracellular signaling domain comprises an immunoreceptor tyrosine-based activation motif (ITAM) and optionally, a costimulatory signaling domain; and a transmembrane domain connecting the extracellular domain and intracellular signaling domain.

25. A lentiviral vector according to claim 24, wherein the CAR encoded by the transgene comprises:

(a) a binding domain that specifically binds one or more of CD19, BCMA, alpha folate receptor, 5T4, Ab integrin, B7-H3, B7-H6, CAIX, CD20, CD22, CD23, CD30, CD33, CD38, CD44, CD44v6, CD44v7/8, CD52, CD70, CD79a, CD79b, CD80, CD123, CD138, CD171, CEA, CSPG4, EGFR, ErbB2 (HER2), EGFRvIII, EGP2, EGP40, EpCAM, FAP, fetal AchR, FLT3, Fra, GD2, GD3, Glypican-3 (GPC3), HLA-A1 + MAGE1, HLA-A2 + MAGE1, HLA-A3 + MAGE1, HLA-A1 + NY-ESO-1, HLA-A2 + NY-ESO-1, HLA-A3 + NY-ESO-1, HLADR, IL-11Ralpha, IL-13 Ralpha2, Lambda, Lewis-Y, Kappa, mesothelin, Muc1, Muc16, NCAM, NKG2d ligands, NY-ESO-1, PRAME, PSCA, PSMA, ROR1, SSX, Survivin, TAG72, TEMs, VEGFR2, BAFF-R, Claudin18.2, CD86, FcRL5, GPRC5, and TACI;

(b) a transmembrane domain selected from a CD28, CD2, CD4, CD8a, CD5, CD3ε, CD3δ, CD3ζ, CD9, CD16, CD22, CD25, CD27, CD33, CD37, CD40, CD45, CD64, CD79A, CD79B, CD80, CD86, CD95 (Fas), CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD154 (CD40L), CD200R, CD223 (LAG3), CD270 (HVEM), CD272 (BTLA), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), CD279 (PD-1), CD300, CD357 (GITR), A2aR, DAP10, FcRα, FcRβ, FcRγ, Fyn,

GAL9, KIR, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPα, pTα, TCRα, TCRβ, TIM3, TRIM, LPA5, and Zap70 transmembrane domain;

(c) an intracellular signaling domain selected from a CD3γ, CD3δ, CD3ε, CD3ζ, CD5, CD22, CD79a, CD278 (ICOS), DAP10, DAP12, FcRγ, and CD66d domain;

(d) a costimulatory domain selected from one or more of a CD27, CD28, CD40L, GITR, NKG2C, CARD1, CD2, CD7, CD27, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX-40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD226, CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, LFA-1, LIGHT, NKG2C, NKD2C, SLP76, TRIM, and ZAP70 domain; and

(e) optionally, an extracellular, non-signaling linker domain between the binding domain and the transmembrane domain, wherein the non-signaling linker domain is selected from:

(i) an immunoglobulin hinge region selected from a wild type or modified IgG1, IgG2, IgG3, IgG4, IgA, and IgD hinge region;

(ii) a hinge region selected from a wild type or modified CD8a, CD4, CD28 and CD7 hinge region;

(iii) all or a portion of an Fc domain selected from one or more of a CH1 domain, a CH2 domain, and a CH3 domain; and

(iv) a stalk region of a type II C-lectin derived from a stalk region of CD23, CD69, CD72, CD94, NKG2A, and NKG2D.

26. A lentiviral vector according to either of claims 24 or 25, wherein the CAR encoded by the transgene is an anti-CD19 CAR comprising:

a binding domain comprising an anti-CD19 scFv comprising a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 46, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 50;

a CD8a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 52;

a CD3ζ effector domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 56; and
a 4-1BB costimulatory domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 54.

27. A lentiviral vector according to claim 26, wherein the CAR encoded by the transgene comprises SEQ ID NO: 42 or SEQ ID NO: 42 absent the signal peptide of SEQ ID NO: 44.

28. A lentiviral vector according to either of claim 24 or 25, wherein the CAR encoded by the transgene is an anti-BCMA CAR comprising:
a binding domain comprising an anti-BCMA scFv comprising a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 62, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 66;
a CD8a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 52;
a CD3ζ effector domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 122; and
a 4-1BB costimulatory domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 54.

29. A lentiviral vector according to claim 26, wherein the CAR encoded by the transgene comprises SEQ ID NO: 58 or SEQ ID NO: 58 absent the signal peptide of SEQ ID NO: 60.

30. A method for producing a lymphocyte targeted lentiviral vector according to any one of the preceding claims, the method comprising:
(a) transfecting a producer cell with a a GagPol plasmid, a Rev plasmid, a transgene plasmid, and a VSV-G env plasmid, wherein;

(i) the GagPol plasmid comprises a one or more polynucleotides encoding a lentiviral gag gene and a lentiviral pol gene and is capable of expressing a lentiviral Gag polyprotein and a lentiviral Pol polyprotein within the producer cell;

(ii) the Rev plasmid comprises a polynucleotide encoding a lentiviral rev gene and is capable expressing a lentiviral Rev protein within the producer cell;

(iii) the transgene plasmid comprises an expression cassette comprising a polynucleotide encoding a CAR; and

(iv) the VSV-G env plasmid comprises a tandem expression cassette, wherein the tandem expression cassette comprises a polynucleotide encoding a mutated VSV-G envelope protein and a polynucleotide encoding a lymphocyte targeting protein and the VSV-G env plasmid is capable of expressing the mutated VSV-G envelope protein and the lymphocyte targeting protein within the producer cell;

(b) culturing the producer cell in a culture medium; and

(c) harvesting lentiviral vector from the culture medium.

31. A method for producing a lymphocyte targeted lentiviral vector according to any one of claims 1-29, the method comprising:

(a) transfecting a producer cell with a a GagPol plasmid, a Rev plasmid, a transgene plasmid, and a VSV-G env plasmid, wherein;

(i) the GagPol plasmid comprises a tandem expression cassette that encodes the Gag polyprotein precursor and Pol polyprotein precursor and a lymphocyte targeting protein and the GagPol plasmid is capable of expressing a lentiviral Gag polyprotein, a lentiviral Pol polyprotein, and the lymphocyte targeting protein within the producer cell;

(ii) the Rev plasmid comprises a polynucleotide encoding a lentiviral rev gene and is capable expressing a lentiviral Rev protein within the producer cell;

(iii) the transgene plasmid comprises an expression cassette comprising a polynucleotide encoding a CAR; and

(iv) the VSV-G env plasmid comprises a a polynucleotide encoding a mutated VSV-G envelope protein and the VSV-G env plasmid is capable of expressing the mutated VSV-G envelope protein within the producer cell;

(b) culturing the producer cell in a culture medium; and

(c) harvesting lentiviral vector from the culture medium.

32. A method for producing a lymphocyte targeted lentiviral vector according to any one of claims 1-29, the method comprising:

(a) transfecting a producer cell with a a GagPol plasmid, a Rev plasmid, a transgene plasmid, and a VSV-G env plasmid, wherein;

(i) the GagPol plasmid comprises a one or more polynucleotides encoding a lentiviral gag gene and a lentiviral pol gene and is capable of expressing a lentiviral Gag polyprotein and a lentiviral Pol polyprotein within the producer cell;

(ii) the Rev plasmid comprises a tandem expression cassette, wherein the tandem expression cassette comprises a polynucleotide encoding a lentiviral rev gene and a polynucleotide encoding a lymphocyte targeting protein, and the Rev plasmid is capable expressing a lentiviral Rev protein and the lymphocyte targeting protein within the producer cell;

(iii) the transgene plasmid comprises an expression cassette comprising a polynucleotide encoding a CAR; and

(iv) the VSV-G env plasmid comprises a polynucleotide encoding a mutated VSV-G envelope protein and the VSV-G env plasmid is capable of expressing the mutated VSV-G envelope protein within the producer cell;

(b) culturing the producer cell in a culture medium; and

(c) harvesting lentiviral vector from the culture medium.

33. A method for producing a lymphocyte targeted lentiviral vector according to any one of claims 1-29, the method comprising:

(a) transfecting a producer cell with a GagPol plasmid, a Rev plasmid, a transgene plasmid, a VSV-G env plasmid, and a lymphocyte targeting protein plasmid;

(i) the GagPol plasmid comprises a one or more polynucleotides encoding a lentiviral gag gene and a lentiviral pol gene and is capable of

expressing a lentiviral Gag polyprotein and a lentiviral Pol polyprotein within the producer cell;

(ii) the Rev plasmid comprises a polynucleotide encoding a lentiviral rev gene and is capable expressing a lentiviral Rev protein within the producer cell;

(iii) the transgene plasmid comprises an expression cassette comprising a polynucleotide encoding a CAR; and

(iv) the VSV-G env plasmid comprises a polynucleotide encoding a mutated VSV-G envelope protein and is capable of expressing the mutated VSV-G envelope protein within the producer cell; and

(v) the lymphocyte targeting protein plasmid comprises a polynucleotide encoding a lymphocyte targeting protein and is capable of expressing the lymphocyte targeting protein within the producer cell;

(b) culturing the producer cell in a culture medium; and

(c) harvesting lentiviral vector from the culture medium.

34. The method according to any one of claims 30-33, wherein the lymphocyte targeting protein encoded by the lymphocyte targeting protein plasmid is a T cell targeting protein.

35. The method according to any one of claims 30-33, wherein the lymphocyte targeting protein encoded by the lymphocyte targeting protein plasmid is a NK cell targeting protein.

36. The method according to any one of claims 30-33, wherein the lymphocyte targeting protein encoded by the lymphocyte targeting protein plasmid is a B cell targeting protein.

37. A method according to any one of claims 30-33 and 34, wherein the lymphocyte targeting protein encoded by the lymphocyte targeting protein plasmid is a T cell targeting protein that specifically binds to:

(a) a T cell marker selected from CD3, CD28, CD80, 4-1BB, AhR, CD3, CD2, CD7, CD4, CD8, CD25, CD44, CD45RA, CD47, CD62L, CD69, CD94, CD95, CD127, CD161, CD183 (CXCR3), CD184 (CXCR4), CD185 (CXCR5), CD193

(CCR3), CD194 (CCR4), CD195 (CCR5), CD196 (CCR6), CD197 (CCR7), CCR10, PD-1, TCRa/b, CD5, CD27, CD45RO, CD45RB, CD57, CD103, CD122, P2RX7, TIGIT, LAG-3, TIM-3, and IL6ST, or any combination thereof;

(b) a γδ T cell marker selected from γδ TCR, Vdelta1, Vdelta2, and NKG2D (KLRK1, CD314, or any combination thereof);

(c) an NK T cell marker selected from Invariant TCR (Va24-Ja18), CD185 (CXCR5), CXCR6, and IL-21R, or any combination thereof; or

(d) a MAIT cell marker selected from Va7.2, Ja33, CXCR6, IL-18R, KLRB1 (CD161), and VLA4 (alpha4beta1 integrin), or any combination thereof.

38. A method according to claim 37, wherein the targeting protein comprises a CD80 protein comprising an extracellular domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 6.

39. A method according to either of claims 37 and 38, wherein the targeting protein comprises a CD80 protein comprising a transmembrane and intracellular domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 8

40. The method according to any one of claim 37, 38, and 39, wherein the targeting protein comprises a CD80 protein comprising SEQ ID NO: 2 or SEQ ID NO: 2 absent the signal peptide of SEQ ID NO: 4.

41. A method according any of claims 30-34 and 37, wherein the targeting protein comprises: (i) an anti-CD3 scFv having a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 14, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 18; or (ii) an anti-CD3 scFv having a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 115, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 113.

42. A method according to any one of claims 30-34, 37, and 41, wherein the targeting protein comprises an anti-CD3 targeting protein comprising: (i) SEQ ID NO: 10 or SEQ ID NO: 10 absent the signal peptide of SEQ ID NO: 12; or (ii) SEQ ID NO: 117 or SEQ ID NO: 117 absent the signal peptide of SEQ ID NO: 12.

43. The method according to any one of claims 30-33 and 35, wherein the lymphocyte targeting protein encoded by the lymphocyte targeting protein plasmid is a NK cell targeting protein that specifically binds to an NK cell marker selected from CD56, NKp46, CD16, KIR(s), NKG2 proteins (e.g., NKG2D (KLRK1, CD314)), KLRB1 (CD161), KLRD1 (cd94), IL2Rb (CD122), IL-21R, SLAMF6 (CD352), SLAMF7 (CD319), and IL-18R, or any combination thereof.

44. The method according to any one of claims 30-33 and 36, wherierin the lymphocyte targeting protein encoded by the lymphocyte targeting protein plasmid is a B cell targeting protein that specifically binds to a B cell marker selected from selected from CD19, CD20, CD21, CD22, CD24, CD38, CD40, CD72, CD32b, CD268, CD269, CD267, CD86, CD80, CD52, CD138, CD27, CD28, CD23, CD84, CD257, CD270, CD37, CD74, and CD269, or any combination thereof.

45. A method according to any of claims 30-44, wherein the mutated VSV-G envelope protein encoded by the VSV-G env plasmid comprises a mutation selected from one or more of:

a mutation at H8, N9, Q10, K47, K50, A51, S183, S179, N180, I182, M184, Y209, I347, T350, T352, E353, and R354;

an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, and an insertion of GGS between N208 and Y209; and

a deletion of residues 1-8.

46. A method according to any of claims 30-45, wherein the mutated VSV-G envelope protein encoded by the VSV-G env plasmid comprises a mutation selected from two or more of:

a mutation at H8, N9, Q10, K47, K50, A51, S183, S179, N180, I182, M184, Y209, I347, T350, T352, E353, and R354;

an insertion of TT between N9 and Q10, an insertion of GGS between H8 and N9, an insertion of GGS between N9 and Q10, an insertion of TT between N208 and Y209, an insertion of GGS between P46 and K47, and an insertion of GGS between N208 and Y209; and

a deletion of residues 1-8.

47. A method according to any of claims 30-46, wherein the mutated VSV-G envelope protein comprises a mutation selected from one or more of H8A, N9A, Q10A, K47A, K47Q, N180A, I182A, Y209A, T352A, T352W, E353A, R354A, and R354Q.

48. A method according to any of claims 30-47, wherein the mutated VSV-G envelope protein encoded by the VSV-G env plasmid comprises a mutation selected from one or more of H8A, K47A, K47Q, Y209A, R354A, and R354Q.

49. A method according to any of claims 30-48, wherein the mutated VSV-G envelope protein comprises:

(a) a K47 mutation and a R354 mutation;

(b) a N180 mutation, a I182 mutation, a T352 mutation, and a E353 mutation;

(c) a T352 mutation and E353 mutation;

(d) a N9 mutation, a Q10 mutation, and a N180 mutation; or

(e) an insertion of GGS between H8 and N9 and an insertion of GGS between N9 and Q10;

(f) an insertion of TT between N9 and Q10; or

(g) an insertion of GGS between P46 and K47.

50. The method according to claim 49, wherein the mutated VSV-G envelope protein comprises:

(a) a K47Q mutation and a R354A mutation;

(b) a N180A mutation, a I182A mutation, a T352A mutation and a E353A mutation;

(c) a T352W mutation and a E353A mutation; or

(d) a N9A mutation, a Q10A mutation, and a N180A mutation.

51. The method according to any one claims 30-50, wherein the mutated VSV-G envelope protein comprises the amino acid sequence of any one of SEQ ID NOS: 74, 93, 95, 97, 99, and 103.

52. A method according to any of claims 30-51, wherein the CAR encoded by the transgene plasmid comprises an extracellular domain comprising a binding domain that specifically binds to a target molecule; an intracellular signaling domain, wherein the intracellular signaling domain comprises an immunoreceptor tyrosine-based activation motif (ITAM) and optionally a costimulatory signaling domain; and a transmembrane domain connecting the extracellular domain and intracellular signaling domain.

53. A method according to any of claims 30-52, wherein the CAR encoded by the transgene plasmid comprises:

(a) a binding domain that specifically binds one or more of CD19, BCMA, alpha folate receptor, 5T4, Ab integrin, B7-H3, B7-H6, CAIX, CD20, CD22, CD23, CD30, CD33, CD38, CD44, CD44v6, CD44v7/8, CD52, CD70, CD79a, CD79b, CD80, CD123, CD138, CD171, CEA, CSPG4, EGFR, ErbB2 (HER2), EGFRvIII, EGP2, EGP40, EpCAM, FAP, fetal AchR, FLT3, Fra, GD2, GD3, Glypican-3 (GPC3), HLA-A1 + MAGE1, HLA-A2 + MAGE1, HLA-A3 + MAGE1, HLA-A1 + NY-ESO-1, HLA-A2 + NY-ESO-1, HLA-A3 + NY-ESO-1, HLADR, IL-11Ralpha, IL-13 Ralpha2, Lambda, Lewis-Y, Kappa, mesothelin, Muc1, Muc16, NCAM, NKG2d ligands, NY-ESO-1, PRAME, PSCA, PSMA, ROR1, SSX, Survivin, TAG72, TEMs, VEGFR2, BAFF-R, Claudin18.2, CD86, FcRL5, GPRC5, and TACI;

(b) a transmembrane domain selected from a CD28, CD2, CD4, CD8a, CD5, CD3ε, CD3δ, CD3ζ, CD9, CD16, CD22, CD25, CD27, CD33, CD37, CD40, CD45, CD64, CD79A, CD79B, CD80, CD86, CD95 (Fas), CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD154 (CD40L), CD200R, CD223 (LAG3), CD270 (HVEM), CD272 (BTLA), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), CD279 (PD-1), CD300, CD357 (GITR), A2aR, DAP10, FcRα, FcRβ, FcRγ, Fyn, GAL9, KIR, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPα, pTα, TCRα, TCRβ, TIM3, TRIM, LPA5, and Zap70 transmembrane domain;

(c) an intracellular signaling domain selected from a CD3γ, CD3δ, CD3ε, CD3ζ, CD5, CD22, CD79a, CD278 (ICOS), DAP10, DAP12, FcRγ, and CD66d domain;

(d) a costimulatory domain selected from one or more of a CD27, CD28, CD40L, GITR, NKG2C, CARD1, CD2, CD7, CD27, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX-40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD226, CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, LFA-1, LIGHT, NKG2C, NKD2C, SLP76, TRIM, and ZAP70 domain; and

(e) optionally, an extracellular, non-signaling linker domain between the binding domain and the transmembrane domain, with the non-signaling linker domain being selected from:

(i) an immunoglobulin hinge region selected from a wild type or modified IgG1, IgG2, IgG3, IgG4, IgA, and IgD hinge region;

(ii) a hinge region selected from a wild type or modified CD8a, CD4, CD28 and CD7 hinge region;

(iii) all or a portion of an Fc domain selected from one or more of a CH1 domain, a CH2 domain, and a CH3 domain; and

(iv) a stalk region of a type II C-lectin derived from a stalk region of CD23, CD69, CD72, CD94, NKG2A, and NKG2D.

54. A method according to any of claims 30-53, wherein the CAR encoded by the transgene plasmid is an anti-CD19 CAR comprising:

(a) a binding domain comprising an anti-CD19 scFv comprising a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,

97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 46, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 50;

(b) a CD8a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 52;

(c) a CD3ζ effector domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 56; and

(d) a 4-1BB costimulatory domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 54.

55. The method oaccording to claim 54, wherein the CAR encoded by the transgene comprises SEQ ID NO: 42 or SEQ ID NO: 42 absent the signal peptide of SEQ ID NO: 44.

56. A method according to any of claims 30-53, wherein the CAR encoded by the transgene plasmid is an anti-BCMA CAR comprising:

(a) a binding domain comprising an anti-BCMA scFv comprising a light chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 62, and a heavy chain variable region that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 66;

(b) a CD8a hinge and transmembrane domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 52;

(c) a CD3ζ effector domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 122; and

(d) a 4-1BB costimulatory domain that has at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 54.

57. The method of claim 56, wherein the CAR encoded by the transgene comprises SEQ ID NO: 58 or SEQ ID NO: 58 absent the signal peptide of SEQ ID NO: 60.

58. The method of any one of claims 30-57, wherein the producer cell is transduced with a defined ratio of a transgene plasmid, GagPol plasmid, and a VSV-G env plasmid, and Rev plasmid, optionally wherein the defined ratio of transgene plasmid, GagPol plasmid, and a VSV-G env plasmid, and Rev plasmid is about about 1:1:1:1 to about 5:4:1:1, respectively.

59. The method of claim 58, wherein the producer cell is transduced with a defined ratio of a transgene plasmid, GagPol plasmid, and a VSV-G env plasmid, and Rev plasmid of about 4:2:1:1.

60. The method of claim 58, wherein the producer cell is transduced with a defined ratio of a transgene plasmid, GagPol plasmid, and a VSV-G env plasmid, and Rev plasmid of about 3.125:3.125:2.5:1.25.

61. The method of any one of claims 30-60, wherein harvesting lentiviral vector from the culture medium comprises centrifugation, anion exchange, chromatography, or any combination thereof.

62. The method of any one of claims 30-61, wherein a LVV product harvested from the culture medium has a viral titer of about 1e7 TU/mL to about 1e12 TU/mL in concentrated LVV product.

63. A method for producing a T cell expressing a heterologous transgene, the method comprising:

transducing the T cell with one or more LVVs according to any one of claims 1, 2, 5, 6, and 9-29, wherein transduction of the T cell results in expression of the heterologous transgene by the T cell.

64. The method according to claim 63, wherein the T cell is a resting T cell.

65. The method of according to claim 63 or 64, wherein transducing the T cell is performed in vivo or ex vivo.

66. The method according to any one of claims 63-65, wherein the one or more LVVs is administered to a subject intravenously, intraperitoneally, intratumorly, intraosseously, or intranodally.

67. The method according to any one of claims 63-66, wherein transducing the T cell comprises contacting T cells within whole blood or peripheral blood mononuclear cells (PBMCs) obtained from a subject with the one or more LVVs.

68. A method according to any one of claims 63-67, wherein transduction is accomplished without use of an exogenous T cell stimulating agent.

69. The method according to claim 68, wherein the exogenous T cell stimulating agent comprises an anti-CD3 antibody or antigen binding fragment thereof, an anti-CD28 antibody or antigen binding fragment thereof, or both.

70. A method for producing a T cell expressing a CAR, the method comprising:

transducing the T cell with one or more LVVs according to any one of claims 24-29 and transduction of the T cell results in expression of the CAR transgene by the T cell.

71. The method according to claim 70, wherein the T cell is a resting T cell.

72. The method of according to claim 70 or 71, wherein transducing the T cell is performed in vivo or ex vivo.

73. The method according to any one of claims 70-72, wherein the one or more LVVs is administered to a subject intravenously, intraperitoneally, intratumorly, intraosseously, or intranodally.

74. The method according to any one of claims70-72, wherein transducing the T cell comprises contacting T cells within whole blood or peripheral blood mononuclear cells (PBMCs) obtained from a subject with the one or more LVVs.

75. A method according to any one of claims 70-74, wherein transduction is accomplished without use of an exogenous T cell stimulating agent.

76. The method of claim 75, wherein the exogenous T cell stimulating agent comprises an anti-CD3 antibody or antigen binding fragment thereof, an anti-CD28 antibody or antigen binding fragment thereof, or both.

77. A method for producing a NK cell expressing a heterologous transgene, the method comprising:

transducing the NK cell with one or more LVVs according to any one of claims 1, 3, 7, and 14-29, wherein transduction of the NK cell results in expression of the heterologous transgene by the NK cell.

78. The method according to claim 77, wherein the NK cell is a resting NK cell.

79. The method of according to claim 77 or 78, wherein transducing the NK cell is performed in vivo or ex vivo.

80. The method according to any one of claims 77-79, wherein the one or more LVVs is administered to a subject intravenously, intraperitoneally, intratumorly, intraosseously, or intranodally.

81. The method according to any one of claims 77-79, wherein transducing the NK cell comprises contacting NK cells within whole blood or peripheral blood mononuclear cells (PBMCs) obtained from a subject.

82. A method according to any one of claims 77-81, wherein transduction is accomplished without use of an exogenous NK cell stimulating agent.

83. A method for producing a NK cell expressing a CAR, the method comprising:

transducing the NK cell with one or more LVVs according to any one of claims 24-29 and transduction of the NK cell results in expression of the CAR transgene by the NK cell.

84. The method according to claim 83, wherein the NK cell is a resting NK cell.

85. The method of according to claim 83 or 84, wherein transducing the NK cell is performed in vivo or ex vivo.

86. The method according to any one of claims 83-85, wherein the one or more LVVs is administered to a subject intravenously, intraperitoneally, intratumorly, intraosseously, or intranodally.

87. The method according to any one of claims 83-85, wherein transducing the NK cell comprises contacting NK cells within whole blood or peripheral blood mononuclear cells (PBMCs) obtained from a subject with the one or more LVVs.

88. A method according to any one of claims 83-87, wherein transduction is accomplished without use of an exogenous NK cell stimulating agent.

89. A method for producing a B cell expressing a heterologous transgene, the method comprising:

transducing the B cell with one or more LVVs according to any one of claims 1, 4, 8 and 14-29, wherein transduction of the B cell results in expression of the heterologous transgene by the B cell.

90. The method according to claim 89, wherein the B cell is a resting B cell.

91. The method of according to claim 89 or 90, wherein transducing the B cell is performed in vivo or ex vivo.

92. The method according to any one of claims 89-91, wherein the one or more LVVs is administered to a subject intravenously, intraperitoneally, intratumorly, intraosseously, or intranodally.

93. The method according to any one of claims 89-91, wherein transducing the B cell comprises contacting B cells within whole blood or peripheral blood mononuclear cells (PBMCs) obtained from a subject.

94. A method according to any one of claims 89-93, wherein transduction is accomplished without use of B cell stimulating agents.

95. A method for producing a B cell expressing a CAR, the method comprising:

transducing the B cell with one or more LVVs according to any one of claims 24-29 and transduction of the NK cell results in expression of the CAR transgene by the B cell.

96. The method according to claim 95, wherein the B cell is a resting B cell.

97. The method of according to claim 95 or 96, wherein transducing the B cell is performed in vivo or ex vivo.

98. The method according to any one of claims 95-97, wherein the one or more LVVs is administered to a subject intravenously, intraperitoneally, intratumorly, intraosseously, or intranodally.

99. The method according to any one of claims 95-97, wherein transducing the B cell comprises contacting B cells within whole blood or peripheral blood mononuclear cells (PBMCs) obtained from a subject with the one or more LVVs.

100. A method according to any one of claims 95-99, wherein transduction is accomplished without use of an exogenous B cell stimulating agent.

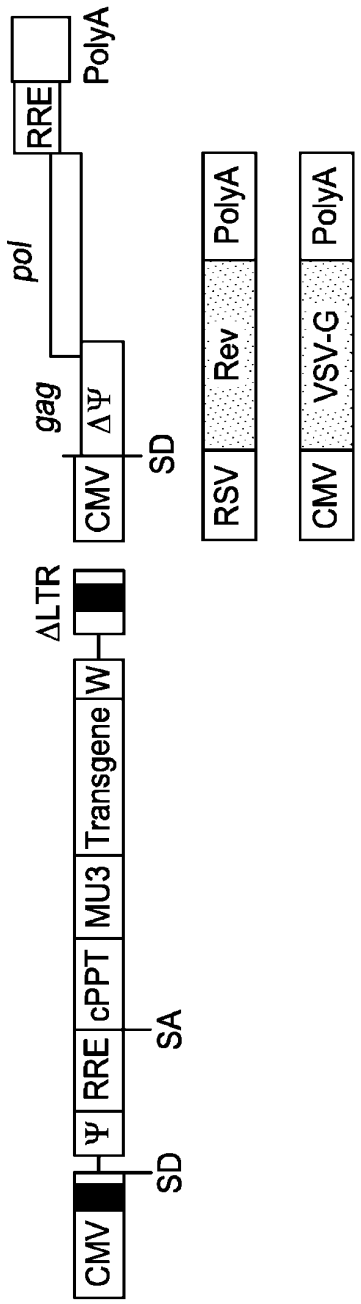

CMV
SD
Ψ
RRE
cPPT
SA
MU3
Transgene
W
ΔLTR
CMV
ΔΨ
gag
pol
RRE
PolyA
RSV
Rev
PolyA
CMV
VSV-G
PolyA

FIG. 1

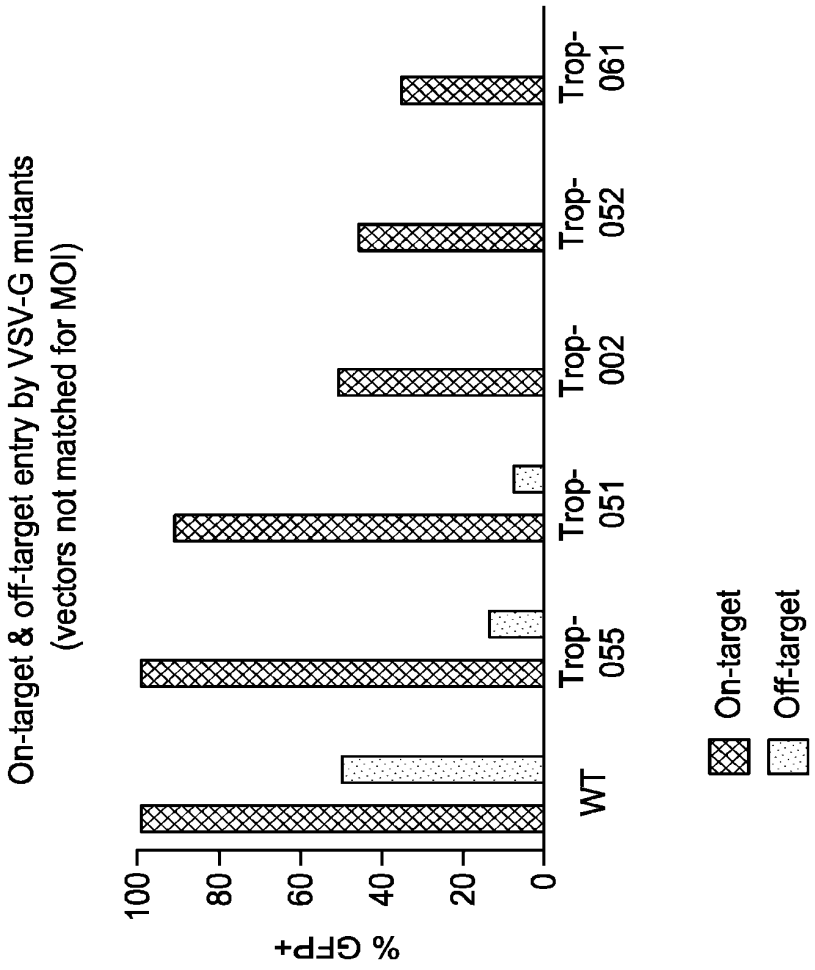

On-target & off-target entry by VSV-G mutants
(vectors not matched for MOI)
% GFP+
100
80
60
40
20
0
WT
Trop-055
Trop-051
Trop-002
Trop-052
Trop-061
On-target
Off-target

FIG. 2A

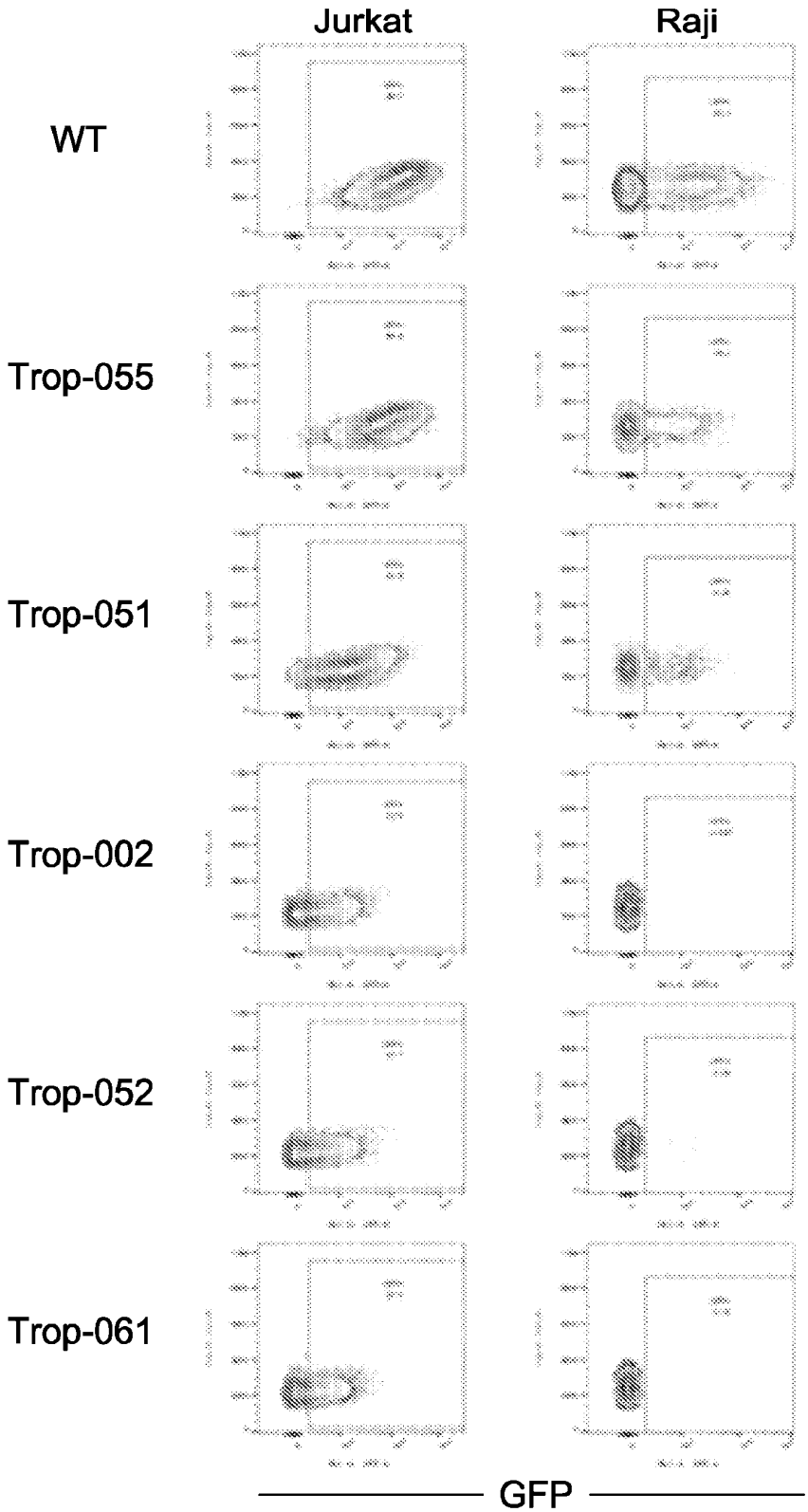

Jurkat
Raji
WT
Trop-055
Trop-051
Trop-002
Trop-052
Trop-061
GFP

FIG. 2B

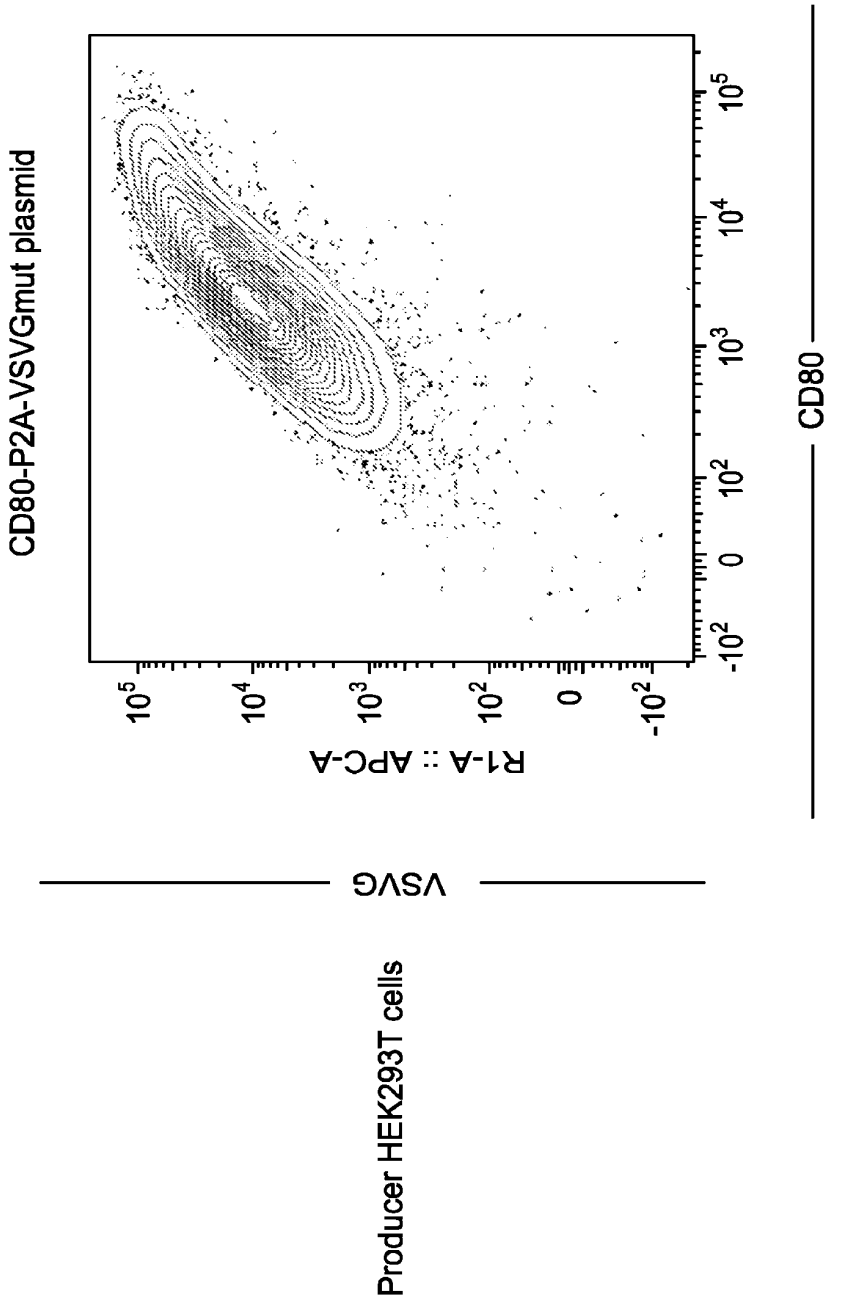

CD80-P2A-VSVGmut plasmid
R1-A :: APC-A
10^5
10^4
10^3
10^2
0
-10^2
-10^2
0
10^2
10^3
10^4
10^5
CD80
VSVG
Producer HEK293T cells

FIG. 3A

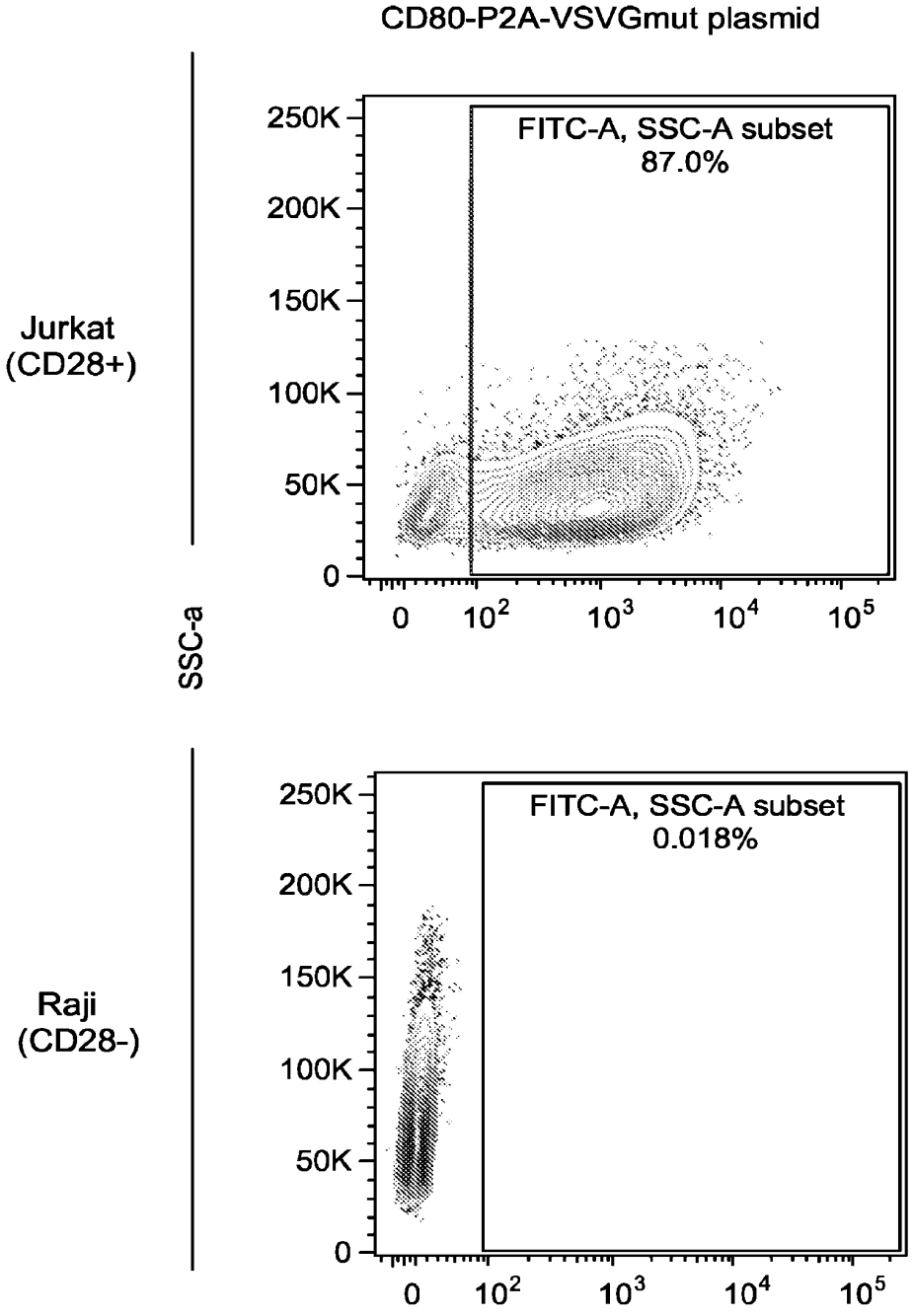

CD80-P2A-VSVGmut plasmid
Jurkat
(CD28+)
Raji
(CD28-)
SSC-a
FITC-A, SSC-A subset
87.0%
FITC-A, SSC-A subset
0.018%
250K
200K
150K
100K
50K
0
0
10^2
10^3
10^4
10^5

FIG. 3B

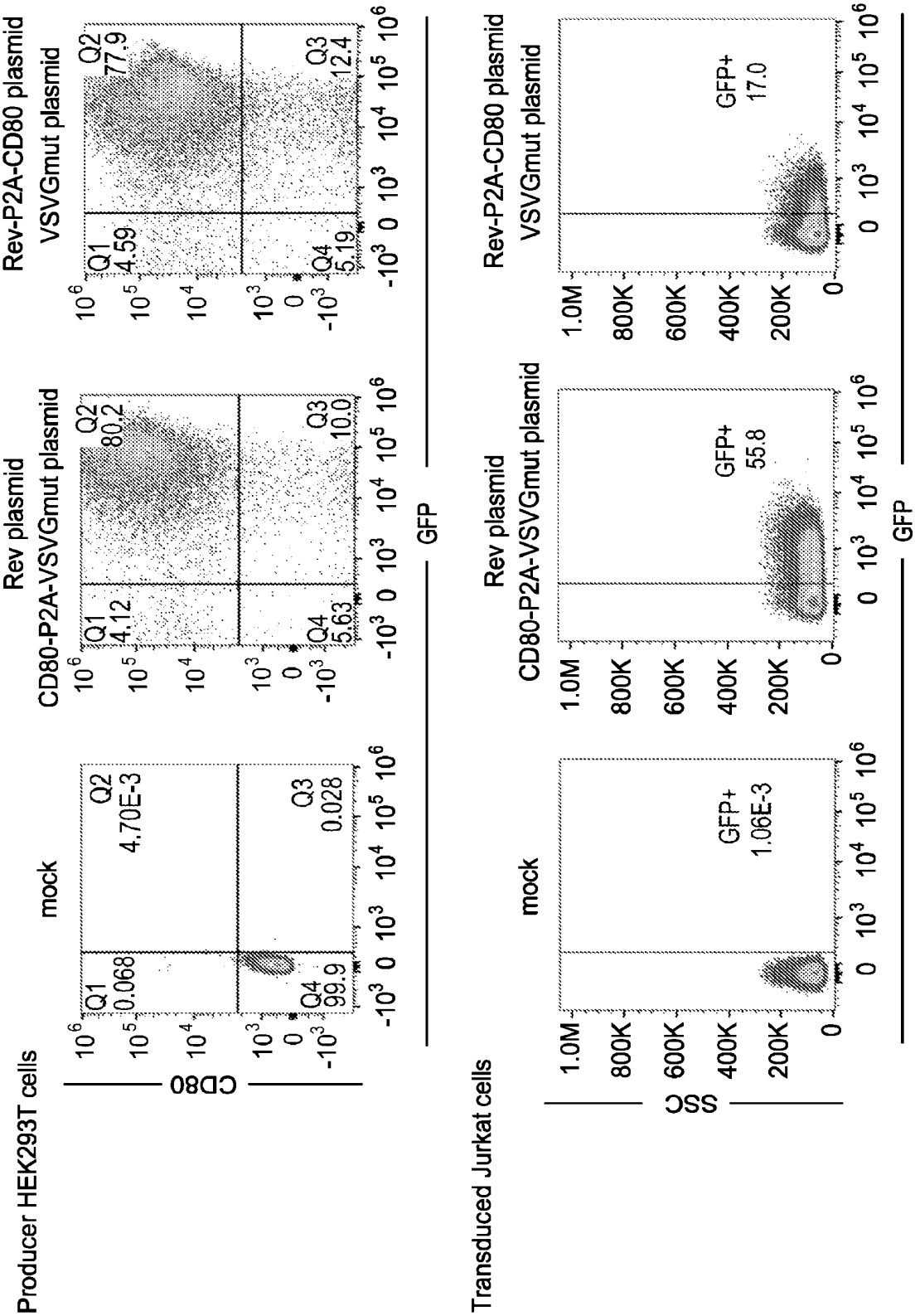

Producer HEK293T cells
Transduced Jurkat cells
mock
Rev plasmid
CD80-P2A-VSVGmut plasmid
Rev-P2A-CD80 plasmid
VSVGmut plasmid
CD80
SSC
GFP
Q1 0.068
Q2 4.70E-3
Q3 0.028
Q4 99.9
Q1 4.12
Q2 80.2
Q3 10.0
Q4 5.63
Q1 4.59
Q2 77.9
Q3 12.4
Q4 5.19
GFP+ 1.06E-3
GFP+ 55.8
GFP+ 17.0

FIG. 4

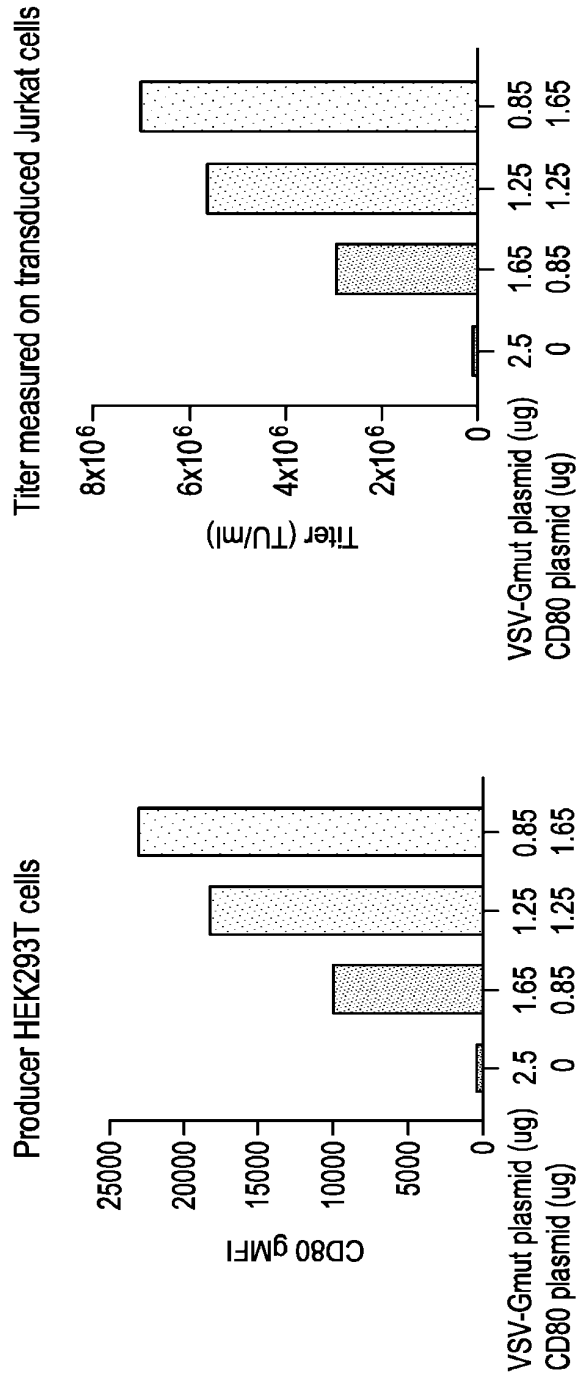

Producer HEK293T cells
CD80 gMFI
25000
20000
15000
10000
5000
0
VSV-Gmut plasmid (ug) 2.5 0 1.65 0.85 1.25 1.25 0.85 1.65
CD80 plasmid (ug)
Titer measured on transduced Jurkat cells
Titer (TU/ml)
8x10^6
6x10^6
4x10^6
2x10^6
0
VSV-Gmut plasmid (ug) 2.5 0 1.65 0.85 1.25 1.25 0.85 1.65
CD80 plasmid (ug)

FIG. 5

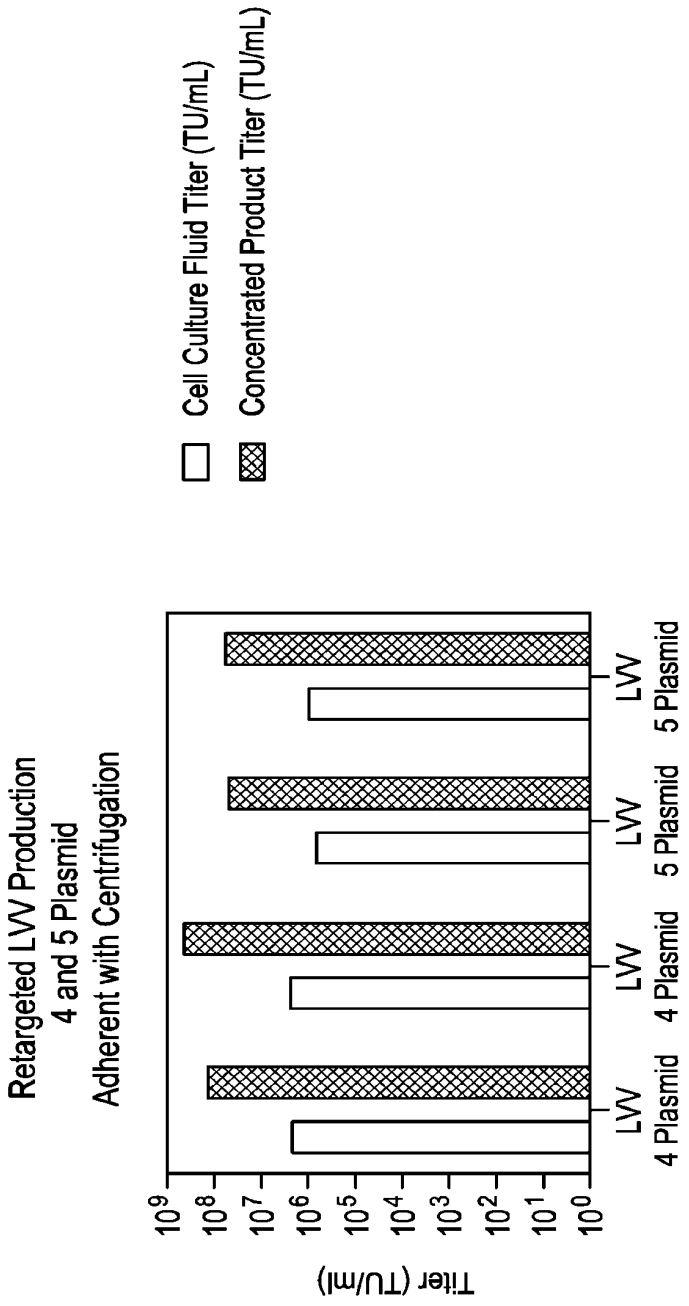

Retargeted LVV Production
4 and 5 Plasmid
Adherent with Centrifugation
Cell Culture Fluid Titer (TU/mL)
Concentrated Product Titer (TU/mL)
Titer (TU/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
LVV 4 Plasmid
LVV 4 Plasmid
LVV 5 Plasmid
LVV 5 Plasmid

FIG. 6A

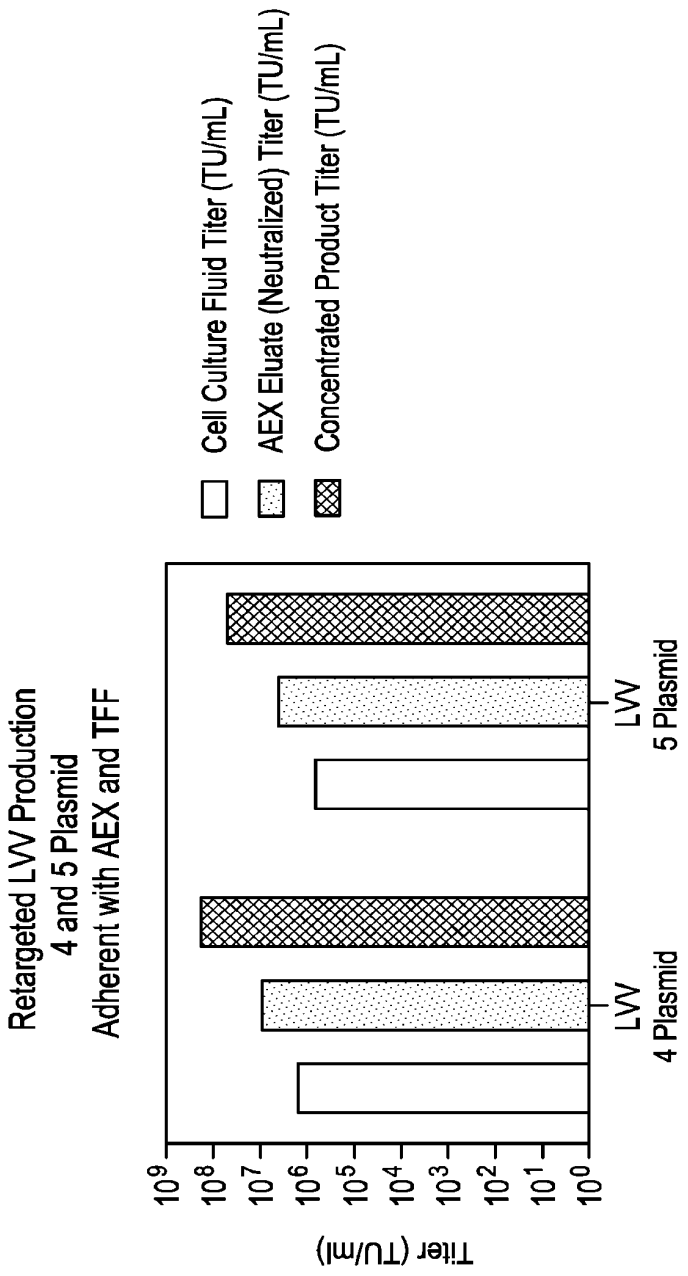

Retargeted LVV Production
4 and 5 Plasmid
Adherent with AEX and TFF
Cell Culture Fluid Titer (TU/mL)
AEX Eluate (Neutralized) Titer (TU/mL)
Concentrated Product Titer (TU/mL)
Titer (TU/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
LVV
4 Plasmid
LVV
5 Plasmid

FIG. 6B

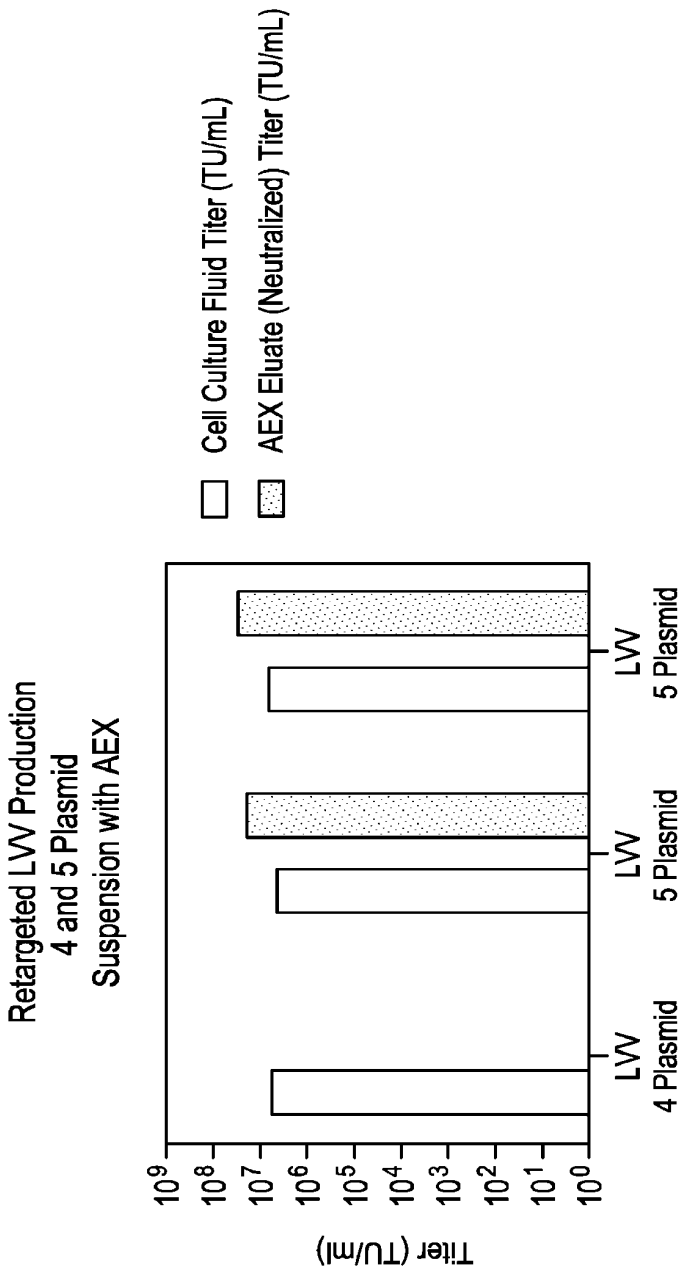

Retargeted LVV Production
4 and 5 Plasmid
Suspension with AEX
Cell Culture Fluid Titer (TU/mL)
AEX Eluate (Neutralized) Titer (TU/mL)
Titer (TU/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
LVV
4 Plasmid
LVV
5 Plasmid
LVV
5 Plasmid

FIG. 6C

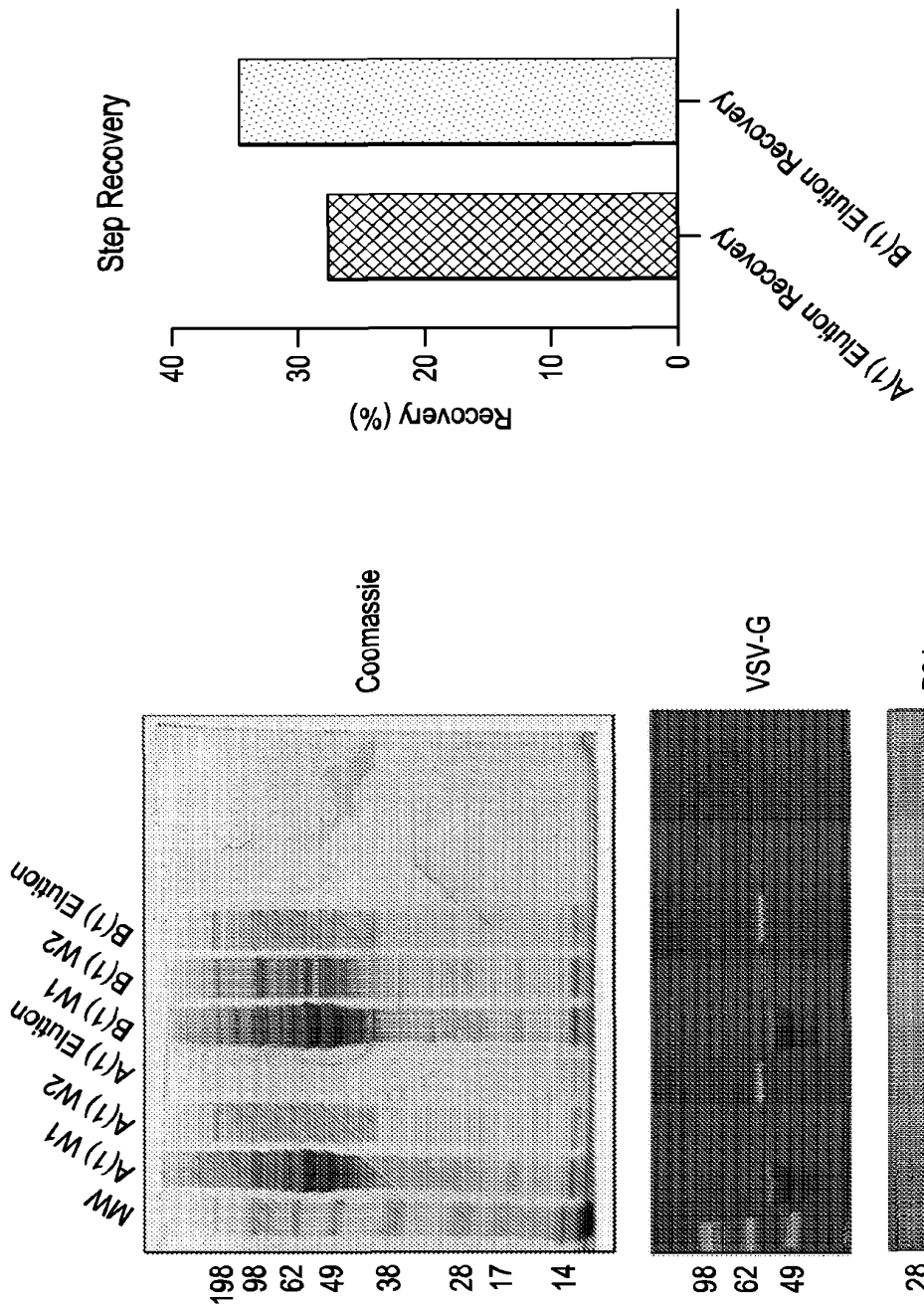

Step Recovery
Recovery (%)
40
30
20
10
0
A(1) Elution Recovery
B(1) Elution Recovery
Coomassie
VSV-G
P24
MW
A(1) W1
A(1) W2
A(1) Elution
B(1) W1
B(1) W2
B(1) Elution
198
98
62
49
38
28
17
14
98
62
49
28
17
14

FIG. 6D

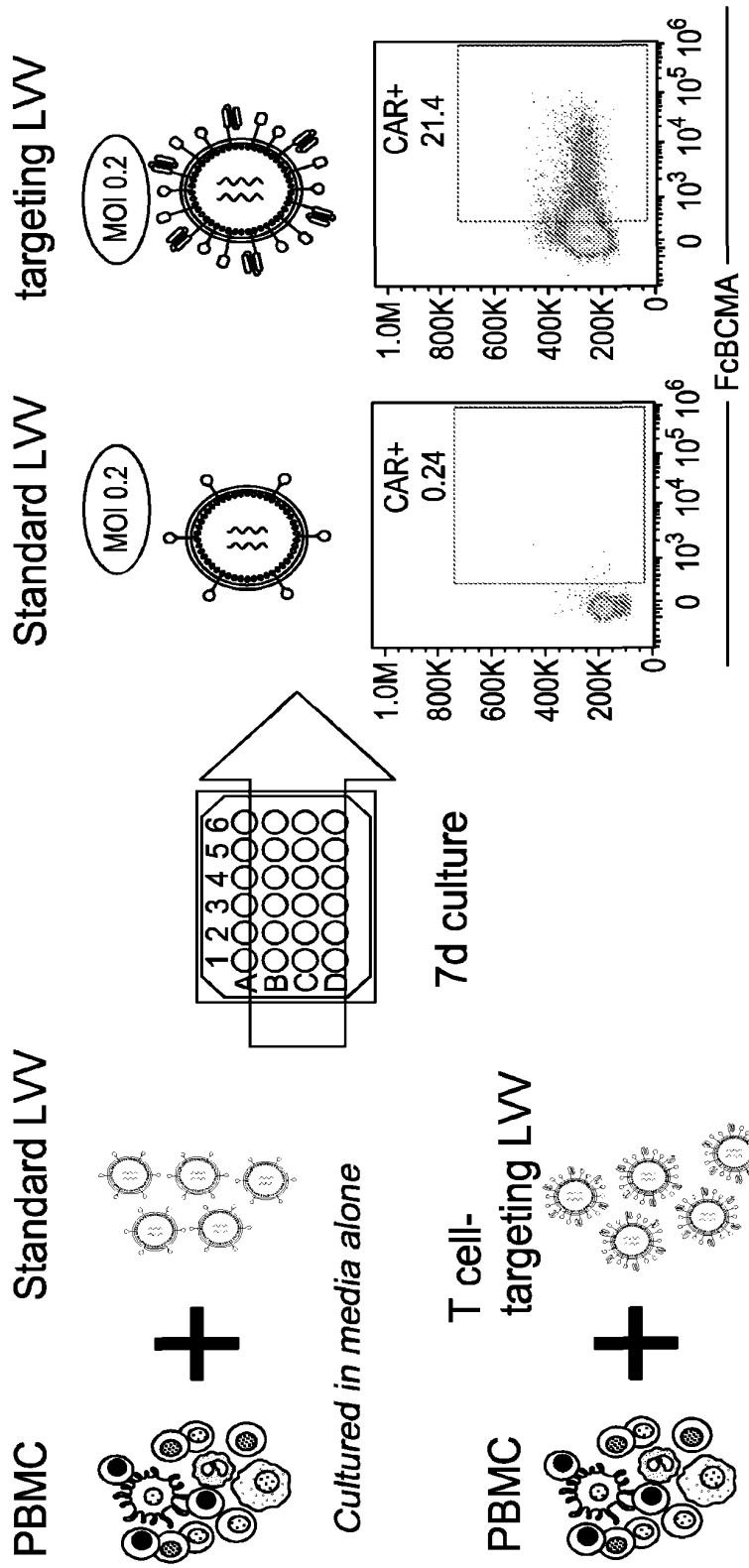

PBMC
Standard LVV
Cultured in media alone
T cell-targeting LVV
PBMC
T cell-targeting LVV
Cultured in media alone
1 2 3 4 5 6
A
B
C
D
7d culture
Standard LVV
MOI 0.2
T cell-targeting LVV
MOI 0.2
CAR+ 0.24
CAR+ 21.4
1.0M
800K
600K
400K
200K
0
0 10^3 10^4 10^5 10^6
FcBCMA

FIG. 7

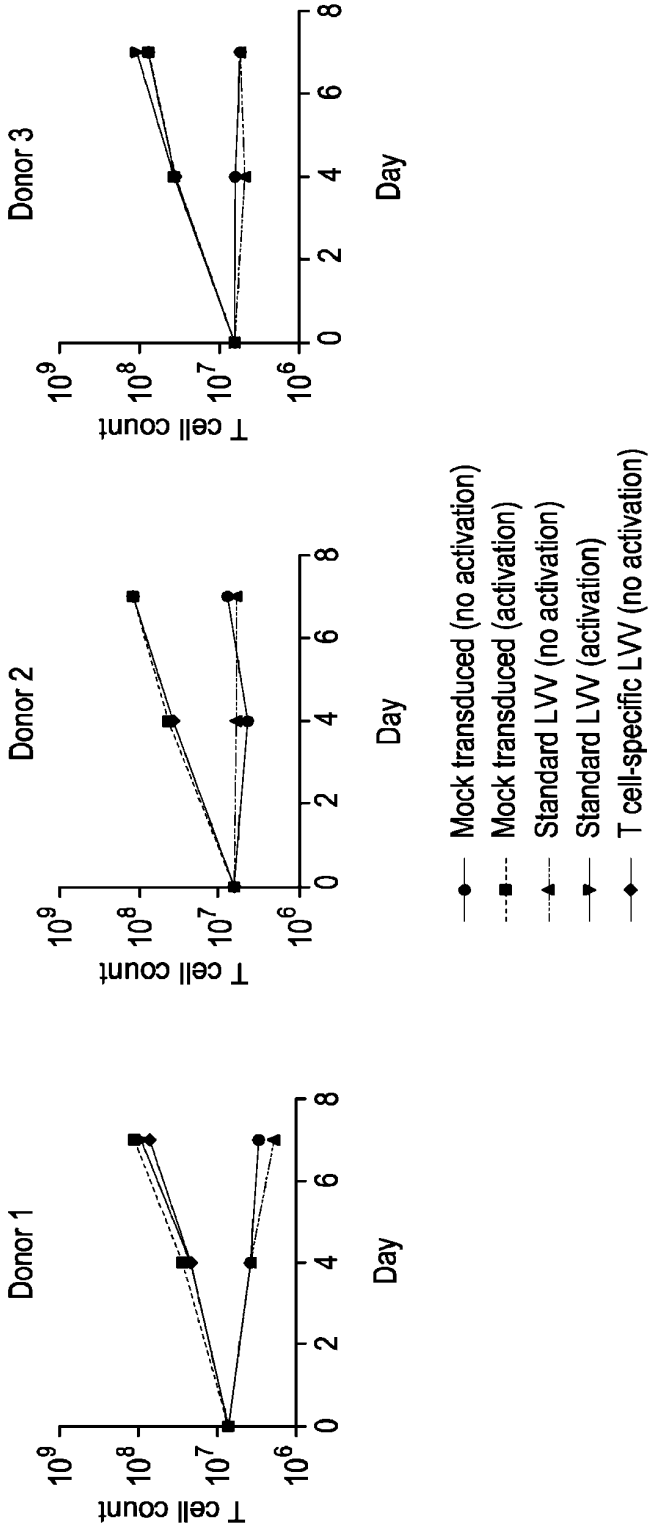

Donor 1
Donor 2
Donor 3
T cell count
Day
Mock transduced (no activation)
Mock transduced (activation)
Standard LVV (no activation)
Standard LVV (activation)
T cell-specific LVV (no activation)

FIG. 8

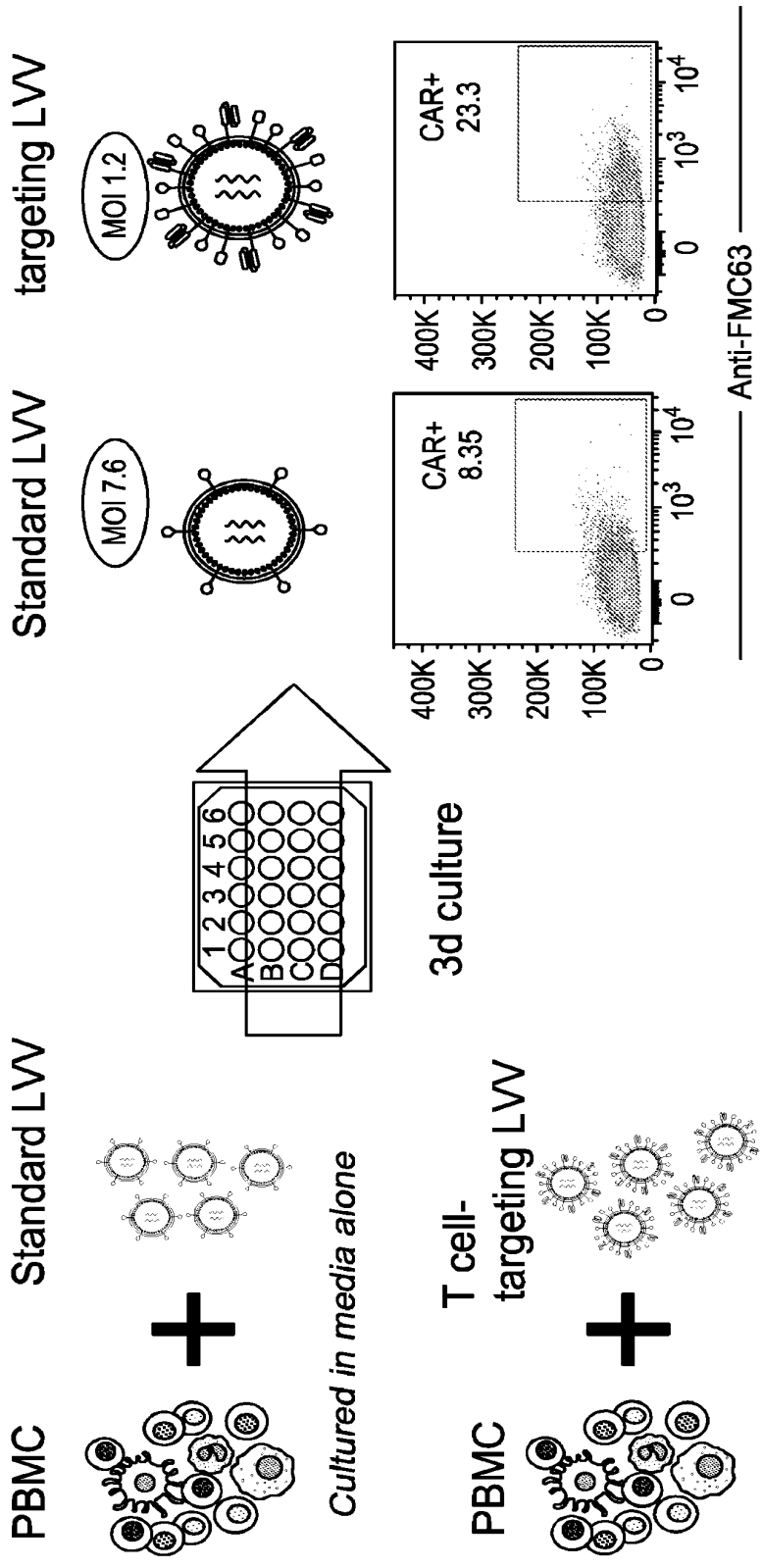

PBMC
Standard LVV
Cultured in media alone
PBMC
T cell-targeting LVV
Cultured in media alone
3d culture
Standard LVV
MOI 7.6
T cell-targeting LVV
MOI 1.2
CAR+ 8.35
CAR+ 23.3
400K
300K
200K
100K
0
10^3
10^4
Anti-FMC63

FIG. 9

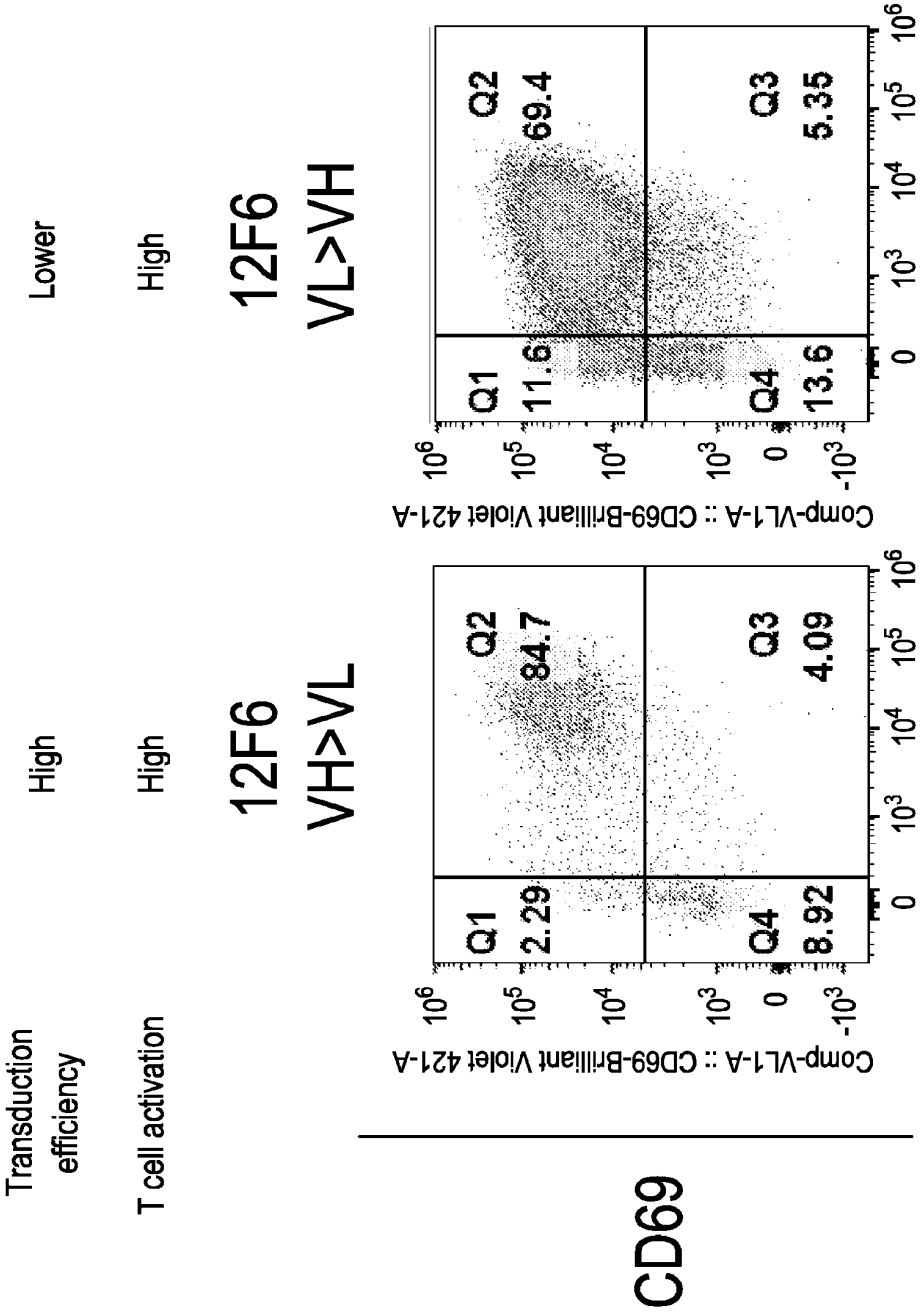

Transduction efficiency
T cell activation
High
High
Lower
High
12F6 VH>VL
12F6 VL>VH
CD69
GFP
Comp-VL1-A :: CD69-Brilliant Violet 421-A
Q1 2.29
Q2 84.7
Q3 4.09
Q4 8.92
Q1 11.6
Q2 69.4
Q3 5.35
Q4 13.6

FIG. 10

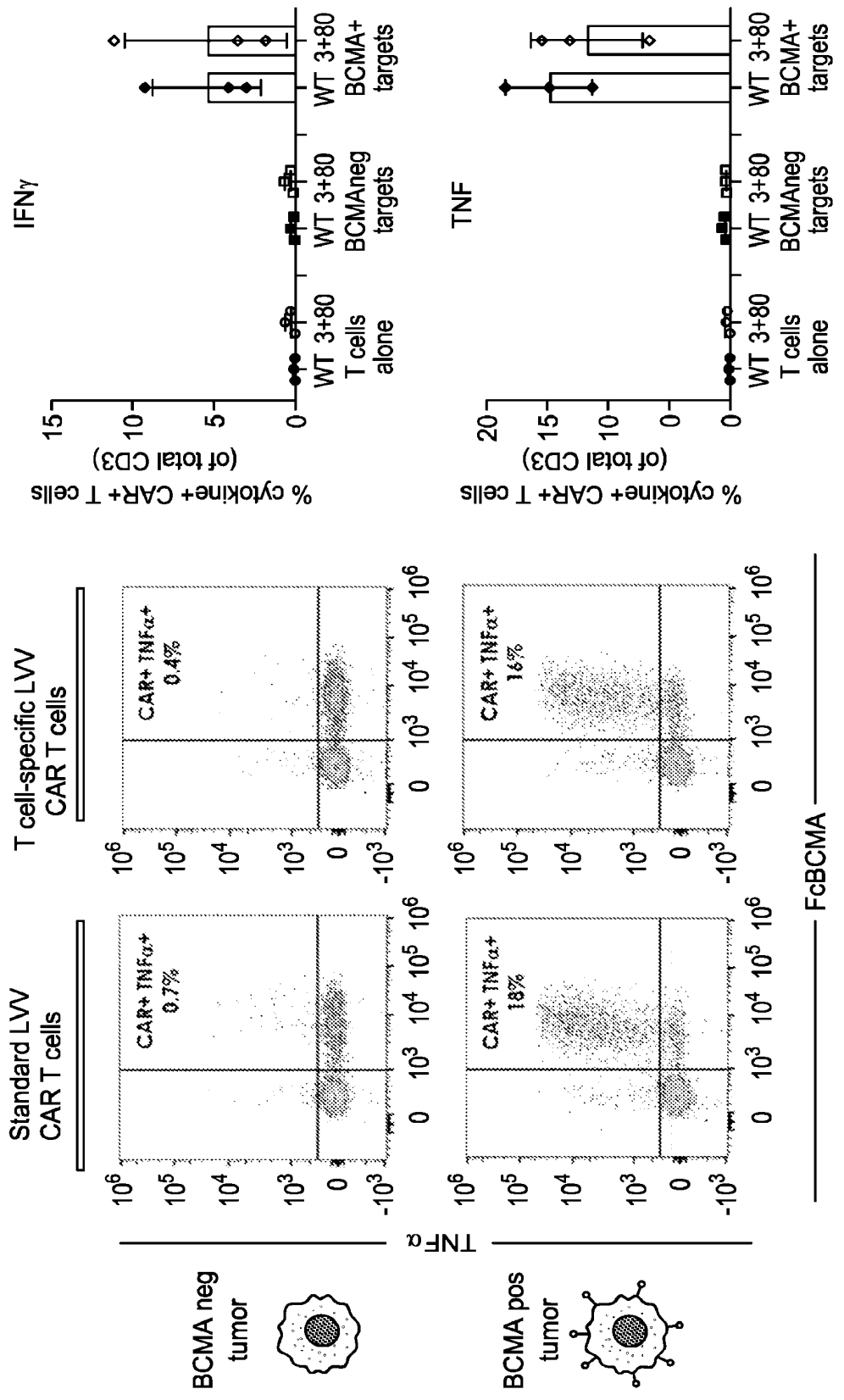

IFNγ
% cytokine+ CAR+ T cells (of total CD3)
WT 3+80 T cells alone
WT 3+80 BCMAneg targets
WT 3+80 BCMA+ targets
TNF
% cytokine+ CAR+ T cells (of total CD3)
WT 3+80 T cells alone
WT 3+80 BCMAneg targets
WT 3+80 BCMA+ targets
Standard LVV CAR T cells
T cell-specific LVV CAR T cells
CAR+ TNFα+ 0.7%
CAR+ TNFα+ 0.4%
CAR+ TNFα+ 18%
CAR+ TNFα+ 16%
BCMA neg tumor
BCMA pos tumor
TNFα
FcBCMA

FIG. 11

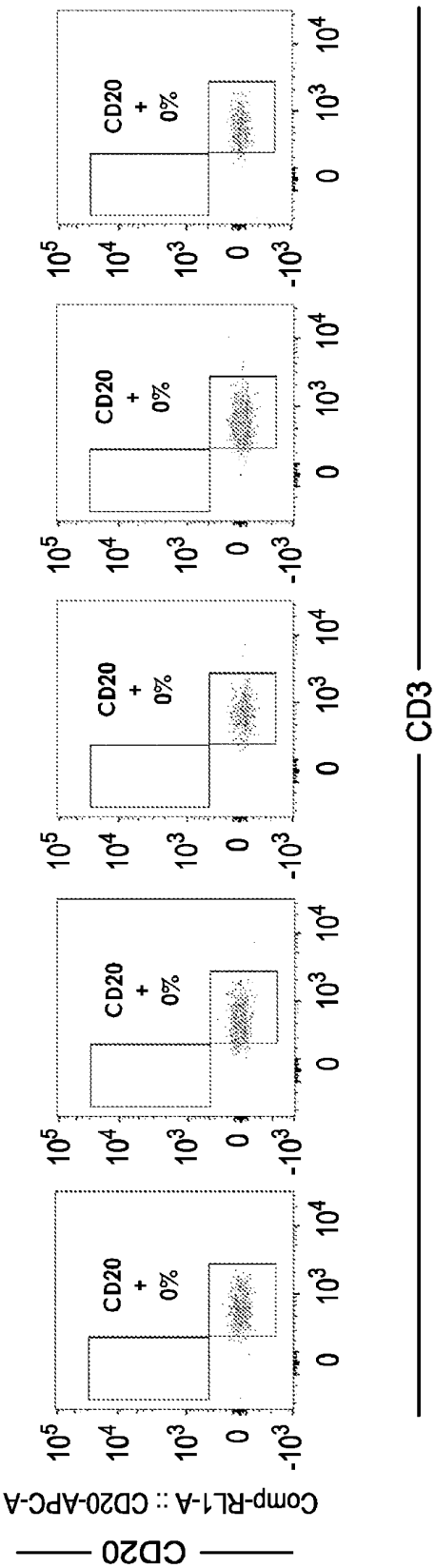


FIG. 12A

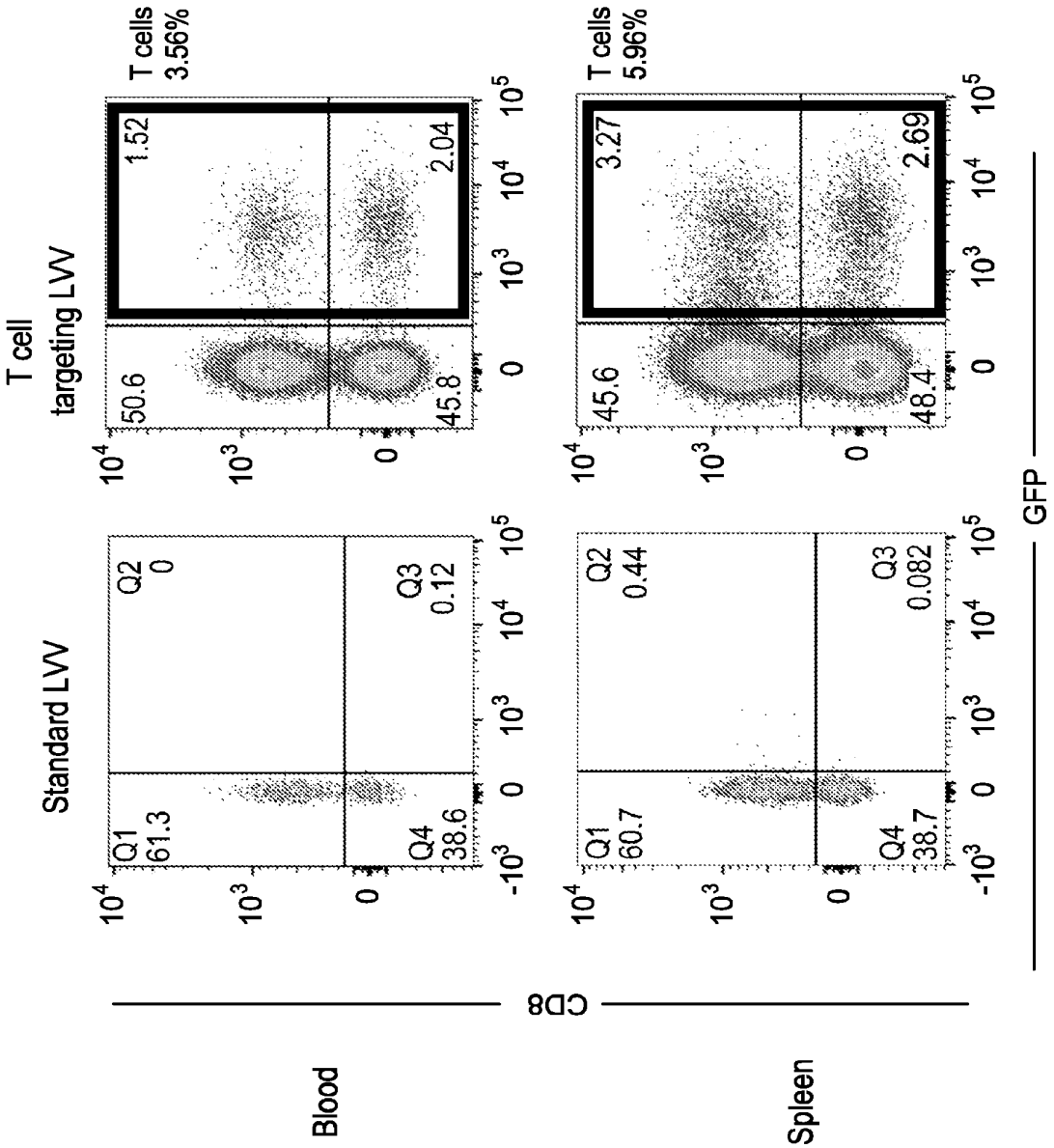

T cell targeting LVV
T cells 3.56%
T cells 5.96%
Standard LVV
Q1 61.3
Q2 0
Q3 0.12
Q4 38.6
50.6
1.52
2.04
45.8
Q1 60.7
Q2 0.44
Q3 0.082
Q4 38.7
45.6
3.27
2.69
48.4
Blood
Spleen
CD8
GFP

FIG. 12B

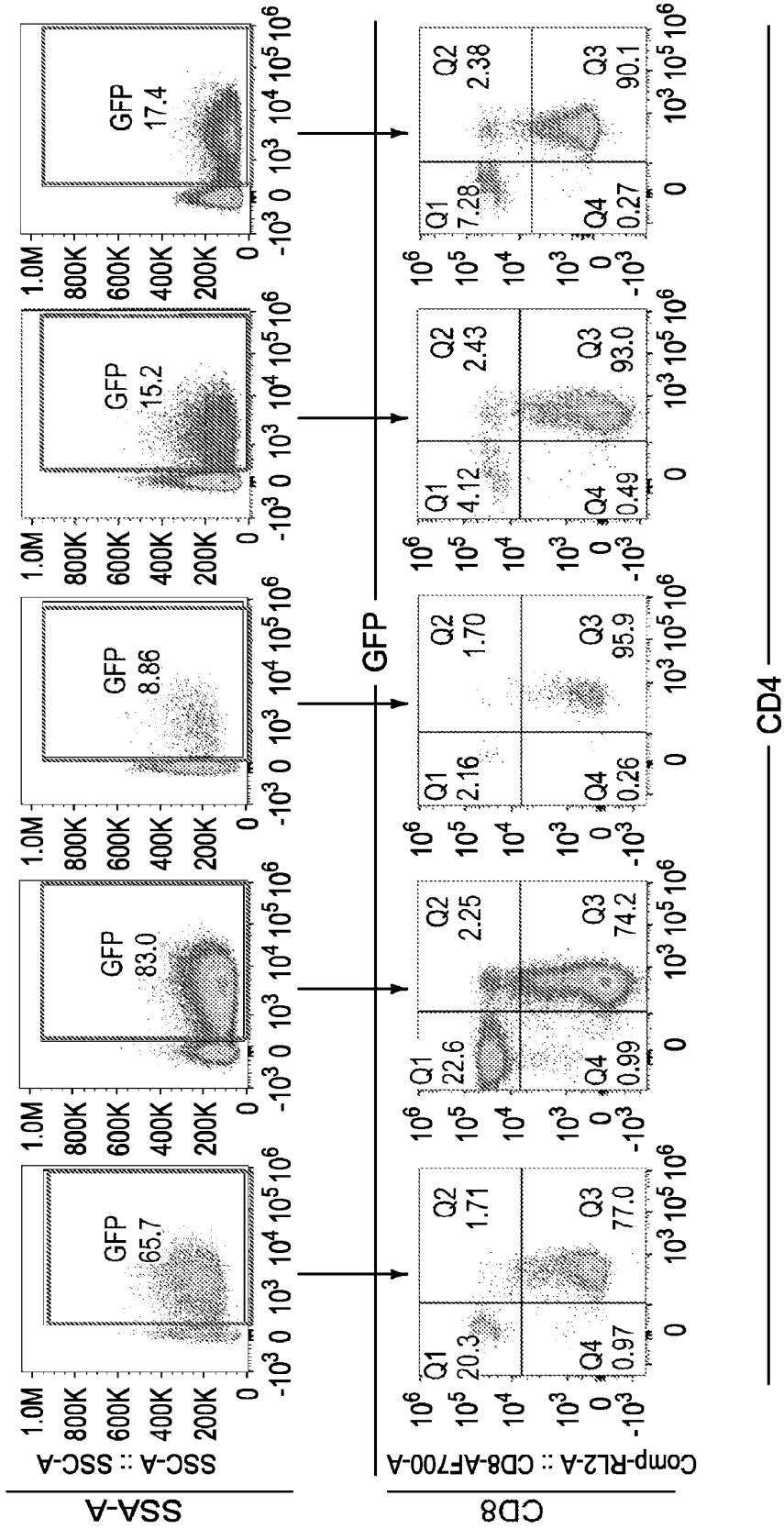

WT LVV GFP
CD80 LVV GFP
D3
D5
D7
GFP
CD4
CD8
SSC-A
SSC-A :: SSC-A
Comp-RL2-A :: CD8-AF700-A
GFP 65.7
GFP 83.0
GFP 8.86
GFP 15.2
GFP 17.4
Q1 20.3
Q2 1.71
Q3 77.0
Q4 0.97
Q1 22.6
Q2 2.25
Q3 74.2
Q4 0.99
Q1 2.16
Q2 1.70
Q3 95.9
Q4 0.26
Q1 4.12
Q2 2.43
Q3 93.0
Q4 0.49
Q1 7.28
Q2 2.38
Q3 90.1
Q4 0.27

FIG. 13

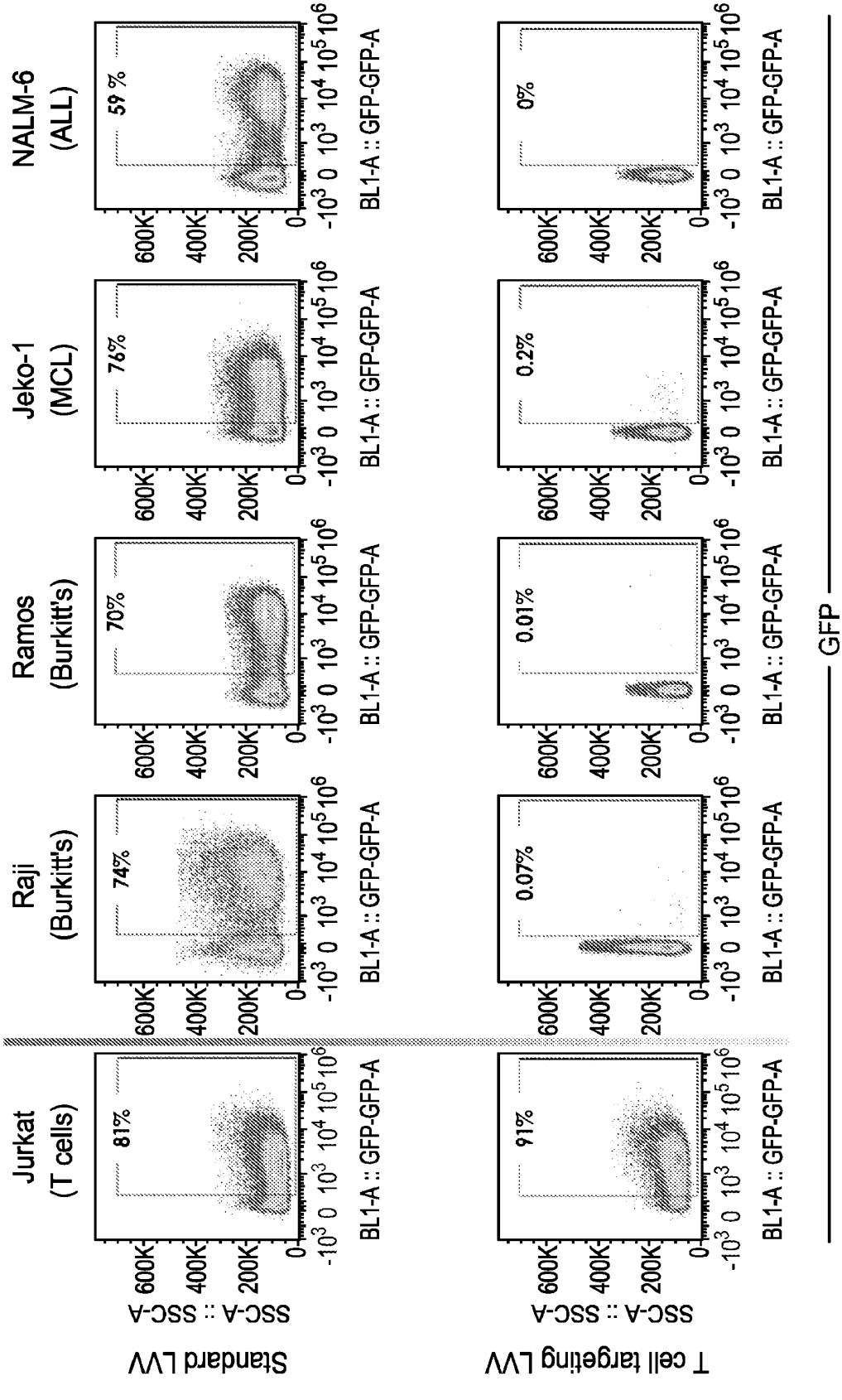

Jurkat (T cells)
Raji (Burkitt's)
Ramos (Burkitt's)
Jeko-1 (MCL)
NALM-6 (ALL)
Standard LVV
T cell targeting LVV
SSC-A :: SSC-A
BL1-A :: GFP-GFP-A
GFP
81%
74%
70%
76%
59 %
91%
0.07%
0.01%
0.2%
0%

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/018027

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/145 C12N15/867
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2020/371088 A1 (BIRNBAUM MICHAEL [US] ET AL) 26 November 2020 (2020-11-26)	1-18, 21-46, 49, 51-100
Y	abstract paragraphs [0004], [0006], [0009], [0010], [0013], [0014], [0019], [0055], [0057], [0064], [0067], [0069], [0097] examples 3-8, 10-12 figures 10, 17-22, 25, 27	19,20, 47,48,50

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance:; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance:; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 2022	21/06/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Chavanne, Franz

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/018027

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOVAN NIKOLIC; BELOT LAURA; RAUX HÉLÈNE; LEGRAND PIERRE; GAUDIN YVES; ALBERTINI AURÉLIE A: "Structural basis for the recognition of LDL-receptor family members by VSV glycoprotein", NATURE COMMUNICATIONS, vol. 9, no. 1, 12 March 2018 (2018-03-12), pages 1-12, XP055518690, DOI: 10.1038/s41467-018-03432-4 abstract pages 7-9 figures 4, 5 -----	19,20, 47,48,50

1

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/018027

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/018027

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2020371088 A1	26-11-2020	AU 2020278470 A1	02-12-2021
		BR 112021023353 A2	01-02-2022
		CA 3140115 A1	26-11-2020
		CN 114127305 A	01-03-2022
		EP 3973065 A1	30-03-2022
		KR 20220012291 A	03-02-2022
		SG 11202112344V A	30-12-2021
		US 2020371088 A1	26-11-2020
		WO 2020236263 A1	26-11-2020

Form PCT/ISA/210 (patent family annex) (April 2005)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 April 2016 (28.04.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/065323 A2

(51) International Patent Classification:
A61K 39/395 (2006.01) *G01N 33/574* (2006.01)

(21) International Application Number:
PCT/US2015/057223

(22) International Filing Date:
23 October 2015 (23.10.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/067,908	23 October 2014 (23.10.2014)	US
62/148,656	16 April 2015 (16.04.2015)	US
62/188,353	2 July 2015 (02.07.2015)	US
62/210,795	27 August 2015 (27.08.2015)	US

(71) Applicant: **SINGH BIOTECHNOLOGY, LLC** [US/US]; Suite 115, 3802 Spectrum Boulevard, Tampa, Florida 33612 (US).

(72) Inventor: **SINGH, Sunanda**; 4708 Rue Bordeaux, Lutz, Florida 33558 (US).

(74) Agents: **LLOYD, Laura M.** et al.; LEECH TISHMAN FUSCALDO LAMPL, LLP, 100 Corson Street, Third Floor, Pasadena, CA, California 91103-3842 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

[Continued on next page]

(54) Title: SINGLE DOMAIN ANTIBODIES DIRECTED AGAINST INTRACELLULAR ANTIGENS

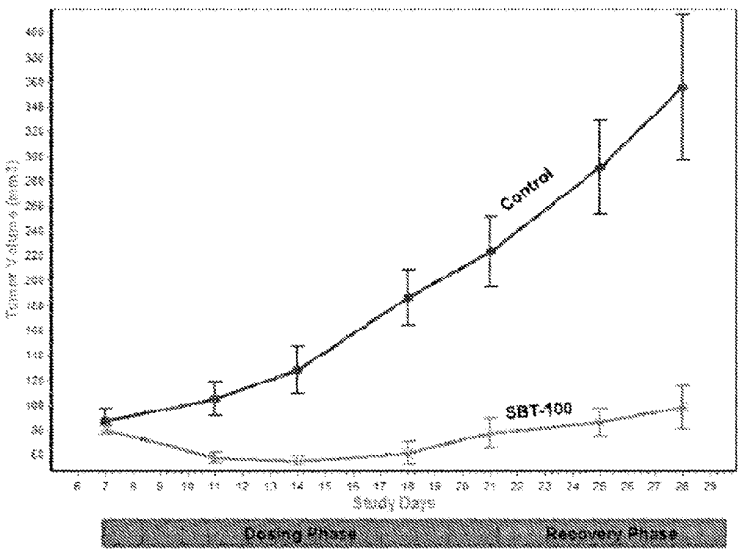


FIG. 7

(57) Abstract: This invention provides compositions and methods to treat a condition or disease without the use of exogenous targeting sequences or chemical compositions. The present invention relates to single-domain antibodies (sdAbs), proteins and polypeptides comprising the sdAbs that are directed against intracellular components that cause a condition or disease. The invention also includes nucleic acids encoding the sdAbs, proteins and polypeptides, and compositions comprising the sdAbs. The invention includes the use of the compositions, sdAbs, and nucleic acids encoding the sdAbs for prophylactic, therapeutic or diagnostic purposes.

WO 2016/065323 A2

— *with sequence listing part of description (Rule 5.2(a))*

SINGLE DOMAIN ANTIBODIES DIRECTED AGAINST INTRACELLULAR ANTIGENS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This International Patent Application claims the benefit of United States Provisional Patent Application No. 62/067,908, filed on October 23, 2014, United States Provisional Patent Application No. 62/148,656, filed on April 16, 2015, United States Provisional Patent Application No. 62/188,353 filed on July 2, 2015, and United States Provisional Patent Application No. 62/210,795, filed on August 27, 2015, the contents of which are incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file titled "Sequence_Listing_STP25.txt," created September 30, 2015, last modified October 22, 2015, which is 83,000 bytes in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND

[0003] The use of single-domain antibodies (sdAbs) as single antigen-binding proteins or as an antigen-binding domain in larger protein or polypeptide offers a number of significant advantages over the use of conventional antibodies or antibody fragments. The advantages of sdAbs include: only a single domain is required to bind an antigen with high affinity and with high selectivity; sdAbs can be expressed from a single gene and require no post-translational modification; sdAbs are highly stable to heat, pH, proteases and other denaturing agents or conditions; sdAbs are inexpensive to prepare; and sdAbs can access targets and epitopes not accessible to conventional antibodies.

[0004] There are a number of diseases or conditions, such as cancer, that are caused by aberrant intracellular or transmembrane components such as nucleotides and proteins. Elimination of the aberrant components can be used to prevent or treat the diseases or conditions. There are a number of pharmacological compounds available for treatment, but the compounds can be ineffective, undeliverable, or toxic to unaffected cells.

[0005] Other treatments include the use of therapeutic proteins or agents that contain an exogenous targeting sequence so that the therapeutic agent can be recognized by receptors in

the cell membrane, enabling the therapeutic agent to cross the cell membrane and enter the cell. Once the therapeutic agent is inside the cell, the therapeutic agent can interact with the target component in order to treat the disease. However, the use of exogenous targeting sequence can limit the cell type that is targeted by the therapeutic agent, and adds to the cost of manufacturing the therapeutic agent.

[0006] For the foregoing reasons, there is a need for compositions and methods to treat or prevent a disease that do not rely on exogenous targeting sequences or chemical compositions in order to enter the cell, and that are effective in targeting only the affected cells in the body.

[0007] The present invention relates to single-domain antibodies (sdAbs), proteins and polypeptides comprising the sdAbs. The sdAbs are directed against intracellular components that cause a condition or disease. The invention also includes nucleic acids encoding the sdAbs, proteins and polypeptides, and compositions comprising the sdAbs. The invention includes the use of the compositions, sdAbs, proteins or polypeptides for prophylactic, therapeutic or diagnostic purposes. The invention also includes the use of monoclonal antibodies directed towards the sdAbs of the invention.

SUMMARY

[0008] One embodiment of the invention is a single-domain antibody (sdAb) directed against an intracellular component. The intracellular component can be, for example, a protein, nucleic acid, lipid, carbohydrate, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6, TNF-alpha, and KRAS.

[0009] In another embodiment, the invention is directed towards an anti-STAT3 sdAb. Optionally, the anti-STAT3 sdAb comprises an amino acid sequence set forth in SEQ ID NO:3 or SEQ ID NO:4.

[0010] In another embodiment, the invention is directed towards an isolated polypeptide comprising an amino acid sequence encoding an anti-STAT sdAb, such as, for example, the polypeptide set forth in SEQ ID NO:3 or SEQ ID NO:4.

[0011] In yet another embodiment, the invention is directed towards a host cell, and the host cell expresses the amino acid sequence of the sdAb such as, for example, the amino acid set forth in SEQ ID NO:3 or SEQ ID NO:4.

[0012] One embodiment of the invention is a pharmaceutical composition comprising a sdAb, or a polypeptide, and a pharmaceutically acceptable carrier. Optionally, the sdAb comprises an anti-STAT3 sdAb comprising an amino acid sequence set forth in SEQ ID NO:3 or SEQ ID NO:4, and the polypeptide comprises an isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:3 or SEQ ID NO:4.

[0013] Another embodiment of the invention is a method to diagnose a disorder mediated by STAT3 in a subject, the method comprising the steps of a) contacting a biological sample with the sdAb, or a polypeptide; b) determining the amount of STAT3 in the biological sample; and c) comparing the amount determined in step (b) with a standard, a difference in amount indicating the presence of the disorder.

[0014] Another embodiment of the invention is a method of preventing or treating a disease or disorder, or preventing recurrence of a disease mediated by STAT3, or for use in the treatment of cancer, or diseases caused by abnormal cellular proliferation, comprising administering an anti-STAT3 sdAb, or a polypeptide, to a subject in need thereof. Optionally, the sdAb comprises an anti-STAT3 sdAb comprising an amino acid sequence set forth in SEQ ID NO:3 or SEQ ID NO:4 and the polypeptide comprises an isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:3 or SEQ ID NO:4.

[0015] One embodiment of the invention is an anti-TNF-alpha sdAb. Optionally, the anti-TNF-alpha sdAb comprises an amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7. The invention also comprises an isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

[0016] Another embodiment of the invention is a host cell expressing the amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

[0017] In another embodiment, the invention is also a pharmaceutical composition comprising a sdAb or a polypeptide and a pharmaceutically acceptable carrier. Optionally, the sdAb comprises an anti-TNF-alpha sdAb comprising an amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7 and the polypeptide comprises an isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

[0018] Another embodiment of the invention is a method to diagnose a disorder mediated by TNF-alpha in a subject, the method comprising the steps of a) contacting a biological

sample with a sdAb or a polypeptide; b) determining the amount of TNF-alpha in the biological sample; and c) comparing the amount determined in step (b) with a standard, a difference in amount indicating the presence of the disorder.

[0019] In one embodiment, the invention describes a method of preventing or treating a disease or disorder or recurrence of a disease or disorder mediated by TNF-alpha, or for use in the treatment of cancer, or diseases caused by abnormal cellular proliferation, comprising administering an anti-TNF-alpha sdAb, or a polypeptide, to a mammal in need thereof. Optionally, the anti-TNF-alpha sdAb comprises an amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7 and the polypeptide comprises isolated polypeptide, the isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

[0020] One embodiment of the invention is an anti-KRAS sdAb. Optionally, the anti-KRAS sdAb comprises an amino acid sequence set forth in SEQ ID NO:2. In one aspect, the invention comprises an isolated polypeptide, wherein the isolated polypeptide comprises an amino acid sequence set forth in SEQ ID NO:2. In another aspect, the invention comprises a host cell expressing the amino acid sequence set forth in SEQ ID NO:2.

[0021] Another embodiment of the invention is a pharmaceutical composition, comprising a sdAb or a polypeptide, and a pharmaceutically acceptable carrier. Optionally, the sdAb comprises an anti-KRAS sdAb comprising an amino acid sequence set forth in SEQ ID NO:2 and the polypeptide comprises isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:2.

[0022] An additional embodiment of the invention is method to diagnose a disorder mediated by KRAS in a subject, the method comprising the steps of a) contacting a biological sample with a sdAb or a polypeptide; b) determining the amount of KRAS in said biological sample; and c) comparing the amount determined in step (b) with a standard, a difference in amount indicating the presence of the disorder. Optionally, the sdAb comprises an anti-KRAS sdAb comprising an amino acid sequence set forth in SEQ ID NO: 2 and the polypeptide comprises isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:2.

[0023] The invention also comprises a method of treating a disease using an anti-KRAS sdAb, the method comprising administering an effective amount of an anti-KRAS sdAb to a subject in need thereof.

[0024] In one embodiment, the invention describes a method of preventing or treating a disease or disorder, or the recurrence of a disease or disorder, mediated by KRAS, or for use in the treatment of cancer, or diseases caused by abnormal cellular proliferation, comprising administering an anti-KRAS sdAb or a polypeptide, to a mammal in need thereof. Optionally, the anti-KRAS sdAb comprises an amino acid sequence set forth in SEQ ID NO: 2 and the polypeptide comprises isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 2.

[0025] In one embodiment, the invention describes a method of administering the sdAb of the invention, the method comprising intravenous administration, intramuscular administration, oral administration, rectal administration, intraocular administration, enteral administration, parenteral administration, subcutaneous administration, transdermal administration, administered as eye drops, administered as nasal spray, administered by inhalation or nebulization, topical administration, and administered as an implantable drug.

[0026] In another embodiment, the invention describes a method of treating a disease, preventing a disease or preventing the reoccurrence of a disease using the sdAb of the invention in combination with one or more compounds. Optionally, the one or more compounds is a transcriptional inhibitor.

[0027] In another embodiment, the invention describes a method of measuring the levels of a sdAb, the method comprising the steps of a) generating a mouse monoclonal antibody directed against one or more domains of the sdAb; b) performing an immunoassay to determine the amount of sdAb in a subject; and c) quantifying the amount of sdAb in the subject.

DRAWINGS

[0028] These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying drawings where:

Figure 1 is a schematic map of VHH13 anti-STAT3 sdAb expression vector pTT21-stt VHH13;

Figure 2 is a schematic map of VHH14 anti-STAT3 sdAb expression vector pTT21-stt VHH14;

Figure 3 depicts the results of an immunoprecipitation assay using anti-STAT3 bacterial VHH13 STAT3 (SEQ ID NO:3) and anti-STAT3 bacterial VHH14 STAT3 (SEQ ID NO:4);

Figure 4 depicts the results of an immunoprecipitation assay using anti-STAT3 bacterial VHH13 STAT3 (SEQ ID NO:3);

Figure 5 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the MDA-MB-231 xenograft model, dosed at 0.5mg/kg/day;

Figure 6 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ. ID. NO. 3) sdAb in MDA-MB-231 xenograft model at doses ranging from 1 mg/kg twice daily to 2 mg/kg twice daily or 2mg/kg/day;

Figure 7 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the MDA-MB-231 xenograft model, dosed at 5mg/kg/twice daily;

Figure 8 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the DU145 xenograft model, dosed at 5mg/kg/twice daily;

Figure 9 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the PANC-1 xenograft model, dosed at 5mg/kg/twice daily;

Figure 10 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the MCF-7 xenograft model, dosed at 1mg/kg/three times daily;

Figure 11 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the BT-474 xenograft model, dosed at 1mg/kg/three times daily;

Figure 12 illustrates the cytotoxicity of TNF-alpha in U937 cells;

Figure 13 illustrates the cytotoxicity of Staurosporine in U937 cells; and

Figure 14 illustrates inhibition of TNF-alpha cytotoxicity by anti-TNF-alpha sdAbs.

DESCRIPTION

[0029] As used herein, the following terms and variations thereof have the meanings given below, unless a different meaning is clearly intended by the context in which such term is used.

[0030] The terms "a," "an," and "the" and similar referents used herein are to be construed to cover both the singular and the plural unless their usage in context indicates otherwise.

[0031] The term "antigenic determinant" refers to the epitope on the antigen recognized by the antigen-binding molecule (such as an sdAb or polypeptide of the invention) and more in particular by the antigen-binding site of the antigen-binding molecule. The terms "antigenic determinant" and "epitope" may also be used interchangeably. An amino acid sequence that can bind to, that has affinity for and/or that has specificity for a specific antigenic determinant, epitope, antigen or protein is said to be "against" or "directed against" the antigenic determinant, epitope, antigen or protein.

[0032] As used herein, the term "comprise" and variations of the term, such as "comprising" and "comprises," are not intended to exclude other additives, components, integers or steps.

[0033] It is contemplated that the sdAbs, polypeptides and proteins described herein can contain so-called "conservative" amino acid substitutions, which can generally be described as amino acid substitutions in which an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Conservative amino acid substitutions are well known in the art. Conservative substitutions are substitutions in which one amino acid within the following groups (a)-(e) is substituted by another amino acid within the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp. Other conservative substitutions include: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

[0034] A "domain" as used herein generally refers to a globular region of an antibody chain, and in particular to a globular region of a heavy chain antibody, or to a polypeptide that essentially consists of such a globular region.

[0035] The amino acid sequence and structure of an sdAb is typically made up of four framework regions or "FRs," which are referred to as "Framework region 1" or "FR1"; as "Framework region 2" or"FR2"; as "Framework region 3" or "FR3"; and as "Framework region 4" or "FR4," respectively. The framework regions are interrupted by three complementarity determining regions or "CDRs," which are referred as "Complementarity Determining Region 1" or "CDR1"; as "Complementarity Determining Region 2" or "CDR2"; and as "Complementarity Determining Region 3" or "CDR3," respectively.

[0036] As used herein, the term "humanized sdAb" means an sdAb that has had one or more amino acid residues in the amino acid sequence of the naturally occurring VHH sequence replaced by one or more of the amino acid residues that occur at the corresponding position in a

VH domain from a conventional 4-chain antibody from a human. This can be performed by methods that are well known in the art. For example, the FRs of the sdAbs can be replaced by human variable FRs.

[0037] As used herein, an "isolated" nucleic acid or amino acid has been separated from at least one other component with which it is usually associated, such as its source or medium, another nucleic acid, another protein/polypeptide, another biological component or macromolecule or contaminant, impurity or minor component.

[0038] The term "mammal" is defined as an individual belonging to the class Mammalia and includes, without limitation, humans, domestic and farm animals, and zoo, sports, and pet animals, such as cows, horses, sheep, dogs and cats.

[0039] As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, PBS (phosphate-buffered saline), and 5% human serum albumin. Liposomes, cationic lipids and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with a therapeutic agent as defined above, use thereof in the composition of the present invention is contemplated.

[0040] A "quantitative immunoassay" refers to any means of measuring an amount of antigen present in a sample by using an antibody. Methods for performing quantitative immunoassays include, but are not limited to, enzyme-linked immunosorbent assay (ELISA), specific analyte labeling and recapture assay (SALRA), liquid chromatography, mass spectrometry, fluorescence-activated cell sorting, and the like.

[0041] The term "solution" refers to a composition comprising a solvent and a solute, and includes true solutions and suspensions. Examples of solutions include a solid, liquid or gas dissolved in a liquid and particulates or micelles suspended in a liquid.

[0042] The term "specificity" refers to the number of different types of antigens or antigenic determinants to which a particular antigen-binding molecule or antigen-binding

protein molecule can bind. The specificity of an antigen-binding protein can be determined based on affinity and/or avidity. The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (KD), is a measure for the binding strength between an antigenic determinant and an antigen-binding site on the antigen-binding protein: the lesser the value of the KD, the stronger the binding strength between an antigenic determinant and the antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (KA), which is 1/KD). As will be clear to one of skill in the art, affinity can be determined depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule and the antigen. Avidity is related to both the affinity between an antigenic determinant and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule. Specific binding of an antigen-binding protein to an antigen or antigenic determinant can be determined by any known manner, such as, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays.

[0043] As used herein, the term "recombinant" refers to the use of genetic engineering methods (for example, cloning, and amplification) used to produce the sdAbs of the invention.

[0044] A "single domain antibody," "sdAb" or "VHH" can be generally defined as a polypeptide or protein comprising an amino acid sequence that is comprised of four framework regions interrupted by three complementarity determining regions. This is represented as FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. An sdAb of the invention also includes a polypeptide or protein that comprises the sdAb amino acid sequence. Typically, sdAbs are produced in camelids such as llamas, but can also be synthetically generated using techniques that are well known in the art. As used herein, the variable domains present in naturally occurring heavy chain antibodies will also be referred to as "VHH domains," in order to distinguish them from the heavy chain variable domains that are present in conventional 4-chain antibodies, referred to as "VH domains," and from the light chain variable domains that are present in conventional 4-chain antibodies, referred to as "VL domains." "VHH" and "sdAb" are used interchangeably herein. The numbering of the amino acid residues of a sdAb or polypeptide is according to the general numbering for VH domains given by Kabat et al. ("Sequence of proteins of immunological interest," US Public Health Services, NIH Bethesda, MD, Publication No. 91). According to this numbering, FR1 of a sdAb comprises the amino acid residues at positions 1-30, CDR1 of a sdAb comprises the amino acid residues at positions 31-36, FR2 of a sdAb

comprises the amino acids at positions 36-49, CDR2 of a sdAb comprises the amino acid residues at positions 50-65, FR3 of a sdAb comprises the amino acid residues at positions 66-94, CDR3 of a sdAb comprises the amino acid residues at positions 95-102, and FR4 of a sdAb comprises the amino acid residues at positions 103-113.

[0045] The term "synthetic" refers to production by *in vitro* chemical or enzymatic synthesis.

[0046] The term "target" as used herein refers to any component, antigen, or moiety that is recognized by the sdAb. The term "intracellular target" refers to any component, antigen, or moiety present inside a cell. A "transmembrane target" is a component, antigen, or moiety that is located within the cell membrane. An "extracelluar target" refers to a component, antigen, or moiety that is located outside of the cell.

[0047] A "therapeutic composition" as used herein means a substance that is intended to have a therapeutic effect such as pharmaceutical compositions, genetic materials, biologics, and other substances. Genetic materials include substances intended to have a direct or indirect genetic therapeutic effect such as genetic vectors, genetic regulator elements, genetic structural elements, DNA, RNA and the like. Biologics include substances that are living matter or derived from living matter intended to have a therapeutic effect.

[0048] As used herein, the phrases "therapeutically effective amount" and "prophylactically effective amount" refer to an amount that provides a therapeutic benefit in the treatment, prevention, or management of a disease or an overt symptom of the disease. The therapeutically effective amount may treat a disease or condition, a symptom of disease, or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of disease, or the predisposition toward disease. The specific amount that is therapeutically effective can be readily determined by an ordinary medical practitioner, and may vary depending on factors known in the art, such as, e.g., the type of disease, the patient's history and age, the stage of disease, and the administration of other therapeutic agents.

[0049] The present invention relates to single-domain antibodies (sdAbs) that are directed against intracellular components, as well as to proteins and polypeptides comprising the sdAbs and nucleotides encoding the proteins and polypeptides. The invention can also relate to sdAbs that are directed against intercellular, transcellular and extracellular targets or antigens. The invention also includes nucleic acids encoding the sdAbs, proteins and polypeptides, and

compositions comprising the sdAbs. The invention includes the use of the compositions, sdAbs, proteins or polypeptides for prophylactic, therapeutic or diagnostic purposes.

[0050] SdAbs have a number of unique structural characteristics and functional properties which make sdAbs highly advantageous for use as functional antigen-binding domains or proteins. SdAbs functionally bind to an antigen in the absence of a light chain variable domain, and can function as a single, relatively small, functional antigen-binding structural unit, domain or protein. This distinguishes sdAbs from the domains of conventional antibodies, which by themselves do not function as an antigen-binding protein or domain, but need to be combined with conventional antibody fragments such as Fab fragments or ScFv's fragment in order to bind an antigen.

[0051] SdAbs can be obtained using methods that are well known in the art. For example, one method for obtaining sdAbs includes (a) immunizing a Camelid with one or more antigens, (b) isolating peripheral lymphocytes from the immunized Camelid, obtaining the total RNA and synthesizing the corresponding cDNAs, (c) constructing a library of cDNA fragments encoding VHH domains, (d) transcribing the VHH domain-encoding cDNAs obtained in step (c) to mRNA using PCR, converting the mRNA to ribosome display format, and selecting the VHH domain by ribosome display, and (e) expressing the VHH domain in a suitable vector and, optionally purifying the expressed VHH domain.

[0052] Another method of obtaining the sdAbs of the invention is by preparing a nucleic acid encoding an sdAb using techniques for nucleic acid synthesis, followed by expression of the nucleic acid *in vivo* or *in vitro*. Additionally, the sdAb, polypeptides and proteins of the invention can be prepared using synthetic or semi-synthetic techniques for preparing proteins, polypeptides or other amino acid sequences.

[0053] The sdAbs of the invention will generally bind to all naturally occurring or synthetic analogs, variants, mutants, alleles, parts and fragments of the target, or at least to those analogs, variants, mutants, alleles, parts and fragments of the target that contain one or more antigenic determinants or epitopes that are essentially the same as the antigenic determinant or epitope to which the sdAbs of the invention bind in the wild-type target. The sdAbs of the invention may bind to such analogs, variants, mutants, alleles, parts and fragments with an affinity and/or specificity that is the same as, or that is higher than or lower than the affinity and specificity with which the sdAbs of the invention bind to the wild-type target. It is also contemplated within the scope of the invention that the sdAbs of the invention bind to

some analogs, variants, mutants, alleles, parts and fragments of the target but not to others. In addition, the sdAb of the invention may be humanized, and may be monovalent or multivalent, and/or multispecific. Additionally, the sdAbs of the invention can bind to the phosphorylated form of the target protein as well as the unphosphorylated form of the target protein. sdAbs can be linked to other molecules such as albumin or other macromolecules.

[0054] In addition, it is within the scope of the invention that the sdAbs are multivalent, that is, the sdAb can have two or more proteins or polypeptides which are directed against two or more different of epitopes of the target. In such a multivalent sdAb, the protein or polypeptide may be directed, for example, against the same epitopes, substantially equivalent epitopes, or different epitopes. The different epitopes may be located on the same target, or it could be on two or more different targets.

[0055] It is also contemplated that the sequence of one or more sdAbs of the invention may be connected or joined with one or more linker sequences. The linker can be, for example, a protein sequence containing a combination of serines, glycines and alanines.

[0056] It is also within the scope of the invention to use parts, fragments, analogs, mutants, variants, alleles and/or derivatives of the sdAbs of the invention, as long as these are suitable for the described uses.

[0057] Since the sdAbs of the invention are mainly intended for therapeutic and/or diagnostic use, they are directed against mammalian, preferably human, targets. However, it is possible that the sdAbs described herein are cross-reactive with targets from other species, for example with targets from one or more other species of primates or other animals (for example, mouse, rat, rabbit, pig or dog), and in particular in animal models for diseases and disorders associated with the disease associated with the targets.

[0058] In another aspect, the invention relates to a nucleic acid that encodes an sdAb of the invention. Such a nucleic acid may be, for example, in the form of a genetic construct.

[0059] In another aspect, the invention relates to host or host cell that expresses or is capable of expressing an sdAb of the invention, and/or that contains a nucleic acid encoding a sdAb of the invention. Sequences of the sdAbs can be used to insert into the genome of any organism to create a genetically modified organism (GMO). Examples include, but are not limited to, plants, bacteria, viruses, and animals.

[0060] The invention further relates to methods for preparing or generating the sdAbs, nucleic acids encoding the sdAbs, host cells expressing or capable of expressing such sdAbs, products and compositions containing the sdAbs of the invention.

[0061] The invention further relates to applications and uses of the sdAb, the nucleic acids encoding the sdAbs, host cells, products and compositions described herein. Such a product or composition may, for example, be a pharmaceutical composition for treatment or prevention of a disease, or a product or composition for diagnostic use. sdAbs can be used in a variety of assays, for example ELISA assays and mass spectrometry assays to measure the serum and tissue levels of the sdAbs.

[0062] In another aspect, a nucleic acid encoding one or more sdAb of the invention can be inserted into the genome of an organism to treat or prevent diseases.

[0063] The present invention generally relates to sdAbs, as well as to proteins or polypeptides comprising or essentially consisting of one or more of such sdAbs, that can be used for prophylactic, therapeutic and/or diagnostic purposes.

[0064] The methods and compositions detailed in the present invention can be used to treat disease described herein, and can be used with any dosage and/or formulation described herein or otherwise known, as well as with any route of administration described herein or otherwise known to one of skill in the art.

[0065] The sdAbs of the invention, in particular the anti-STAT3 VHH, the anti-KRAS VHH, and the anti-TNF-alpha VHH of the present invention, can be used for treatment and prevention of malignant diseases including, but not limited to: multiple myeloma, leukemias (HTLV-1 dependent, erythroleukemia, acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), and large granular lymphocyte leukemia (LGL), lymphomas (EBV-related/Burkitt's, mycosis fungoides, cutaneous T-cell lymphoma, non-Hodgkins lymphoma (NHL), anaplastic large-cell lymphoma (ALCL), breast cancers, triple-negative breast cancers, head and neck cancers, melanoma, ovarian cancers, lung cancers, pancreatic cancers, prostate cancers, sarcomas, osteosarcoma, Kaposi's sarcoma, Ewing's sarcoma, hepatocellular cancers, glioma, neuroblastoma, astrocytoma, colorectal cancers, Wilm's tumors, renal cancers, bladder cancers, endometrial cancers, cervical cancers, esophageal cancers, cutaneous squamous cell cancers, basal cell cancers, and any metastatic cancers. The sdAbs can be used in cancer patients to help prevent or reduce weight loss or cachexia due to cancer.

[0066] The sdAb, in particular the anti-STAT3 and the anti-TNF-alpha sdAbs of the present invention, can also be used for treatment and prevention of diseases such as, but not limited to: autoimmune diseases (e.g., rheumatoid arthritis, ulcerative colitis, Crohn's disease, bacterial induced colitis, asthma, scleroderma, lupus, encephalomyelitis, arteritis, vasculitis, glomerulonephritis, uveitis, uveoretinitis, multiple sclerosis), polycystic kidney disease, dermatologic diseases (e.g., psoriasis, alopecia areata, atopic dermatitis, keloids/hypertrophic scars, lipoma, Padget's disease, and actinic keratosis), Hidradenitis suppurativa, transplantation (e.g., solid organ, bone marrow, hand, face, limbs, any body part), muscular dystrophy and muscle wasting associated with cancers and aging, endometriosis, macular degeneration, retinal degeneration, stroke, epilepsy, traumatic brain and spinal cord injuries, hypertension, cardiac hypertrophy, Alzheimer's disease, pulmonary artery hypertension, type 2 diabetes mellitus, and ankylosing spondylitis. Additionally sdAbs can target orphan diseases. Examples of these rare orphan diseases include, but are not limited to, triple negative breast cancers, pancreatic cancers, AML (acute myeloid leukemia), head and neck cancers, multiple myeloma, and chemo-resistant cancers.

[0067] Viral infections can be treated by targeting intracellular viral proteins in infected cells. Viral proteins, such as HIV reverse transcriptase, can block viral life-cycle. The sdAb of the invention can also target intracellular viral proteins such as Ebola VP24 and thus block Ebola's ability to shut down the host's anti-viral immune response. The sdAbs of the invention can be used to target diseases when there is an overexpression of an intracellular molecule. Huntington's disease can be treated with sdAbs.

[0068] The sdAbs of the invention can be used with one or more compounds. For example, the sdAb of the invention can be used with JAK/STAT inhibitors such as, for example, Curcumin, Resveratrol, Cucurbitacin A, B, E, I, Q, Flavopiridol, Deoxytetrangomycin, Cyclopentenone derivatives, N-Acylhomoserine Lactone, Indirubin derivatives, Meisoindigo, Tyrphostins, Platinum-containing compounds (e.g., IS3-295), Peptidomimetics, antisense oligonucleotides, S3I-201, phosphotyrosin tripeptide derivatives, HIV protease inhibitors (e.g., nelfinavir, indinavir, saquinavir, & ritornavir), JSI-124, XpYL, Ac-pYLPQTV-NH2, ISS 610, CJ-1383, pyrimethamine, Metformin, Atiprimod, S3I-M2001, STX-0119; N-[2-(1,3,4-oxadiazolyl)]-4 quinolinecarboxamide derivative, S3I-1757, LY5; 5,8-dioxo-6(pyridin-3-ylamino)-5,8,-dihydro-naphthalene-1-sulfonamide, withacinstin, Stattic, STA-21, LLL-3, LLL12, XZH-5, SF-1066, SF-1087, 17o, Cryptotanshinone, FLL32, FLL62, C188-9, BP-1108 and BP-1075, Galiellalactone, JQ1, 5, 15 DPP, WP1066, Niclosamide,

SD1008, Nifuroxazide, Cryptotanshinone, BBI quinone, and Ruxolitnib Phosphate. The one or more compounds can increase the therapeutic response and augment the effectiveness of the sdAb of the invention. In addition, the effectiveness of the sdAb can be increased by combining it with peptides, peptidomimetics, and other drugs, such as, for example, but not limited to, cimetidine, atorvastatin, celecoxib, metformin, and cimetidine. In addition, anti-STAT3 sdAbs can convert radioresistant cancers to radiosensitive cancers with respect to radiation therapy.

[0069] It is also contemplated that one or more sdAbs of the invention can be combined, or the sdAbs of the invention can be combined with other sdAbs.

[0070] It is contemplated that certain sdAbs of the invention can cross the cell membrane and enter the cell without the aid of additional targeting protein sequences on the sdAb, and without the aid of exogenous compounds that direct the sdAb to bind to the cell surface receptors and cross the cell membrane.

[0071] After crossing the cell membrane, these sdAbs can target transmembrane or intracellular molecules or antigens. These intracellular or transmembrane targets can be, for example, proteins, carbohydrates, lipids, nucleic acids, mutated proteins, viral proteins, and prions. The sdAb targets may function as enzymes, structural proteins of the cell, intracellular portions of cell membrane molecules, molecules within the membranes of organelles, any type of RNA molecule, any regions of DNA or chromosome, methylated or unmethylated nucleic acids, partially assembled molecules within the synthesis mechanism of the cell, second messenger molecules, and molecules within cell signaling mechanisms. Targets may include all molecules in the cytoplasm, nucleus, organelles, and cell membrane. Molecules destined for secretion or placement in the cell membrane can be targeted within the cytoplasm before leaving the cell.

[0072] The sdAb targets can be in humans, animals, plants, fungi, parasites, protists, bacteria, viruses, prions, prokaryotic cells, and eukaryotic cells. Some examples of inter- and intracellular signaling molecules and protein groups that can be targeted by the sdAbs of the invention are: oncogene products, hormones, cytokines, growth factors, neurotransmitters, kinases (including tyrosine kinase, serine kinase, and threonine kinase), phosphatases, ubiquitin, cyclic nucleotides, cyclases (adenylyl and guanylyl), G proteins, phosphodiesterases, GTPase superfamily, immunoglobulins (antibodies, Fab fragments, binders, sdAbs), immunoglobulin superfamily, inositol phosphate lipids, steroid receptors, calmodulin, CD

group (e.g., CD4, CD8, CD28, etc.), transcription factors, TGF-beta, TNF-alpha and beta, TNF ligand superfamily, notch receptor signaling molecules, hedgehog receptor signaling molecules, Wnt receptor signaling molecules, toll-like receptor signaling molecules, caspases, actin, myosin, myostatin, 12-lipoxygenase, 15-lipoxygenase, lipoxygenase superfamily, reverse transcriptase, viruses and their proteins, amyloid proteins, collagen, G protein coupled receptors, mutated normal proteins, prions, Ras, Raf, Myc, Src, BCR/ABL, MEK, Erk, Mos, Tpl2, MLK3, TAK, DLK, MKK, p38, MAPK, MEKK, ASK, SAPK, JNK, BMK, MAP, JAK, PI3K, cyclooxygenase, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6, Myc, p53, BRAF, NRAS, KRAS, HRAS and chemokines.

[0073] KRAS is a Kirsten ras oncogene homolog from the mammalian ras gene family. KRAS encodes a protein that is a member of the small GTPase superfamily. The protein is implicated in various malignancies, including lung adenocarcinoma, mucinous adenoma, ductal carcinoma of the pancreas, and colorectal carcinoma. Under normal conditions, Ras family members influence cell growth and differentiation events in a subcellular membrane compartmentalization-based signaling system. However, oncogenic Ras can deregulate processes that control both cell proliferation and apoptosis.

[0074] Anti-KRAS sdAbs were developed to target wild-type and mutated KRAS (G12D) in order to disrupt its role in malignant cells such as, for example, cells involved in colorectal cancer, pancreatic cancer, biliary tract cancer, lung cancer, leukemias, and other metastatic malignancies. Without being bound by a particular mechanism, it is thought that the anti-KRAS sdAb binds KRAS and blocks the downstream signaling of KRAS in malignant cells. Additionally, the anti-KRAS sdAb may successfully treat malignancies that are resistant to anti-EGFR biologics (*e.g.*, cetuximab and panitumumab).

[0075] Using methods that are well-known in the art, recombinant human mutant KRAS (G12D) protein was used to generate sdAbs that are directed against or can bind to an epitope of KRAS or mutant KRAS (G12D), or other KRAS mutants. Additionally, sdAbs can be generated to other KRAS mutants. To generate the anti-KRAS sdAbs, recombinant full-length human KRAS (Gene ID: 3845) was expressed in *Escherichia coli*.

[0076] Several sdAbs were obtained and screened. The DNA sequence of one anti-KRAS (G12D) sdAb, named KRAS_13 (SEQ ID NO:1), is shown below:

5'Gaggtgcagctggtggagtctgggggaggctcggtgcagactggagggtctctgagactctcctgtgcagtttctggaaatatcggc agcagctactgcatgggctggttccgccaggctccagggaagaagcgcgaggcggtcgcacgtattgtacgtgatggtgccactggct acgcagactacgtgaagggccgattcaccatctcccgagacagcgccaagaacactctgtatctgcaaatgaacaggctgatacctgag gacactgccatctactactgtgcggcagacctgcccccaggttgtttgactcaggcgatttggaattttggttatcggggccagggaaccct ggtcaccgtctcctca-3'

[0077] The amino acid sequence of the anti-KRAS (G12D) sdAb (SEQ ID NO. 2), KRAS_13, is shown below, with the CDRs underlined:

EVQLVESGGGSVQTGGSLRLSCAVSGNIGSSYCMGWFRQAPGKKREAVARIVRDGAT GYADYVKGRFTISRDSAKNTLYLQMNRLIPEDTAIYYCAADLPPGCLTQAIWNFGYRG QGTLVTVSS

[0078] Additionally, the present invention comprises one or more mouse monoclonal antibodies which are directed against one or more domains of the anti-KRAS sdAb of the invention. The mouse monoclonal antibody can be generated by methods that are known by one of skill in the art, for example, the mouse monoclonal antibody can be produced by a mouse hybridoma. The mouse monoclonal antibody can be used in diagnostic assays, for example, the antibody can be used in an immunoassay such as an ELISA or mass spectrometry assay in order to measure the amount of anti-KRAS sdAb present in a patient's serum. The cytotoxicity of KRAS (G12D) sdAbs on PANC-1 human pancreatic cancer cells was tested, as described below.

[0079] STAT3 is a member of the signal transducers and activators of transcription (STAT) family of proteins that carry both signal transduction and activation of transcription functions. STAT3 is widely expressed and becomes activated through phosphorylation on tyrosine and/or serine as a DNA binding protein in response to a various cytokines and growth factors such as EGF, IL-6, PDGF, IL-2 and G-CSF. The STAT3 phosphoprotein forms homodimers and heterodimers with other members of the STAT family and translocates to the nucleus in order to modulate the transcription of various genes, and as a result plays a key role in many cellular processes such as cell growth, apoptosis, angiogenesis, immune evasion, and survival.

[0080] An anti-STAT3 sdAb can be given to patients and other organisms to treat diseases caused by phosphorylated and non-phosphorylated STAT3, as well as to prevent the

development of disease or recurrence of disease. For example, patients who have undergone organ transplant and bone marrow transplant are at higher risk for cutaneous SCCA and BCCA due to the immunosuppressive medications they take. Administration of an anti-STAT3 sdAb can reduce or eliminate this risk. Patients treated for a malignancy who are at risk for recurrence will benefit from treatment with the anti-STAT3 sdAb. Based on family medical history and HLA-type, some individuals will be at increased risk for some types of autoimmune diseases and may benefit from treatment with sdAbs to reduce risk of developing that autoimmune disease. Breast cancer risk can be reduced with administration of anti-STAT3 medication such as GLG-302, as demonstrated in a recent NCI study.

[0081] In addition to inhibiting STAT3, the anti-STAT3 sdAb can also inhibit STAT1, STAT2, STAT4, STAT5a, STAT5b, and STAT6 due to the high degree of homology between these molecules.

[0082] Recombinant human STAT3 protein was used to produce anti-STAT sdAbs that were directed against or can bind to an epitope of STAT3. To generate the anti-STAT3 sdAbs, recombinant full-length human STAT3 (Gene ID: 6774) was expressed by baculovirus in Sf9 insect cells. The anti-STAT sdAbs were cloned into vectors that can be expressed in both bacterial and mammalian cells, as shown in Figures 1 and 2.

[0083] The anti-STAT3 sdAb of the invention can be used to target STAT3 and all other STAT molecules inside the cell in order to inhibit cell growth, such as, for example, suppression of cancer cell growth. In addition, the anti-STAT3 sdAb can inhibit cell growth in other proliferative diseases such as psoriasis and macular degeneration via VEGF.

[0084] Without being limited to a particular mechanism of action, it is thought that anti-STAT3 sdAb can eliminate cancer induced immune suppression by decreasing STAT3 levels in antigen presenting cells such as, for example, host dendritic cells. STAT3 inhibition promotes anti-cancer response by patient's innate and adaptive immune systems (i.e., dendritic cells, macrophages, neutrophils, T cells, NK cells, and B cells).

[0085] Using methods that are well known in the art, several anti-STAT sdAbs were obtained and screened for the ability to suppress cancer cell growth and induce apoptosis in cancer cell lines, as described below. The cytotoxicity and anti-proliferative activities of the anti-STAT3 sdAbs was tested. In addition, the tolerance of anti-STAT3 sdAbs was tested *in vitro* and *in vivo*. The production of mouse monoclononal antibody directed against one or more domains of the anti-STAT sdAbs is described below.

[0086] The amino acid sequence of one anti-STAT3 sdAb, named VHH13 (SEQ ID NO. 3), is shown below:

HVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
FDCYRGSWFNRYMYNSWGQGTQVTVSS

The three CDRs are underlined.

[0087] The amino acid sequence of a second anti-STAT3 sdAb, named VHH14 (SEQ ID NO. 4), is shown below:

QVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
GWECGETWLDRTAGGHTYWGQGTLVTVSS

[0088] Again, the three CDRs are underlined. The protein sequences of other anti-STAT3 sdAbs that were obtained are as follows:

STAT3_10 (SEQ ID NO. 5):
(1)DVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTQVTVSS

STAT3_34 (SEQ ID NO. 6):
(1) DVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTQVTVSS

STAT3_19 (SEQ ID NO. 7):
(1) HVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTQVTVSS

STAT3_14 (SEQ ID NO. 8):
(1) QVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTLVTVSS

STAT3_35 (SEQ ID NO. 9):
(1) QVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTLVTVSS

STAT3_9 (SEQ ID NO. 10):
(1) QVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTLVTVSS

STAT3_30 (SEQ ID NO. 11):
(1) QVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTLVTVSS

STAT3_23 (SEQ ID NO. 12):
(1) QVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGSHTYWGQGTLVTVSS

STAT3_24 (SEQ ID NO. 13):
(1) EVQLVESGGGSVQAGGSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTLVTVSS

STAT3_36 (SEQ ID NO. 14):
(1) DVQLVESGGGSVQAGDSLRLSCVASTYTGCMGWFRQAPGKEREGVAA
(48) LSSRGFAGHYTDSVKGRFSISRDYVKNAVYLQMNTVKPEDAAMYYCAARE
(98) GWECGETWLDRTAGGHTYWGQGTLVTVSS

STAT3_12 (SEQ ID NO. 15):
(1) QVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTLVTVSS

STAT3_16 (SEQ ID NO. 16):
(1) QVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTNNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTLVTVSS

STAT3_11 (SEQ ID NO. 17):
(1) EVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101)FDCYRGSWFNRYMYNSWGQGTLVTVSS

STAT3_20 (SEQ ID NO. 18):
(1) DVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTLVTVSS

STAT3_2 (SEQ ID NO. 19):
(1) DVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTLVTVSS

STAT3_15 (SEQ ID NO. 20):
(1) DVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTLVTVSS

STAT3_6 (SEQ ID NO. 21):
(1) HVQLVESEGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTLVTVSS

STAT3_33 (SEQ ID NO. 22):
(1) QVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

STAT3_17 (SEQ ID NO. 23):
(1) QVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

STAT3_25 (SEQ ID NO. 24):
(1) EVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMSSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

STAT3_32 (SEQ ID NO. 25):
(1) DVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

STAT3_13 (SEQ ID NO. 26):
(1) HVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

STAT3_39 (SEQ ID NO. 27):
(1) HVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

STAT3_4 (SEQ ID NO. 28):
(1) HVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

STAT3_29 (SEQ ID NO. 29):
(1) HVQLVESGGGSVQAGGSLRLSCAASGANGGRSCMGWFRQVPGKEREGVSG
(51) ISTGGLITYYADSVKGRFTISQDNTKNTLYLQMNSLKPEDTAMYYCATSR
(101) FDCYRGSWFNRYMYNSWGQGTQVTVSS

[0089] The corresponding anti-STAT3 DNA sequences are as follows:

Stat3_VHH-10 (SEQ ID NO. 30): 5'-
gatgtgcagctggtggagtctgggggaggctcggtgcaggctggaggctctctg
agactctcctgtgtagcctctacatacaccggctgcatgggctggttccgccaggctcctggaaaggagcgcgagggagtcgcagctctt
agtagccgtggttttgccgggcactataccgactccgtgaagggccgattctccatctcccgagactacgtcaagaatgcggtgtatctgc
aaatgaacactgtgaaacctgaggacgctgccatgtactactgtgcagcacgggagggatgggagtgcggtgagacctggttggaccg
gaccgccgggggccatacctactggggccaggggacccaggtcaccgtctcctca-3'

Stat3_VHH-14 (SEQ ID NO. 31):5'-caggtgcagctggtggagtctgggggaggctcggtgcaggctggaggctctc
tgagactctcctgtgtagcctctacatacaccggctgcatgggctggttccgccaggctcctggaaaggagcgcgagggagtcgcagct
cttagtagccgtggttttgccgggcactataccgactccgtgaagggccgattctccatctcccgagactacgtcaagaatgcggtgtatct
gcaaatgaacactgtgaaacctgaggacgctgccatgtactactgtgcagcacgggagggatgggagtgcggtgagacctggttggac
cggaccgccgggggccatacctactggggccaggggaccctggtcaccgtctcctca-3'

Stat3_VHH-12 (SEQ ID NO. 32): 5'-caggtgcagctggtggagtctgggggaggctcggtgcaggctggagggtctct
gagactctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgaggg
ggtttctggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaagaaca
cgctgtatctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctcttg
gttcaaccgatatatgtataacagttggggccaggggacccctggtcaccgtctcctca-3'

Stat3_VHH-13 (SEQ ID NO. 33): 5'-catgtgcagctggtggagtctgggggaggctcggtgcaggctggagggtctct
gagactctcctgtgcagcctctggagccaacggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgaggg
ggtttctggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaagaaca
cgctgtatctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctcttg
gttcaaccgatatatgtataacagttggggccaggggacccaggtcactgtctcctca-3'

Stat3_VHH-20 (SEQ ID NO. 34): 5'-gatgtgcagctggtggagtctgggggaggctcggtgcaggctggagggtctct
gagactctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgaggg
ggtttctggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaagaaca
cgctgtatctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctcttg
gttcaaccgatatatgtataacagttggggccaggggacccctggtcaccgtctcctca-3'

Stat3_VHH-23 (SEQ ID NO. 35):5'-caggtgcagctggtggagtctgggggaggctcggtgcaggctggaggctctct
gagactctcctgtgtagcctctacatacaccggctgcatgggctggttccgccaggctcctggaaaggagcgcgagggagtcgcagctc
ttagcagccgtggttttgccgggcactataccgactccgtgaagggccgattctccatctcccgagactacgtcaagaatgcggtgtatct
gcaaatgaacactgtgaaacctgaggacgctgccatgtactactgtgcagcacgggagggatgggagtgcggtgagacctggttggac
cggaccgccgggagccatacctactggggccaggggaccctggtcaccgtctcctca-3'

Stat3_VHH-24 (SEQ ID NO. 36):5'-gaggtgcagctggtggagtctgggggaggctcggtgcaggctggaggctctct
gagactctcctgtgtagcctctacatacaccggctgcatgggctggttccgccaggctcctggaaaggagcgcgagggagtcgcagctc
ttagtagccgtggttttgccgggcactataccgactccgtgaagggccgattctccatctcccgagactacgtcaagaatgcggtgtatctg
caaatgaacactgtgaaacctgaggacgctgccatgtactactgtgcagcacgggagggatgggagtgcggtgagacctggttggacc
gaaccgccgggggccatacctactggggccaggggaccctggtcaccgtctcctca-3'

Stat3_VHH-25 (SEQ ID NO. 37):5'-
gaggtgcagctggtggagtctgggggaggctcggtgcaggctggagggtctctg
agactctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgagggg
gtttctggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggtcgattcaccatctcccaagacaacaccaagaacacg
ctgtatctgcaaatgagcagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctcttggtt
caaccgatatatgtataacagttggggccaggggacccaggtcaccgtctcctca-3'

Stat3_VHH-19 (SEQ ID NO. 38):5'-
catgtgcagctggtggagtctgggggggggctcggtgcaggctggaggctctctga
gactctcctgtgtagcctctacatacaccggctgcatgggctggttccgccaggctcctggaaaggagcgcgagggagtcgcagctctta
gtagccgtggttttgccgggcactataccgactccgtgaagggccgattctccatctcccgagactacgtcaagaatgcggtgtatctgca
aatgaacactgtgaaacctgaggacgctgccatgtactactgtgcagcacgggagggatgggagtgcggtgagacctggttggaccgg
accgccgggggccatacctactggggccaggggacccaggtcaccgtctcctca-3'

Stat3_VHH-32 (SEQ ID NO. 39):5'-gatgtgcagctggtggagtctgggggaggctcggtgcaggctggagggtc
tctgagactctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgag
ggggtttctggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaagaa
cacgctgtatctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctctt
ggttcaaccgatatatgtataacagttggggccaggggacccaggtcaccgtctcctca-3'

Stat3_VHH-33 (SEQ ID NO. 40):5'-caggtgcagctggtggagtctgggggaggctcggtgcaggctggagggtct
ctgagactctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgagg
gggtttctggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaagaac
acgctgtatctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctctg
gttcaaccgatatatgtataacagttggggccaggggacccaggtcaccgtctcctca-3'

Stat3_VHH-36 (SEQ ID NO. 41):5'-
gatgtgcagctggtggagtctgggggaggctcggtgcaggctggagactctctga
gactctcctgtgtagcctctacatacaccggctgcatgggctggttccgccaggctcctggaaaggagcgcgagggagtcgcagctctta
gtagccgtggttttgccgggcactataccgactccgtgaagggccgattctccatctcccgagactacgtcaagaatgcggtgtatctgca
aatgaacactgtgaaacctgaggacgctgccatgtactactgtgcagcacgggagggatgggagtgcggtgagacctggttggaccgg
accgccgggggccatacctactggggccaggggaccctggtcactgtctcctca-3'

Stat3_VHH-11 (SEQ ID NO. 42):5'-gtgcagctggtggagtctgggggaggctcggtgcaggctggagggtctctgag
actctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgtgagggggttt
ctggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaagaacacgctg
tatctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctcttggttcaa
ccgatatatgtataacagttggggccaggggaccctggtcactgtctcctca-3'

Stat3_VHH-6 (SEQ ID NO. 43):5'-gtgcagctggtggagtctgagggaggctcggtgcaggctggagggtctctgaga
ctctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgagggggtttc
tggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaagaacacgctgt
atctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctcttggttcaac
cgatatatgtataacagttggggccaggggaccctggtcaccgtctcctca-3'

Stat3_VHH-1 (SEQ ID NO. 44):5'-gtgcagctggtggagtctgggggaggctcggtgcaggctggagggtctctgaga
ctctcctgtgcagcctctggagccaatggtggtcggagctgcatgggctggttccgccaggttccagggaaggagcgcgagggggtttc
tggtatttcaaccggtggtcttattacatactatgccgactccgtgaagggccgattcaccatctcccaagacaacaccaataacacgctgt
atctgcaaatgaacagcctgaaacctgaggacactgccatgtactactgtgcgacgagtcggtttgactgctatagaggctcttggttcaac
cgatatatgtataacagttggggccaggggaccctggtcactgtctcctca-3'

[0090] Additionally, the present invention comprises one or more mouse monoclonal antibodies which are directed against one or more domains of the anti-STAT3 sdAb of the invention. The mouse monoclonal antibody can be generated by methods that are known by one of skill in the art, for example, the mouse monoclonal antibody can be produced by a mouse hybridoma. The mouse monoclonal antibody can be used in diagnostic assays, for example, the antibody can be used in an immunoassay such as an ELISA in order to measure the amount of anti-STAT3 sdAb present in a patient's serum. It should be appreciated that the method is not limited to anti-STAT3 sdAbs, and could be used to produce a mouse antibody directed towards any of the sdAbs of the present invention.

[0091] The TNF-alpha gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. The cytokine is involved in the regulation of a wide spectrum of biological processes including growth regulation, differentiation, inflammation, viral replication, tumorigenesis, and autoimmune diseases; and in viral, bacterial, fungal, and parasitic infections. Besides inducing hemorrhagic necrosis of tumors, TNF was found to be involved in tumorigenesis, tumor metastasis, viral replication, septic shock, fever, inflammation, cachexia, and autoimmune diseases including Crohn's disease, and rheumatoid arthritis as well as graft-versus-host disease.

[0092] The present invention provides sdAbs, proteins, and polypeptides that are directed against TNF-alpha, in particular against human TNF-alpha inside the cell or cell membrane, so as to prevent the secretion of TNF-alpha by cells.

[0093] It is contemplated that the anti-TNF-alpha sdAbs and polypeptides of the invention can be used for the prevention and/or treatment of diseases and disorders associated with and/or mediated by TNF-alpha, such as inflammation, rheumatoid arthritis, Crohn's disease, ulcerative colitis, inflammatory bowel syndrome, multiple sclerosis, Addison's disease, autoimmune hepatitis, autoimmune parotitis, diabetes type 1, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, male infertility, multiple sclerosis, myasthenia gravis, pemphigus, psoriasis,

rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, and weight loss due to cancer and cachexia.

[0094] TNF-alpha exists in different forms; there are monomeric and multimeric forms, including a trimeric form. It is within the scope of the invention that the sdAbs, proteins and polypeptides of the invention bind to TNF-alpha in its different form, i.e., monomeric form or multimeric forms. Thus, when sdAbs, proteins and polypeptides of the invention are directed to TNF-alpha, it should be understood that this also comprises sdAbs, proteins and polypeptides directed against TNF-alpha in its trimeric form.

[0095] It is known that signal transduction by TNF involves crosslinking by TNF receptors by a trimer of TNF molecules, which contains three receptor binding sites (see, for example, Peppel *et al.*, J. Exp. Med., 174 (1991), 1483-1489).

[0096] Recombinant human TNF-alpha protein was used to generate sdAbs that are directed against or can bind to an epitope of TNF-alpha. To generate the anti-TNF-alpha sdAbs, recombinant full-length human TNF-alpha (Gene ID: 7124) was expressed in *Escherichia coli* and used as the target antigen.

[0097] Thirty-five sdAbs against the TNF-alpha protein were obtained. These anti-TNF-alpha antibodies were divided into three groups based on sequence homology.

[0098] The amino acid sequence of the first anti-TNF-alpha sdAb, named TNF-alpha VHH66 (SEQ ID NO. 45) sdAb, is shown below:

HVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGLTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRG-QGTLVTVSS

The three CDRs are underlined.

[0099] In The amino acid sequence of the second anti-TNF-alpha sdAb, named TNF-alpha VHH69 (SEQ ID NO. 46) sdAb, is shown below:

EVQLVESGGGSVLAGGSLRLSCVASGFTSRYNYMAWFRQAPGKERE
GVATIGTASGSADYYGSVKDRFTISQDNAKNTVYLQMNSLKPEDTA
MYYCAARTYGTISLTPSDYRYWGQGTLVTVSS

The three CDRs are underlined.

[0100] The amino acid sequence of the third anti-TNF-alpha sdAb, named TNF-aphha VHH62 (SEQ ID NO. 47) sdAb, is shown below:

QVQLVESGGGPVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCSWAQGTQGTLVTVSS

[0101] The three CDRs are underlined. Other anti-TNF-alpha sdAbs that were found include the sequences below, again with the CDRs underlined:

TNF_2 (SEQ ID NO.48):
QVQLVESGGGSVEAGRSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGQTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTQVTVSS

TNF_46 (SEQ ID NO. 49):
QVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGQTTHADSVKGRFTISRDNVKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTQVTVSS

TNF_71 (SEQ ID NO. 50):
QVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGLTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTQVTVSS

TNF_21 (SEQ ID NO. 51):
QVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGQTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTQVTVSS

TNF_38 (SEQ ID NO. 52):
EVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGQTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTQVTVSS

TNF_18 (SEQ ID NO. 53):
EVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGLTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTLVTVSS

TNF_37 (SEQ ID NO. 54):
DVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGQTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTLVTVSS

TNF_66 (SEQ ID NO. 55):
HVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGLTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTLVTVSS

TNF_68 (SEQ ID NO. 56):
HVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADGKERE
GVATIDIDGLATHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTLVTVSS

TNF_78 (SEQ ID NO. 57):
HVQLVESGGGSVEAGGSLRLSCAASGFRYAAYCMGWFRQADRKERE
GVATIDIDGQTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTQVTVSS

TNF_67 (SEQ ID NO. 58):
HVQLVESGGGSVQAGGSLRLSCAASGFRYAAYCMGWFRQADGKVRE
GVATIDIDGQTTHADSVKGRFTISRDNAKNTLSLQMNDLKPEDTA
MYYCAADRDRCGSIWTYAYKYRGQGTLVTVSS

TNF_6 (SEQ ID NO. 59):
QVQLVESGGGSVQAGGSLRLSCAASGFIDSFGVMAWFRQAPGKERE
GVAAVYRRAGDTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDSA
MYYCAARTYGSVSSWTGYKYWGQGTQVTVSS

TNF_7 (SEQ ID NO. 60):
DVQLVESGGGSVQAGGSLRLSCAASGFIDSFGVMAWFRQTPGKERE
GVAAVYRRAGDTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDSA
MYYCAARTYGSVSSWTGYKYWGQGTQVTVSS

TNF_13 (SEQ ID NO. 61):
DVQLVESGGGSVQVGGSLTLSCAVSGYTDSYGVMAWFRQAPGKERE
GVASIYRNSGITYYPDSVKGRFTISRDNAKNTVLLQMNSLKPEDSA
TYYCAVRSFGSVSTWAGYVYWGQGTQVTVSS

TNF_60 (SEQ ID NO. 62):
DVQLVESGGGSVQAGGSLRLSCAASGFIDSFGVMAWFRQAPGKERE
GVAAVYRRAGDTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDSA
MYYCAARTYGSVSSWTGYKYWGRGTQVTVSS

TNF_73 (SEQ ID NO. 63):
DVQLVESGGGSVRAGGSLRLSCTASGDTSKSDCMAWFRQAPGKERE
RVGAIYTRNGYTHYADSVNGRFTISQDNAKNALYLQMSGLKPEDTA
MYYCAARFRIYGQCVEDDDIDYWGQGTLVTVSS

TNF_69 (SEQ ID NO. 64):
EVQLVESGGGSVLAGGSLRLSCVASGFTSRYNYMAWFRQAPGKERE
GVATIGTASGSADYYGSVKDRFTISQDNAKNTVYLQMNSLKPEDTA
MYYCAARTYGTISLTPSDYRYWGQGTLVTVSS

TNF_76 (SEQ ID NO. 65):
QVQVVEYGGGSVQAGETVRLSCTASGFTFAEADMGWYRQAPGHEWE
LVSNITTEGITSEASSSYYADSVRGRFTIFDNAKNMVYLQMNSLKHEDTA
VYYCAPDPYAYSTYREYCTWAQGTQGTLVTVSS

TNF_62 (SEQ ID NO. 66):
QVQLVESGGGPVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCSWAQGTQGTLVTVSS

TNF_43 (SEQ ID NO. 67):
QVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTLVTVSS

TNF_15 (SEQ ID NO. 68):
QVQPVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGAQGTLVTVSS

TNF_11 (SEQ ID NO. 69):
QVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCSWAQGTQGTQVTVSS

TNF_17 (SEQ ID NO. 70):
QVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTQVTVSS

TNF_63 (SEQ ID NO. 71):
QVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTLVTVSS

TNF_20 (SEQ ID NO. 72):
HVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTQVTVSS

TNF_58 (SEQ ID NO. 73):
EVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGALVTVSS

TNF_27 (SEQ ID NO. 74):
EVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTLVTVSS

TNF_28 (SEQ ID NO. 75):
EVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCSWAQGTQGTQVTVSS

TNF_4 (SEQ ID NO. 76):
EVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTQVTVSS

TNF_14 (SEQ ID NO. 77):
DVQLVESRGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTLVTVSS

TNF_3 (SEQ ID NO. 78):
DVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHVCE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCSWAQGTQGTQVTVSS

TNF_1 (SEQ ID NO. 79):
DVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGLECE
LVSTITTEGITSEASSYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSEYCTWAQGTQGTLVTVSS

TNF_45 (SEQ ID NO. 80):
DVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSEASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTLVTVSS

TNF_22 (SEQ ID NO. 81):
DVQLVESGGGSVQAGETLRLSCTASGFTFAEADMGWYRQAPGHECE
LVSTITTEGITSVASSYYADSVRGRFTISRDNAKNMVYLQMNSLKPEDTA
VYYCAPDPYAYSTYSDYCTWAQGTQGTQVTVSS

[0102] The *in vitro* growth inhibition of several TNF-alpha sdAbs was tested, as described below. Additionally, the present invention comprises one or more mouse monoclonal antibodies which are directed against one or more domains of the anti-TNF-alpha sdAb of the invention. The mouse monoclonal antibody can be generated by methods that are known by one of skill in the art, as described above. The mouse monoclonal antibody can be used in diagnostic assays, such as, for example, an immunoassay such as an ELISA in order to measure the amount of anti-TNF-alpha sdAb present in a patient's serum.

[0103] The RAF proteins are a family of serine/threonine-specific kinases that serve as a central intermediate in transmitting extracellular signals to the mitogen-activated protein kinase cascade, which controls cell growth, differentiation and survival. BRAF is a member of the RAF family that is activated by members of the Ras family upon growth factor-induced stimulation. Active Ras can induce heterodimerization of cRaf and BRAF and this may explain the observed cooperativity of cRaf and BRaf in cells responding to growth factor signals. Activating mutations in the BRAF gene are present in a large percentage of human malignant melanomas and in a proportion of colon cancers. The vast majority of these mutations result in a valine to glutamic acid change at residue 599 within the activation segment of BRAF.

[0104] Anti-BRAF sdAbs were developed to target wild-type and mutated BRAF in order to disrupt its role in malignant cells such as, for example, cells involved in colon cancer and other malignancies.

[0105] Using methods that are well-known in the art, recombinant human BRAF protein was used to generate sdAbs that are directed against or can bind to an epitope of BRAF.

[0106] Additionally, the present invention comprises one or more mouse monoclonal antibodies which are directed against one or more domains of the anti-BRAF sdAb of the invention. The mouse monoclonal antibody can be generated by methods that are known by one of skill in the art. The mouse monoclonal antibody can be used in diagnostic assays, for example, the antibody can be used in an immunoassay such as an ELISA in order to measure the amount of anti-BRAF sdAb present in a patient's serum.

EXAMPLES

EXAMPLE 1: ANTI-STAT3 VHH13 (SEQ ID NO. 3) SDAB BINDS STAT3

[0107] In this example, the affinity of two VHH targets against STAT3 was measured using Octet based label-free binding assay. Anti-STAT3 VHH13 (SEQ ID NO:3) sdAb, anti-KRAS (negative control) and GST-STAT3 (16kDa monovalent antigen, Creative BioMart #STAT3-1476H) were used as antigen probes in this assay. The GST-STAT3 protein was captured at 20μg/ml in PBS using aminopropylsilane (APS) dip and read biosensors, specifically meant for hydrophobic protein. The probes were then dipped into wells with the GST-STAT3 protein, anti-STAT3 VHH13 (SEQ ID NO:3) sdAb or anti-KRAS at a

concentration as indicated. The association rate (on rate) of the antigen was measured. The sensors were quenched with 1% BSA in water. The probes were dipped into assay buffer (PBS) and the dissociation rate (off rate) was measured.

[0108] The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (KD) was determined from the obtained affinity constant (KA), and KD using 1:1 global fit analysis Fortebio software as shown below in Table 1. Affinity was determined by averaging KD values for curves with R2 values >0.95. The 250 nM anti-STAT3 VHH13 data point was omitted as it is an outlier. It was determined that the anti-STAT3 VHH13 (SEQ ID NO. 3) sdAb affinity was 1.16×10^{-7}. The affinity of anti-KRAS VHH was not determined.

TABLE 1

Local fit analysis, highlighted values used to determine the affinity to be 1.16×10^{-7}

Sensor Type	Sample ID	Loading Sample ID	VHH Conc. (nM)	KD (M)	kon(1/Ms)	koff(1/s)	Full R^2
APS (Aminopropylsilane)	ANTI-STAT3 VHH13	STAT3 20 µg/ml	1000	1.168E-07	3.16E+05	3.69E-02	0.985
APS (Aminopropylsilane)	ANTI-STAT3 VHH13	STAT3 20 µg/ml	500	1.012E-07	4.04E+05	4.09E-02	0.974
APS (Aminopropylsilane)	ANTI-STAT3 VHH13	STAT3 20 µg/ml	250	<1.0E-12	4.69E+01	5.11E-02	0.980
APS (Aminopropylsilane)	ANTI-STAT3 VHH13	STAT3 20 µg/ml	125	1.474E-07	3.09E+05	4.55E-02	0.991
APS (Aminopropylsilane)	ANTI-STAT3 VHH13	STAT3 20 µg/ml	62.5	9.921E-08	2.71E+05	2.69E-02	0.975
APS (Aminopropylsilane)	ANTI-STAT3 VHH13	STAT3 20 µg/ml	31.3	1.53E-06	6.75E+04	1.03E-01	0.656
APS (Aminopropylsilane)	ANTI-kras	STAT3 20 µg/ml	1000	6.75E-08	1.19E+04	8.03E-04	0.917
APS (Aminopropylsilane)	ANTI-kras	STAT3 20 µg/ml	500	2.916E-08	1.65E+04	4.80E-04	0.890
APS (Aminopropylsilane)	ANTI-kras	STAT3 20 µg/ml	250	4.324E-09	8.93E+04	3.86E-04	0.276
APS (Aminopropylsilane)	ANTI-kras	STAT3 20 µg/ml	125	NA	NA	NA	NA
APS (Aminopropylsilane)	ANTI-kras	STAT3 20 µg/ml	62.5	NA	NA	NA	NA
APS (Aminopropylsilane)	ANTI-kras	STAT3 20 µg/ml	31.3	NA	NA	NA	NA

EXAMPLE 2: IMMUNOPRECIPITATION STUDIES

[0109] The specificity of STAT3 sdAbs was assayed in human breast cancer cells. In this example, MDA-MB-231 human breast cancer cells were grown to 50% to 70% confluence. The cells were then disrupted in freshly prepared ice-cold lysis buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 0.1% NP-40, 10% glycerol, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin) for 45 minutes on ice. Lysates were then centrifuged, the supernatant collected, and protein concentration was determined using a modified Lowry method (Bio Rad, Hercules, CA). Total protein (1 mg) was incubated with 1.5 mg of Dynabeads (Invitrogen) with sdAbs

against STAT3, a positive control (STAT3, cat#SC-482, Santa Cruz Biotechnology, Dallas, TX), or negative control (STAT-1, cat# 9172, Cell Signaling, Danvers, MA) for 1 hr at 4 °C. Beads were then washed. Following the final wash, 60 µl of lysis buffer was added, and the resulting supernatant was subject to Western blot analysis. Briefly, samples were separated on 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were blocked, then incubated with appropriate primary and secondary antibodies. Anti-STAT3 antibody, used as a positive control, was from Cell Signaling (Cat# 4904, Danvers, MA). The chemiluminescence reaction was performed using the ECL system from Santa Cruz Biotechnology (Dallas, TX).

[0110] As illustrated in Figure 3, endogenous STAT3 immunoprecipitated with all sdAbs tested at varying amounts. M is the Marker lane containing the marker, lane 1 contained STAT3 VHH13 (SEQ ID NO:3) produced and isolated from mammalian cells, lane 2 contained STAT3 VHH14 (SEQ ID NO:4) produced and isolated from mammalian cells, lane 3 contained STAT3 VHH13 (SEQ ID NO:3) produced and isolated from bacterial cells, lane 4 contained STAT3 VHH14 (SEQ ID NO:4) produced and isolated from mammalian cells, lane 5 was the positive STAT3 antibody, lane 6, used STAT-1 as a negative control, showed no band.

EXAMPLE 3: ANTI-STAT3 BACTERIAL VHH13 BINDS WITH HIGH AFFINITY TO CELL LINES CONTINING CONSTITUTIVELY ACTIVED STAT3

[0111] The specificity of bacterial anti-STAT3 VHH13 (SEQ ID NO:3) using constitutively activated STAT3 in human (PANC-1 and DU145) and murine (4T1) cell lines was assayed. Commercial HeLa cells were also treated with interferon gamma (INFΓ) in order to induce phosphorylated STAT3. The PC-3 STAT3 null cell line was used as a negative control.

[0112] The cells were grown to 50% to 70% confluence, then disrupted in freshly prepared ice-cold lysis buffer as described above for 45 minutes on ice. Lysates were then centrifuged, the supernatant collected, and protein concentration was determined as described above. Total protein (1 mg) was incubated with 1.5 mg of Dynabeads (Invitrogen) containing the bacterial anti-STAT3 VHH13 (SEQ ID NO:3) or negative control (KRAS, Creative Biolabs, Shirley, NY) for 1 hour at 4°C. Beads were then washed. Following the final wash, 60 µl of lysis buffer was added, and the resulting supernatant was subject to Western Blot analysis as described in Example 2.

[0113] As illustrated in Figure 4, endogenous STAT3 was immunoprecipitated by bacterial VHH13 STAT3 (SEQ ID NO:3) in the constitutively activated STAT3 cell lines: PANC-1 (lane 1), DU145 (lane 2), and 4T1 (lane 4). Furthermore, bacterial VHH13 STAT3 (SEQ ID NO:3) bound to the Phospho-STAT3 in HeLa lysate (lane 3). No bands were noted for either PANC-1 KRAS, lane 3, and PC-3 (negative control), lane 6.

EXAMPLE 4: CYTOTOXICITY STUDIES OF ANTI-STAT3 SDABS IN MDA-MB-231 CANCER CELL LINES

[0114] In this example, the anti-proliferative effects of anti-STAT3 sdAbs were assayed using the human breast cancer cell line MDA-MB-231. For the experiments, MDA-MB-231 cells were grown until they reached a confluency of 90%. At that time, cells were washed, trypsinized and counted using a Coulter Counter (Beckman, Brea, CA). The proliferation studies were carried out using the 3-[4,5-dimethylthiaolyl]-2,5-diphenyltetrazolium bromide (MTT) assay. For this, cells were seeded in a 96-well plate at a density of 5 x 10^3 per well as indicated by the manufacturer (Roche Diagnostics Corporation, Indianapolis, IN). Cells were allowed to adhere for 24 hours and then the sdAbs were added at the appropriate concentrations (*i.e.*, 0, 0.5, 1.0, 10.0, or 100 μg/ml). Cells were counted on day 3. For the 5-day treated cells, fresh media containing the sdAbs was refreshed on day 3. At the time of termination, 10 μl of MTT reagent (0.5 mg/mL) was added to each well as indicated by the manufacturer. After a 4 hour incubation period, 100 μl of solubilization solution was added and the plate was placed in the incubator overnight. All the plates were read at 570 nm wavelength using the Biotek plate reader (Winooski, VT).

[0115] All data were analyzed using GraphPad InStat 3 (GraphPad Software, Inc., La Jolla, CA). Treatments groups were compared with vehicle control group using one-way ANOVA. If a significant difference ($p < 0.05$) was observed, the Tukey-Kramer multiple comparison test was conducted.

[0116] Based on the MTT experiment, the bacterial VHH13 anti-STAT3 (SEQ ID NO. 3) sdAb was found to be effective in inhibiting cell growth at days 3 and 5 post-treatment, as shown in Tables 2-5 below.

TABLE 2

Mean Absorbance (570nM) ± S.E. Day 3 Post Treatment with Anti-STAT3 sdAbs in MDA-MB-231 Cells

Treatment	Control	0.5µg/ml	1.0 µg/ml	10.0 µg/ml	100 µg/ml	p-value*
H.VHH13	0.444 ± 0.030	0.504 ± 0.043	0.545 ± 0.060	0.603 ± 0.025	0.272 ± 0.011	**0.001**
H.VHH14	0.404 ± 0.011	0.485 ± 0.040	0.402 ± 0.017	0.588 ± 0.020	0.416 ± 0.030	**0.002**
B.VHH13	0.550 ± 0.036	0.685 ± 0.018	0.716 ± 0.023	0.355 ± 0.033	0.059 ± 0.001	**<0.0001**
B.VHH14	0.593 ± 0.014	0.666 ± 0.022	0.644 ± 0.045	0.456 ± 0.048	0.255 ± 0.005	**<0.0001**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 3

Effects of Anti-STAT3 sdAb Treatment on MDA-MB-231 Cell Proliferation after 3 Days of Treatment

Treatment	µg/ml	% Inhibition	p-value*
H.VHH13	0.5		NS
	1.0		NS
	10.0		NS
	100.0	38.7	P< 0.05
H.VHH14	0.5		NS
	1.0	0.5	NS
	10.0		NS
	100.0		NS
B.VHH13	0.5		NS
	1.0		NS
	10.0	35.5	P< 0.001
	100.0	89.3	P< 0.001
B.VHH13	0.5		NS
	1.0		NS
	10.0	23.1	P< 0.05
	100.0	57.0	P< 0.001

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 4

Mean Absorbance (570nM) ± S.E. Day 5 Post Treatment with Anti-STAT3 sdAb in MDA-MB-231 Cells

Treatment	Control	0.5µg/ml	1.0 µg/ml	10.0 µg/ml	100 µg/ml	p-value*
H.VHH13	1.100 ± 0.088	0.955 ± 0.013	0.963 ± 0.018	0.832 ± 0.028	0.721 ± 0.025	**0.0012**
H.VHH13	0.983 ± 0.023	0.890 ± 0.021	0.935 ± 0.037	0.804 ± 0.015	0.797 ± 0.010	**0.0007**
B.VHH13	0.804 ± 0.046	0.761 ± 0.055	0.653 ± 0.024	0.506 ± 0.030	0.083 ± 0.005	**<0.0001**
B.VHH14	0.677 ± 0.015	0.733 ± 0.038	0.794 ± 0.023	0.640 ± 0.011	0.549 ± 0.023	**<0.0001**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 5

Effects of Anti-STAT3 sdAb Treatment on MDA-MB-231 Cell Proliferation After 5 Days of Treatment

Treatment	µg/ml	% Inhibition	p-value*
H.VHH13	0.5	13.2	NS
	1.0	12.5	NS
	10.0	24.4	P< 0.01
	100.0	34.5	P< 0.001
H.VHH14	0.5	9.5	NS
	1.0	4.9	NS
	10.0	18.2	P< 0.001
	100.0	18.2	P< 0.001
B.VHH13	0.5	5.4	NS
	1.0	18.8	NS
	10.0	37.1	P< 0.001
	100.0	89.7	P< 0.001
B.VHH14	0.5	0	NS
	1.0	0	NS
	10.0	5.5	NS
	100.0	18.2	P< 0.01

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

EXAMPLE 5: CYTOTOXICITY STUDIES OF ANTI-STAT3 SDABS IN HUMAN BREAST (MDA-MB-231) AND PANCREATIC (PANC-1) CANCER CELL LINES

[0117] In this Example, the anti-proliferative effects of anti-STAT3 VHH13 (SEQ ID NO. 3) and the VHH14 (SEQ ID NO. 4) sdAbs were assayed using the human breast cancer cell line MDA-MB-231 and the human pancreatic cancer cell line PANC-1. For the experiments, MDA-MB-231 and PANC-1 cells were grown until they were 90% confluent. At that time, cells were washed, trypsinized and counted using a Coulter Counter (Beckman, Brea, CA). The proliferation studies were carried out using the MTT assay described above. For the 5-day treated cells, fresh media containing the anti-STAT3 sdAbs was refreshed on day 3.

[0118] All data were analyzed using GraphPad InStat 3. Treatments groups were compared with vehicle control group using one-way ANOVA. If a significant difference ($p < 0.05$) was observed, the Tukey-Kramer multiple comparison test was conducted.

[0119] Based on the MTT experiment, both the VHH13 (SEQ ID NO. 3) and the VHH14 (SEQ ID NO. 4) were found to inhibit cell growth in both the MDA-MB-231 and PANC-1 cancer cells, as shown in Tables 6-13 below.

TABLE 6

Mean Absorbance (570nM) ± S.E. Day 3 Post Treatment With sdAbs in the MDA-MB-231 Cells

Treatment	Experiment	Control	10.0 µg/ml	100 µg/ml	p-value*
B.VHH13	1	0.550 ± 0.036	0.355 ± 0.033	0.059 ± 0.001	<0.0001
	1	0.735 ± 0.092	0.489 ± 0.019	0.449 ± 0.054	0.0355
	3	0.627 ± 0.033	0.432 ± 0.060	0.078 ± 0.001	0.0002
	4	0.648 ± 0.090	0.576 ± 0.061	0.063 ± 0.002	0.0011
Overall Mean		**0.640 ± 0.038**	**0.463 ± 0.047**	**0.163 ± 0.10**	**0.0019**
B.VHH14	1	0.593 ± 0.014	0.456 ± 0.048	0.255 ± 0.005	0.0002
	3	0.624 ± 0.046	0.499 ± 0.018	0.357 ± 0.019	0.0025
	3	0.816 ± 0.088	0.502 ± 0.048	0.308 ± 0.021	0.0026
	4	0.729 ± 0.051	0.559 ± 0.041	0.287 ±0.021	0.0007
Overall Mean		**0.691 ± 0.051**	**0.504 ± 0.021**	**0.302 ± 0.043**	**<0.0001**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 7

Mean Absorbance (570nM) ± S.E. Day 5 Post Treatment with Anti-STAT3 sdAbs in MDA-MB-231 Cells

Treatment	Experiment	Control	10.0 µg/ml	100 µg/ml	p-value*
B.VHH13	1	0.804 ± 0.046	0.506 ± 0.030	0.083 ± 0.005	<0.0001
	1	0.561 ± 0.024	0.417 ± 0.011	0.266 ± 0.015	<0.0001
	3	0.970 ± 0.048	0.814 ± 0.052	0.105 ± 0.005	<0.0001
	1	0.757 ± 0.118	0.665 ± 0.036	0.083 ± 0.005	0.011
Overall Mean		**0.773 ± 0.084**	**0.601 ± 0.088**	**0.135 ± 0.044**	**0.0005**
B.VHH14	3	0.677 ± 0.015	0.640 ± 0.011	0.549 ± 0.023	0.0047
	1	0.456 ± 0.037	0.338 ± 0.023	0.274 ± 0.032	0.0166
	3	0.983 ± 0.019	0.930 ± 0.044	0.578 ± 0.039	0.0004
	1	1.092 ± 0.053	0.842 ± 0.052	0.499 ± 0.036	0.0004
Overall Mean		**0.802 ± 0.145**	**0.688 ± 0.131**	**0.475 ± 0.0690**	**0.2022**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 8

Mean Absorbance (570nM) ± S.E. Day 3 Post Treatment with Anti-STAT3 sdAbs in the PANC-1 Cells

Treatment	Experiment	Control	10.0 µg/ml	100 µg/ml	p-value*
B.VHH13	1	0.756 ± 0.045	0.432 ±0.015	0.307 ±0.012	<0.0001
	2	1.347 ± 0.189	0.491 ± 0.087	0.169 ± 0.094	0.0019
	2	1.025 ± 0.056	0.493 ± 0.029	0.166 ± 0.028	<0.0001
Overall Mean		**1.043 ± 0.171**	**0.472 ± 0.020**	**0.214 ± 0.047**	**0.0034**
H.VHH13	1	1.541 ± 0.097	1.066 ± 0.153	0.732 ± 0.015	0.0046
	2	1.611 ± 0.119	1.353 ± 0.119	0.762 ± 0.654	0.3527
	1	1.074 ± 0.040	0.897 ± 0.154	0.700 ± 0.082	0.1092
Overall Mean		**1.409 ± 0.169**	**1.105 ± 0.133**	**0.731 ± 0.181**	**0.0238**
H.VHH14	1	1.195 ± 0.205	0.920 ± 0.133	0.808 ± 0.239	0.4161
	1	1.423 ± 0.038	1.183 ± 0.114	0.993 ± 0.088	0.0338
	1	1.293 ± 0.169	1.163 ± 0.044	0.916 ± 0.088	0.1330
Overall Mean		**1.304 ± 0.066**	**1.089 ± 0.085**	**0.906 ± 0.054**	**0.0188**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 9

Mean Absorbance (570nM) ± S.E. Day 5 Post Treatment with Anti-STAT3 sdAbs in PANC-1 Cells

Treatment	Experiment	Control	10.0 µg/ml	100 µg/ml	p-value*
B.VHH13	1	0.687 ± 0.047	0.433 ± 0.036	0.243 ± 0.024	0.0004
	1	1.670 ± 0.196	0.869 ± 0.053	0.211 ± 0.006	0.0004
	3	1.389 ± 0.044	0.627 ± 0.073	0.203 ± 0.013	<0.0001
Overall Mean		**1.249 ± 0.292**	**0.643 ± 0.126**	**0.219 ± 0.012**	**0.0208**
H.VHH13	1	1.462 ± 0.150	1.128 ± 0.105	0.839 ± 0.117	0.0349
	3	1.792 ± 0.202	1.341 ± 0.095	0.911 ± 0.079	0.0113
	1	1.605 ± 0.289	1.161 ± 0.140	0.820 ± 0.005	0.0638
Overall Mean		**1.620 ± 0.096**	**1.210 ± 0.066**	**0.857 ± 0.028**	**0.0007**
H.VHH13	1	1.992 ± 0.105	1.859 ± 0.033	0.095 ± 0.003	<0.0001
	3	1.517 ± 0.050	1.165 ± 0.015	1.169 ±0.050	0.0015
	1	1.579 ± 0.134	1.081 ± 0.103	0.998 ± 0.049	0.0136
Overall Mean		**1.696 ± 0.149**	**1.368 ± 0.247**	**0.754 ± 0.333**	**0.0967**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 10

Mean Growth Inhibition Post 3 Days of Anti-STAT3 sdAbs Treatment on MDA-MB-231 Cell Proliferation

Treatment	Experiment	P-value[a]	10.0 µg/ml	P-value[b]	100 µg/ml	P-value[b]
B.VHH13	1	P< 0.0001	35.5	P< 0.001	89.3	P< 0.001
	2	P= 0.03	33.5	ns	38.9	P< 0.05
	3	P= 0.0001	31.1	P< 0.05	87.6	P< 0.001
	4	P= 0.0001	11.1	ns	90.3	P< 0.01
Overall Average % Inhibition			**27.8**		**76.5**	
B.VHH14	1	P< 0.001	23.1	P< 0.05	57.0	P< 0.001
	4	P= 0.03	20.0	ns	42.8	P< 0.01
	4	P= 0.03	38.5	P< 0.05	62.3	P< 0.01
	3	P= 0.006	23.3	ns	60.6	P< 0.001
Overall Average % Inhibition			**26.2**		**55.7**	

a. One-way Analysis of Variance (ANOVA); b. Post test=Tukey-Kramer Multiple Comparisons Test

TABLE 11

Mean Growth Inhibition Post 5 days of Anti-STAT3 sdAbs Treatment on MDA-MB-231 Cell Proliferation

Treatment	Experiment	P-value[a]	10.0 µg/ml	P-value[b]	100 µg/ml	P-value[b]
B.VHH13	1	P< 0.0001	37.1	P< 0.001	89.7	P< 0.001
	2	P< 0.0001	25.7	P< 0.001	52.6	P< 0.001
	1	P< 0.0001	16.1	ns	89.7	P< 0.001
	1	P= 0.001	12.2	ns	88.5	P< 0.01
Overall Average % Inhibition			**22.8**		**80.0**	
	2					
B.VHH14	2	P< 0.0001	5.5	ns	18.9	P< 0.05
	2	P= 0.02	25.7	ns	39.9	P< 0.05
	3	P= 0.0004	5.4	ns	41.2	P< 0.001
	2	P= 0.0004	22.9	P< 0.05	54.3	P< 0.001
Overall Average % Inhibition			**14.9**		**38.6**	

a. One-way Analysis of Variance (ANOVA); b. Post test=Tukey-Kramer Multiple Comparisons Test

TABLE 12

Mean Growth Inhibition Post 3 Days of Anti-STAT3 sdAbs Treatment on PANC-1 Cell Proliferation

Treatment	Experiment	P-value[a]	10.0 µg/ml	P-value[b]	100 µg/ml	P-value[b]
B.VHH13	1	P< 0.0001	42.9	P< 0.001	59.4	P< 0.001
	2	P= 0.03	63.5	P< 0.05	87.5	P< 0.01
	3	P< 0.0001	51.9	P< 0.001	83.8	P< 0.001
Overall Average % Inhibition			**52.8**		**76.9**	
	3	3				
H.VHH13	1	P= 0.005	30.8	P< 0.05	52.5	P< 0.01
	1	P= 0.002	16.0	ns	52.7	P< 0.01
	1	P= 0.11	16.5	ns	34.8	ns
Overall Average % Inhibition			**21.1**		**76.9**	
	1	1				
H.VHH14	3	P= 0.42	23.0	ns	32.4	ns
	1	P= 0.03	16.9	ns	30.2	P< 0.05
	3	P= 0.13	10.1	ns	29.2	ns
Overall Average % Inhibition			**21.1**		**76.9**	

a. One-way Analysis of Variance (ANOVA); b. Post test = Tukey-Kramer Multiple Comparisons Test

TABLE 13

Mean Growth Inhibition Post 5 Days of Anti-STAT3 sdAbs Treatment on PANC-1 Cell Proliferation

Treatment	Experiment	P-value[a]	10.0 µg/ml	P-value[b]	100 µg/ml	P-value[b]
B.VHH13	1	P= 0.0004	37.0	P< 0.01	64.6	P< 0.001
		P= 0.0004	48.0	P< 0.01	87.4	P< 0.001
		P< 0.0001	54.9	P< 0.001	85.4	P< 0.001
Overall Average % Inhibition			**46.6**		**79.1**	
H.VHH13		P= 0.03	22.8	ns	42.6	P< 0.05
		P= 0.01	25.2	ns	49.2	P< 0.01
1	1	P= 0.06	27.7	ns	48.9	ns
Overall Average % Inhibition			**25.2**		**46.9**	
H.VHH13		P= 0.08	26.8	ns	14.8	ns
		P= 0.002	25.2	P< 0.01	22.9	P< 0.01
		P= 0.02	31.5	P< 0.05	36.8	P< 0.05
Overall Average % Inhibition			**27.2**		**24.8**	

a. One-way Analysis of Variance (ANOVA); b. Post test = Tukey-Kramer Multiple Comparisons Test

EXAMPLE 6: ANTI-PROLIFERATIVE ACTIONS OF STAT3 SDABS IN THE HUMAN BREAST CANCER AND HUMAN PROSTATE CANCER CELL LINES

[0120] The anti-proliferative effects of the STAT3 VHH13 (SEQ ID NO. 3) sdAb were assayed in the human breast cancer cell line MDA-MB-231 and the human prostate cancer cell lines DU145. For the experiments, cancer cells were grown until they reached 90% confluence. At that time, cells were washed, trypsinized, and counted using a Coulter Counter (Beckman, Brea, CA). The proliferation studies done using the MTT assay as described above.

[0121] The anti-proliferative properties of anti-STAT3 bacterial VHH13 (SEQ ID NO. 3) sdAb on MDA-MB-231 cells were compared to its actions on DU145 cells. As shown in Table 14, MDA-MB-231 cells treated with the anti-STAT3 (SEQ ID NO:3) sdAbs showed an average growth inhibition of 29.6 and 91.2 at 50.0 and 100 µg/ml, respectively. In the DU145 cells, a similar growth inhibition (31.2 and 92.1% for 50.0 and 100 µg/ml, respectively) was seen as set forth in Table 15.

TABLE 14

Anti-proliferative Actions of Anti-STAT3 Bacterial VHH13 sdAbs on MDA-MB-231 Breast Cancer Cells

	Experiment 1 Absorbance (% Inhibition)	Experiment 2 Absorbance (% Inhibition)	Experiment 2 Absorbance (% Inhibition)	Average Absorbance (% Inhibition)	p-value*
control	0.93	1.25	1.46	1.21	
50 μg	0.82 (12.0)	0.99 (20.5)	0.64 (56.2)	0.82 (32.6)	NS
100 μg	0.07 (93.1)	0.12 (90.1)	0.14 (90.5)	0.11 (91.0)	**<0.001**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

TABLE 15

Anti-proliferative Actions of Anti-STAT3 Bacterial VHH13 sdAbs on DU145 Prostate Cancer Cells

	Experiment 1 Absorbance (% Inhibition)	Experiment 2 Absorbance (% Inhibition)	Experiment 3 Absorbance (% Inhibition)	Average Absorbance (% Inhibition)	p-value*
control	1.05	1.58	1.61	1.41	
50 μg	0.68 (35.7)	1.2 (55.5)	1.03 (35.8)	0.98 (30.5)	NS
100 μg	0.13 (87.4)	0.12 (95.7)	0.06 (96.1)	0.10 (92.7)	**<0.001**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

EXAMPLE 7: ANTI-PROLIFERATIVE EFFECTS OF STAT3 VHH13 (SEQ ID NO. 3) SDABS ON HUMAN CANCER CELL LINES

[0122] To test the anti-proliferative effects of the STAT3 VHH13 (SEQ ID NO. 3) sdAbs using the human cancer cell lines: MDA-MB-231, MDA-MB-468, MCF-7, BT474, and DU145 as shown in Table 16.

[0123] All human cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). Cell lines were maintained and cultured in RPMI 1640 media (MDA-MB-231, MDA-MB-468, MCF-7, BT474) or MEM-E (DU145) containing 10% fetal bovine serum, 2 mM L-glutamine and 1% antibiotic-antimycotic solution (10 units/mL penicillin, 10 μg/mL streptomycin and 25 μg/mL amphotericin B). Cells were kept at 37°C in a humidified atmosphere of 5% CO_2. Cell culture supplies were obtained from Life Technologies, Inc., (Grand Island, NY). The MTT reagent was purchased from Sigma Aldrich (St. Louis, MO).

[0124] For the experiments, cancer cells were grown until they reached 90% confluency. At that time, cells were washed, trypsinized and counted using a Coulter Counter (Beckman, Brea, CA). The proliferation studies were carried out using the MTT assay as described above.

[0125] The anti-proliferative properties of Anti-STAT3 Bacterial VHH13 (SEQ ID NO:3) sdAbs were evaluated on five breast cancer cells of representing various classifications (Table 34). As shown in Table 17, all cell lines at 72 hours post treatment showed significant growth inhibition. The greatest growth inhibition was noted at 100 and 200 μg/ml dose for all cell lines. The half maximal inhibitory concentration (IC_{50}) for growth in the cell lines tested were: 10.1 ± 2.4, 12.36 ± 1.5, 14.8 ± 1.6, and 25.2 ± 14.7 for the MDA-MB-231, MDA-MB-468, MCF-7, and BT474 cell lines, respectively. These data suggest that the triple negative breast cancer cell lines require the lowest concentration of VHH13 (SEQ ID NO:3) sdAbs to achieve the IC_{50} as compared to estrogen/progesterone positive cell lines (i.e., MCF-7) or HER2 amplified cell lines (*i.e.*, BT474).

TABLE 16

Breast Cancer Cell Line Characteristics

Cell line	Disease	Immunoprofile	Classification
MDA-MB-231	adenocarcinoma	ER^-, PR^-, $HER2^-$	Basal; Claudin-low
MDA-MB-468	adenocarcinoma	ER^-, PR^-, $Her2^-$	Basal
MDA-MB-453	metastatic carcinoma	ER, PR, $HER2^-$	Unclassified
BT474	ductal carcinoma	Her2 amplified	Luminal B
MCF-7	adenocarcinoma	ER^+, PR^+, $HER2^+$	Luminal A

TABLE 17

Inhibition of Breast Cancer Cell Lines by Anti-STAT3 VHH13 (SEQ ID NO. 3) sdAbs

Cell Line	Treatment (μg/ml)	Mean Abs	% Inhibition	p-value
BT474	0	0.634		
	0.39	0.322	49.3	P< 0.001
	0.78	0.462	27.2	P< 0.001
	1.56	0.502	20.8	P< 0.01
	3.13	0.446	29.7	P< 0.001
	6.25	0.469	26.1	P< 0.001
	12.5	0.363	42.7	P< 0.001
	25	0.256	59.6	P< 0.001
	50	0.145	77.2	P< 0.001
	100	0.046	92.8	P< 0.001
	200	0.040	93.8	P< 0.001
MCF-7	0	0.590		
	0.39	0.818	0	
	0.78	0.785		
	1.56	0.823	0	
	3.13	0.689	0	
	6.25	0.435	22.1	NS
	12.5	0.327	41.6	P< 0.01
	25	0.212	62.1	P< 0.001
	50	0.057	89.9	P< 0.001

Cell Line	Treatment (µg/ml)	Mean Abs	% Inhibition	p-value
	100	0.038	93.2	P< 0.001
	200	0.040	92.9	P< 0.001
MDA-MB-468	0	0.253		
	0.39	0.311	0	
	0.78	0.289	0	
	1.56	0.201	20.6	
	3.13	0.223	11.9	
	6.25	0.230	9.1	
	12.5	0.130	48.6	P< 0.001
	25	0.067	73.5	P< 0.001
	50	0.042	83.4	P< 0.001
	100	0.038	85.0	P< 0.001
	200	0.040	84.4	P< 0.001
MDA-MB-231	0	0.502		
	0.39	0.603		
	0.78	0.576	0	
	1.56	0.570		
	3.13	0.445	11.4	P< 0.001
	6.25	0.312	37.8	P< 0.001
	12.5	0.224	55.4	P< 0.001
	25	0.196	60.9	P< 0.001
	50	0.130	74.2	P< 0.001
	100	0.041	91.8	P< 0.001
	100	0.042	91.7	P< 0.001

[0126] The actions of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was also evaluated in the human prostate cancer cell line DU145, as shown in Table 18. The anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb showed dose-dependent growth inhibition in all cancer cells tested.

TABLE 18

Effect of Anti-STAT3 VHH13 sdAbs on Prostate Cancer Cell Lines

	Treatment (mg/ml)	Mean Abs	% Inhibition	p-value
DU145	0	0.771		
DU145	0.39	0.906	0	
DU145	0.78	1.023	0	
DU145	1.56	0.967	0	
DU145	3.13	0.783	0	
DU145	6.25	0.770	0	
DU145	12.5	0.560	27.4	P< 0.05
DU145	25	0.359	53.5	P< 0.001
DU145	50	0.161	79.1	P< 0.001
DU145	100	0.039	95.0	P< 0.001
DU145	200	0.039	95.0	P< 0.001

EXAMPLE 8: MAXIMUM TOLERATED DOSE OF ANTI-STAT3 BACTERIAL VHH13 (SEQ ID NO:3) IN BALB/C MICE

[0127] In this Example, the tolerance of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was assayed in test animals using the human breast cancer cell line MDA-MB-231. For the experiment, a total of 9 BALB/C nude female mice (6 to 7 weeks old) were divided into three groups according to body weights. (Table 19) Mice (n=3) received either vehicle (PBS) or anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb at 250 or 500 µg/kg body weight/day for five days. During the study, mortality/morbidity was performed twice daily. Body weights were recorded on days 1, 4, and 6 of the study as well as on the day of study termination (Day 13). Toxicity was assessed by body weight measurements and mouse behavior compared to vehicle control mice. Upon completion of treatment phase, animals were followed for an additional week to note any abnormalities in body weights and/or general health post treatment.

TABLE 19

Experimental Design of Maximum Tolerated Dose Study

Group	# Mice	Treatment	Dose	Route	Frequency
1	3	PBS Vehicle	---	IP	5 days
2	3	Bacterial VHH13	250 µg/kg b.w.	IP	5 days
2	3	Bacterial VHH13	500 µg/kg b.w.	IP	5 days

[0128] As illustrated in Table 20, there was no significant difference in body weights among the groups, and anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was not associated with any drug-related deaths at either dosing level. Additionally, no behavior changes were observed in the animals treated with anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb as compared to the control mice.

TABLE 20

Mean body weights ± S.E

Group	Randomization	Day 1	Day 4	Day 6	Day 13
Vehicle	17.1 ± 0.06	17.1 ± 0.07	17.8 ± 0.12	18.1 ± 0.09	18.8 ± 0.20
250 µg/kg	17.1 ± 0.06	17.2 ± 0.03	17.2 ± 0.15	17.5 ± 0.15	18.1 ± 0.21
500 µg/kg	17.1 ± 0.17	17.1 ± 0.09	17.8 ± 0.18	18.0 ± 0.20	18.5 ± 0.18
p-value*	**> 0.9999**	**0.52**	**0.05**	**0.07**	**0.11**

*One Way Analysis of Variance (ANOVA); Tukey-Kramer Multiple Comparison Test

EXAMPLE 9: ACTIVITY OF BACTERIAL ANTI-STAT3 VHH13 (SEQ ID NO:3) IN NUDE BALB/C MICE XENOGRAFT AND HUMAN BREAST CANCER AND HUMAN PANCREATIC CANCER CELLS

[0129] In this example, the activity of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was evaluated in mice using the human breast cancer cell line MDA-MB-231. Briefly, the activity of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was evaluated using: the MDA-MB-231 human breast cancer xenograft model and the PANC-1 human pancreatic cancer xenograft model. Dosing schedules were as follows: Group 1 (n=6; PBS; IP) daily for 14 days [QDx14]; and Group 2 (n-12; 500 μg/kg bw; IP), every day for 14 days [QDx14]. An observation period of 5 days followed the drug administration.

[0130] The human breast cancer cell lines (MDA-MB-231 and PANC-1) were obtained from American Type Culture Collection (ATCC) (Manassas, VA). The MDA-MB-231 cells were growth in MEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and Penicillin-Streptomycin-Glutamine (Life Technologies, Grand Island, NY). The PANC-1 cells were grown in RPMI 1640 media (Life Technologies, Grand Island, NY) supplemented with 10% FBS and Penicillin-Streptomycin-Glutamine. All cells were grown in the presence of 5% CO_2 at 37°C in an incubator.

[0131] Athymic nude-Foxn1^{nu} male mice aged 4 to 5 weeks were purchased from Harlan Laboratories (Indianapolis, IN). Animals were quarantined for one week and housed five mice per cage, with a 12-hr light-dark cycle, and a relative humidity of 50%. Drinking water and diet were supplied to the animals *ad libitum*. All animals were housed under pathogen-free conditions and experiments were performed in accordance with the IIT Research Institute Animal Use and Care Committee. For the MDA-MB-231 xenograft study, cells (4 x 10^6) in a 100-μL final volume of MEM media were injected subcutaneously into right flanks of mice. For the PANC-1 xenograft study, cells (5 x 10^6) in a 100-μL final volume of RPMI media were injected subcutaneously into right flanks of mice. Tumor measurements for both models were initiated as soon as the tumors were palpable. Thereafter, tumors were measured twice weekly. Animals were randomized when tumors reach a range size of 75 to 175 mm^3, control (n = 6) and a treatment (n=12) groups were randomized using the stratified random sampling algorithm. Treatment (anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb) or Vehicle (PBS) was initiated the day following randomization. The treatment was well tolerated and associated with no drug-related deaths. No significant body weight loss was noted.

[0132] For the MDA-MB-231 xenograft study, the randomization Mean (±SE) tumor size was: 103.01 ± 11.89 and 102. 61 ± 9.60 for control and treatment groups respectively. Mean body weights (± SE) at randomization were: 32.08 ± 0.76 and 30.27 + 0.75 for Group 1 and Group 2, respectively. Table 21 shows the mean body weights (± SE) for the entire study.

TABLE 21

Mean body weights ±S.E.

Treatment	Day 1	Day 6	Day 9	Day 12	Day 16	Day 20
Vehicle	31.0 ± 0.83	32.1 ± 0.76	31.9 ± 0.66	32.1 ± 0.68	32.0 ± 0.71	32.5 ± 0.88
Anti-STAT3 VHH13	29.2 ± 0.71	30.3 ± 0.75	30.4 ± 0.79	29.9 ± 0.72	30.6 ± 0.74	30.6 ± 0.77
p-value*	**0.16**	**0.16**	**0.27**	**.09**	**0.28**	**0.17**

* Two-tail T-Test

[0133] On day 14 of dosing, the mean tumor size (±SE) for the control was 179.11 ± 19.39 versus 118.86 ± 15.94 for treatment group. Mean body weights (± SE) at termination were: 31.98 ± 0.71 and 30.55 ± 0.74 for Group 1 and Group 2, respectively. Table 22 summarizes the tumor volumes (± SE) for entire study. The % mean tumor growth inhibition in the treatment group was 33.64%. The tumor doubling times were as follows: Group 1: 44.27 days; and Group 2: 61.06 days. Figure 5 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the MDA-MB-231 xenograft model. Anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb showed significant growth inhibition (p= 0.047). Thus, anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb has chemotherapeutic activity in the MDA-MB-231 human breast cancer model system.

TABLE 22

Individual Tumor Measurements (mm^3) for the MDA-MB-231 Xenograft Model

Group	Animal #	Day 1	Day 6	Day 9	Day 12	Day 16	Day 20
1	1	117.43	141.72	135.00	139.31	127.93	133.19
	2	130.30	142.83	206.15	256.99	244.06	243.00
	3	78.00	105.97	114.04	144.06	154.50	158.94
	4	118.24	162.41	171.39	225.59	181.32	217.97
	5	71.10	109.03	133.13	168.80	187.73	164.45
Mean		**103.01**	**132.39**	**151.94**	**186.95**	**179.11**	**183.51**
S.E.		**11.89**	**10.82**	**16.42**	**23.28**	**19.39**	**20.28**
2	6	123.94	114.91	129.22	176.04	170.09	162.98
	7	85.93	101.06	112.60	112.24	139.56	96.43
	8	147.34	148.72	169.69	185.08	170.07	256.71
	9	115.91	103.64	108.37	141.21	144.51	119.42
	10	73.23	82.59	110.13	91.22	166.77	285.88
	11	163.73	178.23	183.79	165.52	214.28	129.51
	12	75.54	83.94	103.68	119.88	104.26	99.48

Group	Animal #	Day 1	Day 6	Day 1	Day 12	Day 16	Day 20
	13	70.04	89.24	102.60	75.25	57.65	95.23
	14	101.62	65.09	82.02	68.01	61.41	61.83
	15	67.83	62.21	59.00	77.04	65.49	82.73
	16	131.93	75.28	76.21	53.55	73.66	51.61
	17	74.28	109.06	111.92	89.94	58.56	100.07
Mean		**102.61**	**101.16**	**112.44**	**112.92**	**118.86**	**128.49**
S.E.		**9.6**	**9.8**	**10.3**	**12.9**	**15.9**	**21.1**
P-value		**0.98**	**0.08**	**0.06**	**0.01**	**0.05**	**0.14**

[0134] For the PANC-1 xenograft study, the randomization Mean (+SE) tumor sizes were 107.01 ± 4.54 in the control and 110. 58 ± 6.18 in the treatment groups. Mean body weights (± SE) at randomization were: 29.0 ± 0.81 and 28.5 ± 0.70 for Group 1 and Group 2, respectively. Mean body weights (± SE) at termination were: 31.2 ± 0.99 and 30.1 ± 0.75 for Group 1 and Group 2, respectively. Table 23 summarizes the mean body weights (± SE) for entire study. On day 14 of dosing, the mean tumor size (±SE) for control was 287.30 ± 33.94 versus 318.74 + 29.76 for treatment group. Table 24 summarizes the tumor volumes (± SE) for entire study.

TABLE 23

Mean body weights ±S.E.

Treatment	2/19	2/24	2/27	3/2	3/6	3/10
Vehicle Control	31.0 ± 0.83	32.1 ± 0.76	31.9 ± 0.66	32.1 ± 0.68	32.0 ± 0.71	32.5 ± 0.88
Anti-STAT3	29.2 ± 0.71	30.3 ± 0.75	30.4 ± 0.79	29.9 ± 0.72	30.6 ± 0.74	30.6 ± 0.77

[0135] The tumor doubling times were as follows: Group 1: 22.44 days; and Group 23.02 days. Anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb showed no significant growth inhibition in the PANC-1 human pancreatic cancer model system.

TABLE 24

Individual Tumor Measurements (mm^3) for the PANC-1 xenograft Model

Group	Animal #	2/19	2/24	2/27	3/2	3/6	2/19
1	1	99.77	117.96	134.67	161.27	160.79	195.58
	2	117.54	137.14	221.14	241.27	303.70	321.45
	3	120.30	210.99	276.05	322.17	394.96	732.07
	4	111.65	135.91	215.87	340.97	334.08	382.06
	5	90.88	96.35	165.26	156.28	223.17	314.97
	6	107.05	156.56	192.98	324.34	307.13	573.99
Mean		**107.87**	**142.49**	**201.00**	**257.72**	**287.30**	**420.02**
S.E.		**11.11**	**16.01**	**20.00**	**34.35**	**33.94**	**80.34**
2	7	96.31	193.71	275.06	317.53	395.37	540.66
	8	89.24	90.03	112.43	125.51	189.63	235.08
	9	80.62	148.97	196.38	187.24	299.84	530.46
	10	108.03	144.14	234.46	240.39	288.75	421.61
	11	77.66	116.21	313.19	290.38	411.66	197.67
	12	129.68	143.20	290.67	224.92	261.44	343.04
	13	108.99	182.30	239.00	254.64	342.19	464.00
	14	123.27	171.03	223.34	226.88	248.69	324.30
	15	144.53	136.03	198.47	226.04	247.97	273.58
	16	120.96	136.48	226.43	338.06	564.71	883.81
	17	112.69	144.76	167.12	225.70	223.06	326.19
	18	134.95	189.64	193.14	248.01	351.63	364.44
Mean		**110.58**	**149.71**	**222.47**	**242.11**	**318.74**	**408.74**
S.E.		**6.18**	**8.79**	**15.90**	**16.30**	**29.76**	**53.25**
P-value		**0.78**	**0.67**	**0.43**	**0.64**	**0.53**	**0.91**

EXAMPLE 10: MDA-MB-231 XENOGRAFT STUDY

[0136] In this Example, the efficacy of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the MDA-MB-231 human breast xenograft model was further evaluated. The dosing schedules were as follows: Group 1 (n=4; PBS; IP) twice a day for 14 days [BIDx14]; Group 2 (n=4; 1 mg/kg bw; IP), twice a day for 14 days [BIDx14]; Group 3 (n=4; 2 mg/kg bw; IP) twice a day for 14 days [BIDx14]; and Group 4 (n=4; 2 mg/kg bw; IP) once a day for 14 days [QDx14]. An observation period of 7 days followed administration.

[0137] The human breast cancer cell lines MDA-MB-231 and athymic nude-Foxn1^{nu} female mice were described above.

[0138] MDA-MB-231 cells at a density of 5×10^6 were injected subcutaneously into the right flank of the mice at a final volume of 100-µL in MEM media. Tumor measurements were initiated as soon as the tumors were palpable. Thereafter, tumors were measured twice weekly. Animals are randomized when tumors reach a range size of 55 to 150 mm^3 using the stratified

random sampling algorithm. Treatment (anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb) or Vehicle (PBS) was initiated the day following randomization.

[0139] The randomization Mean (±SE) tumor size was: 92.08 ± 13.24, 82.38 ± 5.17, 77.47 ± 7.17, and 104.71 ± 14.64 for Groups 1, 2, 3, and 4 respectively. As shown in Table 25, mean body weights (± SE) at randomization were: 23.65 ± 0.72, 23.45 ± 0.66, 23.10 ± 0.20, and 22.45 ± 1.25 for Groups 1, 2, 3, and 4, respectively.

[0140] As shown in Table 26, at day 14 of dosing, the mean tumor size (±SE) for control group was 221.51 ± 57.32 versus 67.12 ± 10.66, 58.27 ± 22.54, and 131.44 ± 22.86, for treatment group 2, 3, and 4, respectively. At the time of termination (day 42) mean tumor size (± S.E.) was: 255.42 ± 65.46, 55.98 ± 6.94, 41.15 ± 13.21, and 145.51 ± 52.32, for groups 1, 2, 3, and 4, respectively. Mean body weights (± SE) at termination were: 24.80 ± 0.49, 23.25 ± 1.20, 24.00 ± 0.32, and 23.2 ± 1.46 for Groups 1, 2, 3, and 4, respectively. The max mean % net weight loss (day) was: 0.7 (36), 1.5 (23), 1.8 (36), and 2.2 (29) for Groups 1, 2, 3, and 4, respectively.

[0141] Also as shown in Table 26, the mean growth inhibition in the treatment groups was 78.3, 75.2, and 55.9, for Groups 2, 3, and 4, respectively. The tumor doubling times were: Group 1: 20.56 days; Group 2: 34.54 days; Group 3: 30.07 days; and Group 4: 27.17 days. There was a growth delay of 13.99, 9.52, and 6.61 days for Groups 2, 3 and 4, respectively. The % treatment/control values for treatment groups were: Group 2: -33.75 (tumor stasis); Group 3: -54.4 (tumor regression); and Group 4: 10.28 (tumor inhibition). Figure 6 illustrates the growth inhibition of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the MDA-MB-231 xenograft model.

[0142] Administration of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was associated with a significant growth inhibition in Group 2 (p=0.02) [1 mg/kg; BID X 14] and Group 3 (p= 0.02) [2 mg/kg; BID x 14]. Furthermore, three out of four tumors showed significant regression. Based on these data, it is concluded that anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb has chemotherapeutic activity in the MDA-MB-231 human breast cancer model system.

TABLE 25

Mean Body Weights ± S.E.

		Date/Study Day						
		Dosing					Recovery	
		6/23	6/26	6/29	7/2	7/6	7/9	7/15
Group	Schedule	20	23	26	29	33	26	42
1	PBS; BID x14	23.65 ± 0.72	23.85 ± 0.60	24.18 ± 0.67	24.05 ± 0.63	24.30 ± 0.67	24.13 ± 0.72	24.80 ± 0.49
2	1 mg/kg; BID x 14	23.45 ± 0.66	23.10 ± 0.68	23.13 ± 0.74	23.13 ± 0.95	23.08 ± 1.01	23.13 ± 1.09	23.25 ± 1.20
3	2 mg/kg; BID x 14	23.10 ± 0.20	23.10 ± 0.14	23.20 ± 0.07	23.85 ± 0.39	23.80 ± 0.24	23.38 ± 0.23	24.00 ± 0.32
4	2 mg/kg; QD x 14	22.45 ± 1.25	22.35 ± 1.32	22.58 ± 1.46	22.08 ± 1.44	22.73 ± 1.47	22.55 ± 1.46	23.20 ± 1.38

TABLE 26

Individual Tumor Measurements (mm^3) for the MDA-MB-231 Xenograft Model

Group 1	Animal #	6/23/15 (20)	6/26/15 (23)	6/29/15 (26)	7/2/15 (29)	7/6/15 (33)	7/9/15 (36)	7/15/15 (42)
	001	93.38	119.07	159.80	197.91	210.95	243.31	265.61
	002	116.07	241.31	313.16	339.13	362.30	390.48	426.32
	002	55.67	83.45	98.22	135.50	198.19	204.96	218.29
	002	104.82	112.09	118.44	111.07	114.61	115.31	111.45
Mean Absolute		*92.49*	*138.98*	*172.41*	*195.90*	*221.51*	*238.51*	*255.42*
Mean Relative		*100.00%*	*150.27%*	*186.41%*	*211.82%*	*239.51%*	*257.89%*	*276.17%*
S.E. Mean		*13.12*	*34.97*	*48.64*	*51.12*	*51.56*	*57.32*	*65.46*
% Inhibition Mean								
Median Absolute		*99.10*	*115.58*	*139.12*	*166.71*	*204.57*	*224.13*	*241.95*
Median Relative		*100.00%*	*116.62%*	*140.38%*	*168.22%*	*206.42%*	*226.16%*	*244.14%*
S.E. Median		*13.66*	*37.49*	*52.30*	*53.83*	*52.48*	*57.91*	*65.92*
% Inhibition Median								

5

Group 2	Animal #	6/23/15 (20)	6/26/15 (23)	6/29/15 (26)	7/2/15 (29)	7/6/15 (33)	7/9/15 (36)	7/15/15 (42)
	005	73.15	54.54	59.17	57.21	56.20	37.13	39.17
	006	80.11	76.56	80.34	88.75	99.09	87.42	72.18
	007	97.22	79.99	78.44	59.90	55.90	53.66	60.35
	007	81.21	53.58	54.34	67.43	57.30	29.02	52.23
Mean Absolute		*82.92*	*66.17*	*66.27*	*68.32*	*68.32*	*51.81*	*55.98*
Mean Relative		*100.00%*	*79.79%*	*82.09%*	*82.39%*	*80.95%*	*62.48%*	*67.51%*
S.E. Mean		*5.09*	*7.03*	*6.62*	*7.14*	*10.66*	*12.93*	*6.94*
% Inhibition Mean		*10.34%*	*52.39%*	*60.52%*	*65.12%*	*69.70%*	*78.28%*	*78.08%*
Median Absolute		*80.66*	*65.55*	*68.80*	*63.66*	*56.75*	*45.40*	*56.29*
Median Relative		*100.00%*	*81.27%*	*85.30%*	*78.93%*	*70.36%*	*56.28%*	*69.79%*
S.E. Median		*5.25*	*7.04*	*6.63*	*7.63*	*12.23*	*13.45*	*6.94*
% Inhibition Median		*18.61%*	*43.28%*	*50.54%*	*61.81%*	*72.26%*	*79.75%*	*76.74%*
Group 2	**Animal #**	**6/23/15 (20)**	**6/26/15 (23)**	**6/29/15 (26)**	**7/2/15 (29)**	**7/6/15 (33)**	**7/9/15 (36)**	**7/15/15 (42)**
	009	56.41	43.61	33.13	31.76	34.11	50.33	18.94
	010	84.06	85.18	61.75	80.69	110.72	89.11	73.89
	011	82.87	54.78	34.92	54.38	78.47	78.68	51.30
	012	86.73	44.01	23.09	16.99	9.78	18.71	20.48
Mean Absolute		*77.52*	*56.89*	*38.22*	*45.95*	*58.27*	*58.27*	*41.15*
Mean Relative		*100.00%*	*73.39%*	*49.31%*	*59.28%*	*75.17%*	*76.38%*	*53.09%*
S.E. Mean		*7.08*	*9.78*	*8.26*	*13.90*	*22.54*	*15.79*	*13.21*
% Inhibition Mean		*16.19%*	*59.06%*	*77.83%*	*76.54%*	*73.69%*	*75.18%*	*83.89%*
Median Absolute		*83.46*	*56.89*	*38.22*	*45.95*	*58.27*	*64.51*	*35.89*
Median Relative		*100.00%*	*59.18%*	*40.76%*	*51.60%*	*67.44%*	*77.29%*	*43.00%*
S.E. Median		*7.87*	*10.69*	*8.61*	*14.00*	*22.56*	*14.09*	*22.56*
% Inhibition Median		*15.78%*	*57.27%*	*15.78%*	*74.17%*	*72.49%*	*71.22%*	*85.17%*

Group 4	Animal #	6/23/15 (20)	6/26/15 (23)	6/29/15 (26)	7/2/15 (29)	7/6/15 (33)	7/9/15 (36)	7/15/15 (42)
	013	88.56	108.35	105.80	102.94	183.39	159.78	291.06
	014	78.73	51.51	54.20	70.39	84.29	55.83	42.03
	015	113.20	85.29	69.30	103.16	103.20	87.15	130.64
	016	141.91	130.82	87.49	145.68	154.89	117.63	118.31
Mean Absolute		*105.60*	*93.99*	*79.20*	*105.54*	*131.44*	*105.10*	*145.51*
Mean Relative		*100.00%*	*89.01%*	*75.00%*	*99.94%*	*124.47%*	*99.52%*	*137.79%*
S.E. Mean		*14.11*	*16.94*	*11.18*	*15.44*	*22.86*	*22.17*	*52.32*
% Inhibition Mean		*-14.18%*	*32.37%*	*54.06%*	*46.13%*	*40.66%*	*55.94%*	*43.03%*
Median Absolute		*100.88*	*96.82*	*78.40*	*103.05*	*103.05*	*102.39*	*124.47*
Median Relative		*100.00%*	*95.98%*	*77.71%*	*102.15%*	*127.92%*	*101.49%*	*123.38%*
S.E. Median		*14.37*	*17.02*	*11.19*	*15.50*	*15.50*	*22.22*	*53.72*
% Inhibition Median		*-1.80%*	*16.23%*	*43.65%*	*38.19%*	*36.92%*	*54.32%*	*48.55%*

EXAMPLE 11: EFFICACY OF ANTI-STAT3 BACTERIAL VHH13 (SEQ ID NO:3) SDAB ON THREE HUMAN CANCER XENOGRAFT MODELS

[0143] In this Example, the efficacy of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was evaluated in the MDA-MB-231 Human Breast, PANC-1 Pancreatic, and DU145 Prostate cancer xenograft models.

[0144] Athymic Nude-Foxn1^{nu} mice, MDA-MB-231 breast cancer cells, PANC-1 pancreatic cancer and the DU145 prostate cancer cell lines were described above. The body weight of the mice ranged from 17 to 19 g (34 females) and 21 to 23 g (16 males) on Day 1 of the study.

[0145] Cells in early passages (4 to 10) were used for implantation into the mice and were harvested during log phase growth. MDA-MB-231 (5×10^6), DU145 (5×10^6), and PANC-1 (1.5×10^6) were injected subcutaneously into the right flank of the mice at a final volume of 100-µL of media. Tumor measurements were initiated as soon as the tumors were palpable. Thereafter, tumors were measured twice weekly.

[0146] Animals were randomized using the stratified random sampling algorithm when tumors reach a range size of: 74-120 mm^3 (MDA-MB-231), 89-146 mm^3 (DU145), or 60-160 mm^3 (PANC-1). Treatment (containing anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb and referred to herein as SBT-100) or Vehicle (PBS) was initiated the day following randomization, referred to as day 1.

[0147] Anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was supplied as a pre-formulated solution at a concentration of 0.651 mg/ml and was stored at -20°C until ready to use. The stock solution was diluted in sterile PBS pH 7.6 to provide a 5 mg/kg in a dosing volume of 10 mL/kg. The working solution was prepared every 7 days, aliquoted onto seven vials and stored at 4°C. On each day of treatment, only the needed vial was brought to room temperature. All leftover sdAb material was retained at 4°C as need for the next dose. At day 8, any remaining sdAb material was discarded and a fresh batch prepared.

[0148] Two groups (control and SBT-100) of mice per tumor model were dosed according to the protocol shown in Table 27. Dosing schedules were as follows: Group 1 (n=4; PBS) twice a day for 14 days [BIDx14]; Group 2 (n=4; SBT-100, 5 mg/kg bw), twice a day for 14 days [BIDx14]. Both the vehicle (PBS pH 7.6) and SBT100 were administered intraperitoneally (i.p.) twice a day, six hours apart for fourteen days. Dosing was conducted according to individual animal weights. A recovery period of 7 days followed administration.

TABLE 27

Experimental Design of Xenograft Study

Model	# of cells inoculated/mouse	Group	# Mice	Agent	Dose (mg/Kg)	Route	Schedule
MDA-MB-231	5×10^6	1	4	Control (PBS)	0	IP	BIDx14
		2	4	SBT-100	5	IP	BIDx14
PANC-1	1.5×10^6	1	4	Control (PBS)	0	IP	BIDx14
		2	4	SBT-100	5	IP	BIDx14
DU145	5×10^6	1	4	Control (PBS)	0	IP	BIDx14
		2	4	SBT-100	5	IP	BIDx14

[0149] Study Log Study Director Animal Study Management Software (San Francisco, CA) was used to randomize animals, collect data (e.g., dosing, body weights, tumors measurements, clinical observations), and conduct data analyses.

[0150] In the MDA-MB-231 tumor xenograft model, animals were randomized on day 23 post-inoculation with a mean (±SE) tumor size of: 77.98 ± 21.58 and 84.71 ± 5.56 for Groups 1 and 2, respectively. Mean body weights (± SE) at randomization were: 20.04 ± 0.62 and 23.7 ± 1.84 for Groups 1 and 2, respectively. Table 28 summarizes the mean body weights (± SE) for entire study. At last day of dosing (Day 14), the mean tumor size (±SE) for control group was 168.28 ± 51.57 versus 83.81 ± 22.65 for SBT-100 treated mice. Table 29 summarizes the tumor volumes (± SE) for entire study. At the time of termination (day 28) mean tumor size (± S.E.) was: 270.49 ± 112.35 and 91.72 ± 33.17, for Groups 1 and 2, respectively. Mean body weights (± SE) at termination were: 25.36 ± 1.07 and 24.25 ± 1.68 for Groups 1 and 2, respectively. At the end of the study, the mean tumor growth inhibition in the SBT-100 treated group was 85.8% (p= 0.006). Figure 7 illustrates the mean tumor volume. The tumor doubling times were 25.78 days versus 111.6 days for Group 1 and Group 2, respectively. The % treatment/control for Group 2 was 13.35 (tumor inhibition).

TABLE 28

Mean Body Weights for Mice in MDA-MB-231

		Phase						
		Dosing					Recovery	
Group	Animal ID	8/28	9/1	9/4	9/8	9/11	9/15	9/18
Control	001	23.40	24.80	24.60	25.00	24.70	23.30	25.10
Control	002	22.40	22.50	22.60	22.70	22.80	20.50	23.10
Control	003	23.70	24.80	25.20	24.60	24.30	23.30	25.30
Control	004	23.70	24.70	25.10	25.30	24.90	22.50	25.40
Mean		**23.30**	**24.20**	**24.38**	**24.45**	**24.18**	**22.53**	**24.70**
Median		**23.55**	**24.75**	**24.85**	**24.90**	**24.80**	**23.10**	**25.15**
SD		**0.62**	**1.13**	**1.21**	**1.18**	**0.95**	**1.30**	**1.07**
% Change		**0.00**	**3.82**	**4.66**	**4.89**	**3.73**	**-3.38**	**5.97**
SBT-100	005	21.70	21.70	21.70	22.40	22.60	21.40	23.20
SBT-100	006	25.00	24.30	24.30	24.70	25.30	24.40	25.60
SBT-100	007	22.60	23.00	23.10	23.10	23.60	22.80	23.70
SBT-100	008	25.50	25.30	25.50	26.10	25.60	25.50	26.10
Mean		**23.7**	**23.575**	**23.65**	**24.075**	**24.375**	**23.55**	**24.25**
Median		**23.8**	**23.65**	**23.7**	**23.9**	**24.55**	**23.6**	**24.35**
SD		**1.84**	**1.56**	**1.63**	**1.66**	**1.46**	**1.84**	**1.68**
% Change		**0.00**	**-0.46**	**-0.15**	**1.65**	**2.96**	**-0.63**	**2.38**

TABLE 29

Tumor Volumes for MDA-MB-231

		Pre-Dosing			Phase Dosing					Recovery	
Group	Animal ID	8/21	8/24	8/27	8/28	9/1	9/4	9/8	9/11	9/15	9/18
Control	001	51.60	55.80	80.94	76.35	82.66	94.11	140.79	129.90	162.81	184.15
Control	002	78.19	77.22	121.13	120.73	146.12	179.21	208.15	234.06	306.79	428.64
Control	003	57.04	57.81	75.32	81.06	93.35	114.27	161.67	242.88	295.93	408.67
Control	004	42.92	51.67	108.64	92.23	116.96	142.60	191.58	213.43	286.91	328.19
Mean		**56.54**	**60.63**	**96.99**	**92.59**	**109.75**	**132.55**	**171.84**	**205.10**	**263.89**	**337.11**
Median		**54.32**	**56.80**	**93.74**	**86.64**	**105.10**	**128.44**	**186.72**	**223.76**	**291.42**	**355.93**
SD		**13.71**	**11.38**	**21.68**	**19.91**	**27.42**	**36.92**	**41.63**	**51.57**	**67.78**	**112.36**
SBT-100	005	72.26	64.45	90.92	74.07	86.91	49.44	66.70	73.64	93.33	116.07
SBT-100	006	61.50	63.08	80.67	79.60	71.82	87.08	87.64	116.80	116.97	120.46
SBT-100	007	37.41	35.16	61.93	61.85	50.02	50.32	46.10	53.85	66.57	80.57
SBT-100	008	43.80	56.95	96.22	79.84	58.28	60.19	54.10	80.57	79.47	49.78
Mean		**53.74**	**54.91**	**94.71**	**81.37**	**58.49**	**56.76**	**64.11**	**83.81**	**89.06**	**91.72**
Median		**52.65**	**60.02**	**93.45**	**79.77**	**58.02**	**55.25**	**61.40**	**77.81**	**86.40**	**98.32**
SD		**16.90**	**13.97**	**5.58**	**7.49**	**8.16**	**8.43**	**18.21**	**22.65**	**21.66**	**33.17**
%TC		0.0	32.3	84.9	81.6	13.6	5.9	9.2	20.7	17.8	13.4
p-value		0.000	0.542	0.351	0.332	0.013	0.007	0.003	0.005	0.003	0.009

[0151] In the DU145 tumor xenograft model, animals were randomized on day 17 post-inoculation with a mean (±SE) tumor size of: 111.87 ± 20.53 and 111.23 ± 25.16 for Groups 1 and 2, respectively. Mean body weights (± SE) at randomization were: 29.10 ± 1.94 and 30.68 ± 1.56 for Groups 1 and 2, respectively. Table 30 summarizes the mean body weights (± SE) for entire study. At last day of dosing (Day 14), the mean tumor size (±SE) for control group was 621.81 ± 276.25 versus 364.14 ± 51.64 for SBT-100 treated mice. Table 31 summarizes the tumor volumes (± SE) for entire study. At the time of termination (day 28) mean tumor size (± S.E.) was: 819.42 ± 351.88 and 601.83 ± 131.51, for Groups 1 and 2, respectively. Mean body weights (± SE) at termination were: 29.20 ± 2.33 and 29.60 ± 1.04 for Groups 1 and 2, respectively. At the end of the study, the mean tumor growth inhibition in the SBT-100 treated group was 26.6% (p= 0.29). Figure 8 illustrates the mean tumor volume. The tumor doubling times were 14.57 days versus 18.19 days for Group 1 and Group 2, respectively. The % treatment/control for Group 2 was 74.8.

TABLE 30

Mean Body Weights for Mice in DU145

		Phase						
		Dosing					Recovery	
Group	Animal ID	9/4	9/8	9/11	9/15	9/18	9/22	9/25
Control	001	29.60	28.10	29.30	28.40	28.30	29.00	29.90
Control	002	29.70	30.10	31.20	30.10	30.40	29.60	30.00
Control	003	30.80	30.10	31.00	31.70	31.20	31.10	31.10
Control	004	25.30	25.70	25.60	25.20	25.10	25.20	25.80
Mean		**28.10**	**28.50**	**29.53**	**28.85**	**28.00**	**28.95**	**28.20**
Median		**29.55**	**28.10**	**30.15**	**29.25**	**29.35**	**29.45**	**29.95**
SD		**1.94**	**2.09**	**2.13**	**2.78**	**2.29**	**2.08**	**2.33**
% Change		**0.00**	**-2.67**	**1.48**	**-0.89**	**-0.37**	**-0.10**	**0.27**
SBT-100	005	30.90	30.20	27.90	29.80	29.90	30.50	30.10
SBT-100	006	28.40	26.20	27.30	26.90	27.50	29.10	28.50
SBT-100	007	31.70	31.20	31.50	30.40	30.70	31.20	30.80
SBT-100	008	31.70	29.70	30.20	28.20	28.10	28.80	29.00
Mean		**30.68**	**29.33**	**28.23**	**28.83**	**29.05**	**29.90**	**29.60**
Median		**31.30**	**29.95**	**29.05**	**29.59**	**29.00**	**29.80**	**29.55**
SD		**1.58**	**2.17**	**1.97**	**1.58**	**1.50**	**1.14**	**1.04**
% Change		**9.00**	**-4.47**	**-4.74**	**-5.00**	**-5.23**	**-2.39**	**-3.40**

TABLE 31

Tumor Volumes for DU145

		Pre-Dosing				Phase: Dosing					Recovery	
Group	Animal ID	8/24	8/27	8/31	9/3	9/4	9/8	9/11	9/15	9/18	9/22	9/25
Control	001	39.18	41.27	58.41	82.80	93.22	121.16	209.75	319.41	409.15	430.31	450.89
Control	002	45.05	35.99	64.98	95.50	103.93	138.42	225.62	327.76	478.14	524.46	596.97
Control	003	46.65	22.37	88.76	127.98	141.49	213.24	384.15	930.74	1,023.13	1,094.09	1,198.53
Control	004	17.06	36.44	65.73	101.20	109.33	227.23	289.78	338.79	575.68	926.80	1,027.90
Mean		**36.98**	**34.02**	**64.47**	**111.87**	**119.22**	**174.26**	**276.84**	**479.93**	**621.91**	**743.96**	**819.42**
Median		**42.11**	**36.22**	**65.36**	**111.74**	**121.08**	**174.33**	**257.79**	**323.27**	**527.49**	**730.69**	**813.93**
SD		**13.57**	**9.12**	**20.58**	**30.53**	**24.32**	**53.70**	**80.91**	**302.77**	**275.25**	**311.67**	**351.88**
SBT-100	005	33.80	23.32	35.67	86.02	89.21	181.92	145.67	396.82	325.85	474.31	468.83
SBT-100	006	59.44	41.00	64.21	98.56	121.39	148.44	206.10	357.62	391.02	518.25	586.67
SBT-100	007	42.30	36.11	77.80	144.08	145.78	115.05	106.70	248.12	316.24	454.78	529.83
SBT-100	008	69.37	50.18	71.23	116.28	118.70	134.16	147.82	330.22	423.48	604.72	790.96
Mean		**51.23**	**37.40**	**59.75**	**111.23**	**118.77**	**137.39**	**151.59**	**328.22**	**364.14**	**513.01**	**591.83**
Median		**50.87**	**38.06**	**63.72**	**107.42**	**129.05**	**141.30**	**146.69**	**338.92**	**358.43**	**496.29**	**558.75**
SD		**16.13**	**11.25**	**19.99**	**25.16**	**23.17**	**16.76**	**40.98**	**58.97**	**51.58**	**66.65**	**131.51**
% TIC		**0.9**	**-25.9**	**29.1**	**78.8**	**81.7**	**85.4**	**42.1**	**70.6**	**56.9**	**69.9**	**74.8**
p-value		**0.226**	**0.643**	**0.747**	**0.979**	**0.980**	**0.238**	**0.034**	**0.372**	**0.116**	**0.197**	**0.291**

[0152] In the PANC-1 tumor xenograft model, animals were randomized on day 22 post-inoculation with a mean (±SE) tumor size of: 78.74 ± 40.21 and 93.84 ± 36.31 for Groups 1 and 2, respectively. Mean body weights (± SE) at randomization were: 22.50 ± 1.47 and 24.23 ± 1.63 for Groups 1 and 2, respectively. Table 32 summarizes the mean body weights (± SE)

for entire study. At last day of dosing (Day 14), the mean tumor size (±SE) for control group was 204.95 ± 178.90 versus 159.03 ± 28.01 for SBT-100 treated mice. Table 33 summarizes the tumor volumes (± SE) for entire study. At the time of termination (day 28) mean tumor size (± S.E.) was: 284.77 ± 288.88 and 203.02 ± 30.34, for groups 1 and 2, respectively. Mean body weights (± SE) at termination were: 27.38 ± 1.07 and 26.23 ± 1.19 for Groups 1 and 2, respectively. At the end of the study, the mean tumor growth inhibition in the SBT-100 treated group was 41.78% (p= 0.35). Figure 9 illustrates the mean tumor volume. The tumor doubling times were 18.51 days versus 35.70 days for Group 1 and Group 2, respectively. The % treatment/control for Group 2 was 52.79.

TABLE 32

Mean Body Weights for Mice in PANC-1

		Phase						
		Dosing					Recovery	
Group	Animal ID	9/8	9/11	9/15	9/18	9/22	9/25	9/29
Control	001	26.50	26.60	25.80	27.10	25.70	26.10	27.20
Control	002	24.30	24.60	23.90	25.10	24.40	25.00	25.60
Control	003	27.60	26.60	26.30	26.20	25.10	27.50	28.20
Control	004	25.10	25.30	24.20	25.10	24.70	25.80	26.90
Mean		**25.88**	**25.83**	**25.04**	**25.88**	**25.08**	**26.10**	**26.98**
Median		**25.80**	**25.90**	**25.00**	**25.65**	**25.20**	**26.00**	**27.05**
SD		**1.47**	**0.97**	**1.18**	**0.97**	**0.81**	**1.03**	**1.07**
% Change		**0.00**	**-0.39**	**-3.15**	**0.12**	**-2.41**	**1.05**	**4.33**
SBT-100	005	22.80	22.80	21.40	22.50	22.60	22.80	24.00
SBT-100	006	26.00	25.10	24.90	25.70	25.10	25.40	27.10
SBT-100	007	23.10	22.30	22.40	22.70	23.10	23.50	25.70
SBT-100	008	26.20	25.00	25.30	25.40	26.20	25.40	27.30
Mean		**24.23**	**23.80**	**23.48**	**24.08**	**24.25**	**24.30**	**26.03**
Median		**24.15**	**23.80**	**23.65**	**24.10**	**24.45**	**24.40**	**26.40**
SD		**1.63**	**1.46**	**1.87**	**1.71**	**1.89**	**1.28**	**1.19**
% Change		**0.00**	**-1.71**	**-3.14**	**-0.83**	**0.13**	**0.29**	**8.29**

TABLE 33

Tumor Volumes for PANC-1

		Pre-Dosing			Phase Dosing					Recovery	
Group	Animal ID	8/31	9/3	9/8	9/8	9/11	9/15	9/18	9/22	9/25	9/29
Control	001	54.91	58.79	94.22	94.37	94.69	123.90	135.77	206.74	220.31	223.91
Control	002	46.39	78.43	91.98	91.62	88.44	130.01	151.06	140.52	165.62	202.22
Control	003	0.00	27.50	57.30	55.60	95.77	107.02	142.23	140.55	168.68	197.27
Control	004	0.00	0.00	152.17	139.59	227.62	388.54	502.06	514.93	574.44	781.46
Mean		29.26	32.54	78.74	80.91	104.18	151.29	169.83	204.95	226.81	384.77
Median		23.19	42.15	88.11	87.99	97.23	126.96	146.64	173.55	194.59	213.06
SD		29.45	33.13	40.21	43.19	66.52	130.48	179.63	178.90	200.56	288.80
SBT-100	005	39.60	64.78	78.44	78.07	87.54	93.17	112.88	145.09	173.82	248.84
SBT-100	006	40.31	37.27	68.57	73.13	76.46	113.30	130.48	182.56	208.58	188.42
SBT-100	007	88.71	91.27	147.61	148.02	123.96	116.01	157.50	171.29	175.68	200.37
SBT-100	008	48.72	55.19	82.73	83.19	86.90	102.40	135.65	132.19	136.63	175.94
Mean		53.59	62.12	93.84	95.85	86.21	106.24	126.65	158.03	173.93	203.02
Median		44.51	59.97	79.59	80.62	81.68	107.89	121.73	155.69	174.80	195.19
SD		21.82	23.52	36.31	38.69	27.84	10.48	23.06	26.01	28.10	30.34
%TC		0.0	0.0	42.6	44.2	27.4	34.4	39.0	49.7	51.0	52.8
p-value		0.174	0.310	0.927	0.917	0.296	0.272	0.298	0.350	0.343	0.358

EXAMPLE 12: EFFICACY OF ANTI-STAT3 BACTERIAL VHH13 (SEQ ID NO:3) SDAB IN THE ER+/PR+ (MCF-7) HUMAN BREAST TUMOR XENOGRAFT MODEL

[0153] This Example demonstrates the efficacy of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb in the MCF-7 human breast tumor xenograft model in nude mice.

[0154] Female athymic nude mice (Crl:NU(Ncr)-*Foxn1*^{nu}, Charles River) were twelve weeks old with a body weight (BW) range of 23.0 to 30.1 g on Day 1 of the study. The animals were fed and housed as described above.

[0155] MCF-7 human breast carcinoma cells were obtained and cultured as described above, and used for the mouse xenograph. Three days prior to tumor cell implantation, estrogen pellets (0.36 mg estradiol, 60-day release, Innovative Research of America, Sarasota, FL) were implanted subcutaneously between the scapulae of each test animal using a sterilized trocar.

[0156] The tumor cells used for implantation were harvested during log phase growth and resuspended in phosphate buffered saline (PBS) at a concentration of 1 x 10^8 cells/mL. On the day of implantation, each test mouse received 1 x 10^7 MCF-7 cells (0.1 mL cell suspension) implanted subcutaneously in the right flank and tumor growth was monitored as the average size approached the target range of 100–150 mm^3. Twenty-one days later, designated as Day 1 of the study, the animals were sorted into two groups each consisting of four mice with

individual tumor volumes ranging from 108 to 144 mm^3 and group mean tumor volumes from 117 to 123 mm^3.

[0157] Anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was provided as a pre-formulated ready to dose solution at a concentration 0.41867 mg/mL in 1 mL aliquots and were stored at -20 °C until needed. The 0.41867 mg/mL solution provided 1 mg/kg dosage in a dosing volume of 23.88 mL/kg. On each day of treatment, only needed vials of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb were thawed to room temperature. All leftover dosing suspensions were retained at 4 °C as needed for the next dose.

[0158] Two groups of athymic nude mice were dosed according to the protocol shown in Table 34. All vehicle (control) and anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb doses were administered intraperitoneally (i.p.) three times daily, six hours apart for fourteen days, with two doses delivered on Day 1 and one dose delivered on the morning of Day 15 (tid x 14, first day 2 doses). The dosing volume for vehicle and anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was 0.478 mL per 20 grams of body weight (23.88 mL/kg) and was scaled to the body weight of each individual animal. Group 1 received the vehicle and served as the benchmark group for tumor engraftment and progression, as well as the control. Group 2 was given anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb at 1 mg/kg.

TABLE 34

Protocol Design for the Study

Group	n	Treatment Regimen			
		Agent	mg/kg	Route	Schedule
1	4	vehicle	-	ip	tid x 14 first Day 2 doses
2	4	VHH13	1	ip	tid x 14 first Day 2 doses

[0159] Tumors were measured twice weekly, and each animal was euthanized when its neoplasm reached the predetermined endpoint volume (1000 mm^3) or at the end of the study, day 39, whichever came first. When a tumor reached the endpoint volume, the animal was documented as euthanized for tumor progression (TP), with the date of euthanasia. The time to endpoint (TTE) for each mouse was calculated by the following equation:

$$TTE = \frac{\log_{10}(\text{endpoint volume}) - b}{m}$$

where TTE is expressed in days, endpoint volume is expressed in mm^3, b is the intercept, and m is the slope of the line obtained by linear regression of a log-transformed tumor growth data set. The data set consists of the first observation that exceeded the endpoint volume used in analysis and the three consecutive observations that immediately preceded the attainment of this endpoint volume. The calculated TTE is usually less than the TP date, the day on which the animal was euthanized for tumor size. Animals that did not reach the endpoint volume were assigned a TTE value equal to the last day of the study (D39). Any animal classified as having died from treatment-related (TR) causes was to be assigned a TTE value equal to the day of death. Any animal classified as having died from non-treatment-related (NTR) causes was to be excluded from TTE calculations.

[0160] Treatment efficacy was determined from tumor growth delay (TGD), which is defined as the increase in the median TTE, in days, for a treatment group compared to the control group:

$$TGD = T - C$$

The percent increase in the median TTE, relative to the control group, is

where:

$$\%TGD = \frac{T - C}{C} \times 100$$

T = median TTE for a treatment group, and

C = median TTE for the designated control group.

[0161] Treatment efficacy in each group may be indicated by the median tumor volume, MTV(n), which was defined as the median tumor volume on the last day of the study (D39) in the number of animals remaining (n) whose tumors had not attained the endpoint volume.

[0162] Treatment efficacy may also be determined from the incidence and magnitude of regression responses observed during the study. Treatment may cause partial regression (PR) or complete regression (CR) of the tumor in an animal. In a PR response, the tumor volume was 50% or less of its D1 volume for three consecutive measurements during the course of the study, and equal to or greater than 13.5 mm^3 for one or more of these three measurements. In a CR response, the tumor volume was less than 13.5 mm^3 for three consecutive measurements during the course of the study. Any animal with a CR response at the end of the study was additionally classified as a tumor-free survivor (TFS).

[0163] Animals were weighed daily for the first five days, then twice weekly for the remainder of the study. The mice were observed frequently for health and overt signs of any adverse treatment related TR side effects, and noteworthy clinical observations were recorded. Individual body weight loss was monitored per protocol, and any animal with weight loss exceeding 30% for one measurement, or exceeding 25% for three measurements, was to be euthanized for health as a TR death. If group mean body weight recovered, dosing may resume in that group, but at a lower dose or less frequent dosing schedule. Acceptable toxicity was defined as a group mean BW loss of less than 20% during the study and not more than one TR death among ten treated animals, or 10%. Any dosing regimen resulting in greater toxicity is considered above the maximum tolerated dose (MTD). A death was to be classified as TR if it was attributable to treatment side effects as evidenced by clinical signs and/or necropsy, or may also be classified as TR if due to unknown causes during the dosing period or within 14 days of the last dose. A death was classified as NTR if there was evidence that the death was related to the tumor model, rather than treatment-related. NTR deaths are further categorized as NTRa (due to accident or human error), NTRm (due to necropsy-confirmed tumor dissemination by invasion or metastasis), and NTRu (due to unknown causes).

[0164] Prism 6.07 (GraphPad) for Windows was employed for graphical analyses. Statistics were not employed due to small sample size.

[0165] A scatter plot was constructed to show TTE values for individual mice, by group; this plot shows NTR deaths, which were excluded from all other figures. Individual animal, group median and mean tumor volumes were plotted as functions of time. When an animal exited the study because of tumor size or TR death, its final recorded tumor volume was included with the data used to calculate the median volume at subsequent time points. A Kaplan-Meier plot was constructed to show the percentage of animals in each group remaining on study versus time. Tumor growth curves were truncated after two TR deaths occurred in the same group. Group mean BW changes over the course of the study were graphed as percent change, ± SEM, from Day 1. Tumor growth and BW change curves were truncated after more than half the assessable mice in a group exited the study. Figure 10 illustrates the mean tumor volume in the study.

[0166] Table 35 provides the mean BW losses, TR and NTR deaths for the mice. Clinical signs were recorded when observed, as shown in Tables 36-38. No TR deaths occurred during the study. Bodyweight losses were variable, severe for one animal in each group, and resulted

from estrogen effects. Clinical observations including weight loss, enlarged uterine horns, and bladder crystals were present in both groups and were also attributable to estrogen effects. Estrogen toxicity resulted in two non-treatment related deaths in each group. The treatment evaluated in the study was acceptably tolerated.

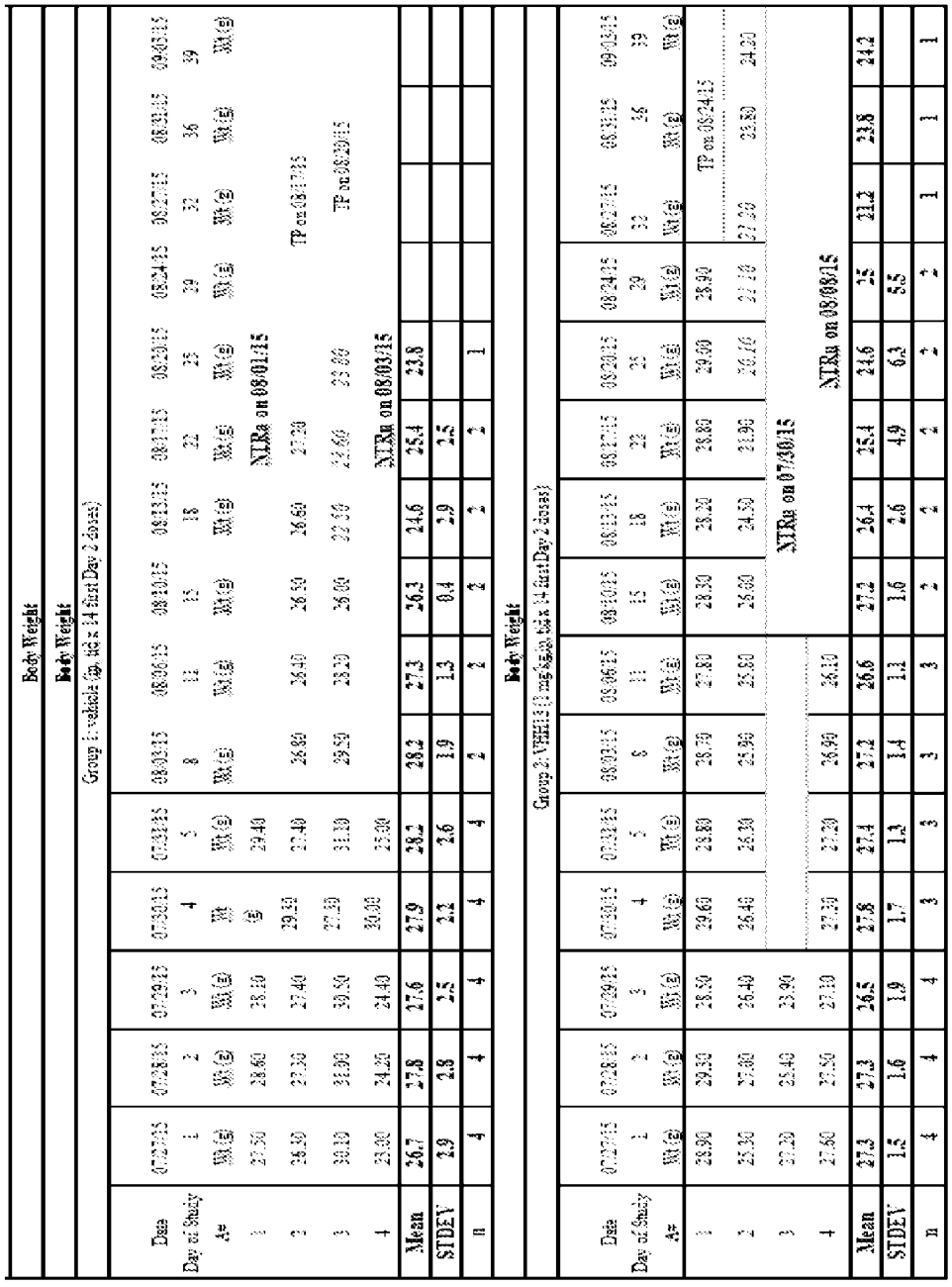
TABLE 35

Response Summary

		Treatment Regimen				Median			MTV (cu.	Regressions			Mean BW	Deaths	
Group	n	Agent	mg/kg	Route	Schedule	TTE	T-C	%TGD	D30	PR	CR	TFS	Nadir	TR	NTR
1	2	vehicle	-	ip	qdx14 days Day 2 Acute	23.2	---	---	---	0	0	0	-15.6% Day 28	0	2
2	2	[illegible]	3	ip	qdx14 days Day 2 Acute	32.8	9.7	42	---	0	0	0	-31.8% Day 32	0	2

TABLE 36

Body Weight



Body Weight																							
Body Weight																							
Group 1: vehicle (qd, tid x 14 first Day 2 dosed)																							
Date	07/27/15	07/28/15	07/29/15	07/30/15	07/31/15	08/03/15	08/06/15	08/10/15	08/13/15	08/17/15	08/20/15	08/24/15	08/27/15	08/31/15	09/03/15									
Day of Study	1	2	3	4	5	8	11	15	18	22	25	29	32	36	39								
Ani	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)								
1	27.50	28.60	28.10	29.40	29.40																		
2	26.30	27.30	27.40	29.20	27.40	26.80	26.40	26.50	26.60	27.20													
3	30.10	31.00	30.50	27.30	31.10	29.50	28.20	26.00	22.50	24.60	23.80												
4	23.00	24.20	24.40	30.00	25.00																		
Mean	26.7	27.8	27.6	27.9	28.2	28.2	27.3	26.3	24.6	25.4	23.8												
STDEV	2.9	2.8	2.5	2.2	2.6	1.9	1.3	0.4	2.9	2.5													
n	4	4	4	4	4	2	2	2	2	2	1												
Body Weight																							
Group 2: VEH (10 mg/kg, qd, tid x 14 first Day 2 dosed)																							
Date	07/27/15	07/28/15	07/29/15	07/30/15	07/31/15	08/03/15	08/06/15	08/10/15	08/13/15	08/17/15	08/20/15	08/24/15	08/27/15	08/31/15	09/03/15									
Day of Study	1	2	3	4	5	8	11	15	18	22	25	29	32	36	39								
Ani	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)	Wt (g)								
1	28.90	29.50	28.50	29.60	28.80	28.70	27.80	28.30	28.20	28.80	29.00	28.90	TP on 08/24/15										
2	25.30	27.00	26.40	26.40	26.30	25.90	25.80	26.00	24.50	24.90	26.10	27.30	23.80	24.30									
3	27.20	25.40	23.90																				
4	27.60	27.50	27.10	27.30	27.20	26.90	26.10																
Mean	27.3	27.3	26.5	27.8	27.4	27.2	26.6	27.2	26.4	25.4	24.6	25	23.2	23.8	24.2								
STDEV	1.5	1.6	1.9	1.7	1.3	1.4	1.1	1.6	2.6	4.9	6.3	5.5											
n	4	4	4	3	3	3	3	2	2	2	2	2	1	1	1								

NTRa on 08/01/15

NTRa on 08/03/15

NTRa on 07/30/15

NTRa on 08/08/15

TP on 08/17/15

TP on 08/20/15

TABLE 37

Tumor Measurement

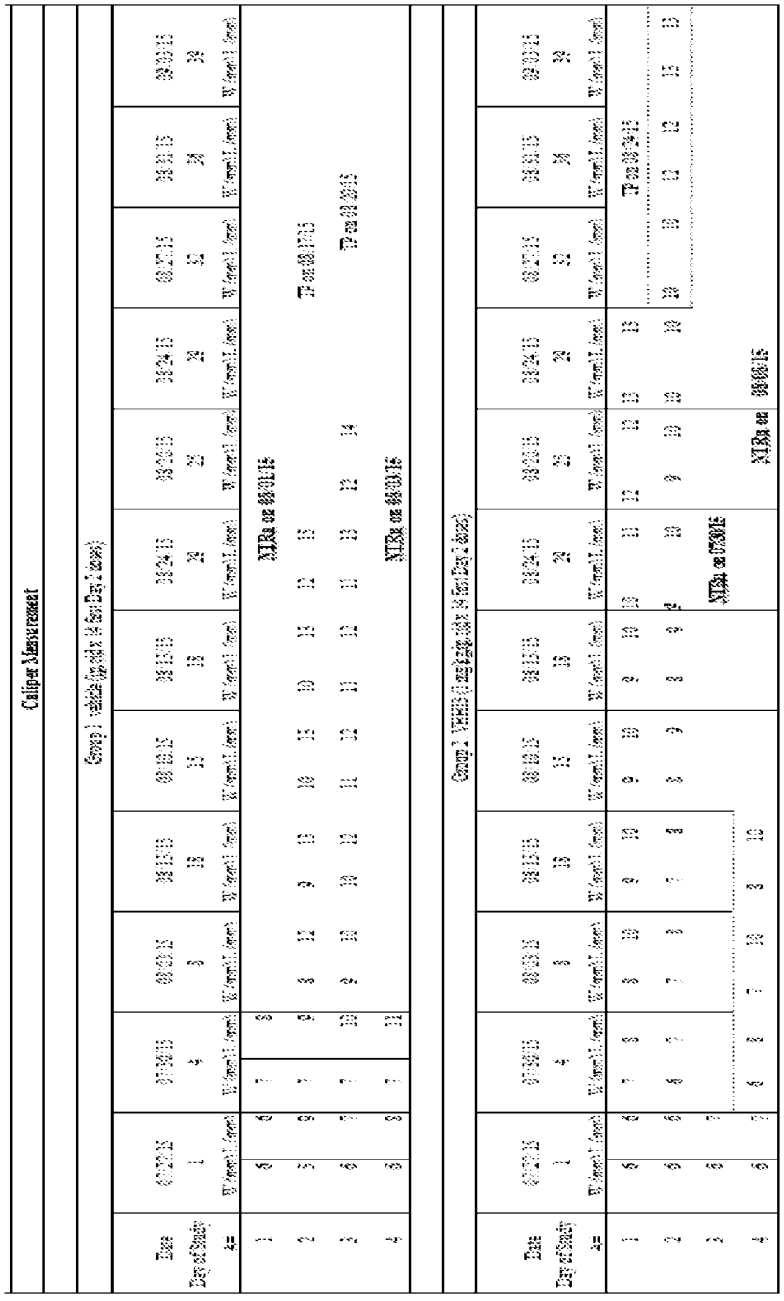


TABLE 38

Tumor Volume

Tumor Volume																				
Group 1: vehicle (ip, tid x 14 first Day 2 doses)																				
Date	07/27/15		14		08/03/15	08/06/15	08/10/15	08/13/15	08/17/15	08/20/15	08/24/15	08/27/15	08/31/15	09/03/15						
Day of Study			07/30/15		8	11	15	18	22	25	29	32	36	39						
Aᵃ	TV		TV (mm³)		TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)						
1	(mm³)		196						NTRᵤ on 08/01/15											
2	108		221		384	527	650	650	1080			TP on 08/17/15								
3	118		245		405	600	726	726	787	1008		TP on 08/20/15								
4	126		270						NTRᵤ on 08/03/15											
Mean	122.6		232.8		394.5	563.3	688	688	933.3	1008										
SEM	8.1		15.9		10.5	36.8	38	38	146.8											
n	4		4				22222													
Group 2: VHH13 (1 mg/kg,ip, tid x 14 first Day 2 doses)																				
Date	07/27/15		14		08/03/15	08/06/15	08/10/15	08/13/15	08/17/15	08/20/15	08/24/15	08/27/15	08/31/15	09/03/15						
Day of Study			07/30/15		8	11	15	18	22	25	29	32	36	39						
Aᵃ	TV (mm³)				TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)	TV (mm³)						
1	108		196		320	405	405	405	550	864	1099	TP on 08/24/15								
2	108		126		196	196	288	288	405	405	500	500	864	1099						
3	126							NTRᵤ on 07/30/15												
4	126		144		245	320														
Mean	117		155.3		253.7	307	346.5	346.5	477.5	634.5	799.3	500	864	1098.5						
SEM	5.2		21		36.1	60.7	58.5	58.5	72.5	229.5	299.3									
n	4		3		3					32222111										

[0167] Because two out of the four mice in the control group and also in the treatment group died of estrogen toxicity, no statistical conclusion could be determined. With the data available, the median tumor growth and mean tumor volume were reduced in the treatment group when compared to the control group. This difference was present during the 14 days of treatment but also to day 25 of the study. It took the control group 25 days to reach a tumor volume of 1000 mm^3, whereas the treatment group took 36 days to reach a tumor volume of 1000 mm^3. This suggests that anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb slows the growth of MCF-7 tumor *in vivo*. Throughout the study both the control group and the treatment group maintained similar weights. This suggests that the anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb did not cause toxicity with respect to weight loss.

EXAMPLE 13: TREATMENT OF HUMAN HER2+ (BT474) BREAST CANCER WITH ANTI-STAT3 BACTERIAL VHH13 (SEQ ID NO:3) SDAB IN XENOGRAFT MICE

[0168] In this Example, the efficacy of anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was determined in the BT474 human breast tumor xenograft in CB.17 SCID mice.

[0169] Two groups of 8-12 week old CB.17 SCID mice containing xenographs of 1 mm^3 BT474 tumor fragments in their flank were treated according to the protocol shown in Table 39 when the tumors reached an average size of 100-150 mm^3. All vehicle (PBS control) and anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb (shown in Table 39 as SB-01) doses were administered intraperitoneally (i.p.) three times daily, six hours apart for fourteen days, with two doses delivered on Day 1 (tid x 14, first day 2 doses). The dosing volume for vehicle and anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was 0.478 mL per 20 grams of body weight (23.88 mL/kg) and was scaled to the body weight of each individual animal. Group 1 received the vehicle and served as the benchmark group for tumor engraftment and progression, as well as the control. Group 2 was given anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb at 1 mg/kg.

TABLE 39

Study protocol

Gr.	N	Regimen 1				
		Agent	Vehicle	mg/kg	Route	Schedule
1#	4	vehicle		-	ip	tid x 14 first day 2 doses
2	4	SB-01		1	ip	tid x 14 first day 2 doses

[0170] During the first 14 days of the study, the treatment group received anti-STAT3 B VHH13 and the control group only received the vehicle. As shown in Table 40, during this time, the treatment group maintained and gained weight throughout the study while the control group had lower weights throughout the study. This suggests that the treatment group did not experience toxicity from anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb with respect to weight loss. Both groups mean tumor volume and median tumor volume were similar, and exactly the same on day 15 of the study. On day 59 of the study, both groups reached a tumor volume of 700 cubic mm^3. This suggests that the anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb did not reduce the growth of BT474 tumors in vivo when compared to the control group. Figure 11 illustrates the group mean tumor volume.

TABLE 40

BT474 Response Summary

Group	n	Treatment Regimen 1					Median TTE	T-C	%TGD	Stat Sign	MTV (n), Day 60	PR	CR	TFS	BW Nadir	TR	NTRm	NTR
		Agent	Vehicle	mg/kg	Route	Schedule												NTR
1#	4	vehicle		-	ip	tid x 14 first day 2 doses	49.2	--	-		288 (2)	0	0	0	-9.1% (3)	0	0	0
2	4	SB-01		1	ip	tid x 14 first day 2 doses	60.0	10.8	22		550 (3)	0	0	0	--	0	0	0

- Control Group

EXAMPLE 14: PRODUCTION OF MOUSE MONOCLONAL ANTIBODY DIRECTED AGAINST ANTI-STAT3 BACTERIAL VHH13 (SEQ ID NO:3) SDAB

[0171] In this Example, mouse monoclonal antibodies were generated towards the sdAb of the invention. The animals used were BALB/c female mice, 8-10 week. A water-soluble adjuvant was used (CBL). The HAT and the HT used were from Sigma-Aldrich.

[0172] Anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb was used to immunize three mice and make hybridoma cell lines. The mice were immunized three times each with water-soluble adjuvant. In one mouse, the serum titer reached 1/51200. The mouse was sacrificed and hybridoma cell lines were made by fusing spleen cells with myeloma cell line Sp2/0.

[0173] The fused cells were seeded into 96 well plates by limited dilution. The fused cells were cultured in the presence of HAT, and 651 single clones were tested. Of the 651 single clones, 27 positive clones were identified that specifically bound to anti-STAT3 bacterial VHH13 (SEQ ID NO:3) sdAb antigen.

EXAMPLE 15: CYTOTOXICITY OF KRAS (G12D) SINGLE DOMAIN ANTIBODIES ON PANC-1 HUMAN PANCREATIC CANCER CELLS

[0174] This Example demonstrates the anti-proliferative effects of the anti-KRAS (G12D) (SEQ ID NO:2) sdAb using the human pancreatic cancer cell line PANC-1. For the experiments, the PANC-1 cells were grown until they reached a confluency of 90%. At that time, proliferation studies were carried out using the MTT assay as described above.

[0175] The anti-proliferative properties of anti-KRAS (G12D) (SEQ ID NO:2) sdAB on PANC-1 cells three days post treatment are shown in Table 41. PANC-1 cells treated with the anti-KRAS (G12D) (SEQ ID NO:2) sdAb showed an average growth inhibition of 19.9 and 37.7 at 50.0 and 100 µg/ml, respectively.

TABLE 41

Anti-proliferative Actions of Anti-KRAS (G12D) (SEQ ID NO:2) sdAb on PANC-1 Cancer Cells

	Mean Abs ± SE	**% Inhibition**
control	0.281± 0.017	
50 µg/ml	0.225± 0.006	19.9
100 µg/ml	0.175± 0.016	37.7

[0176] Thus, the anti-KRAS (G12D) (SEQ ID NO:2) sdAb showed dose-dependent growth inhibition in the PANC-1 human pancreatic cancer cells.

EXAMPLE 16: IN VITRO GROWTH INHIBITION BY TNF-ALPHA SDAB

[0177] This Example demonstrates the method development to determine TNF-alpha concentration and evaluation of the inhibition of TNF-alpha function. The concentration of TNF-alpha required to show measurable modulation of activity in the U937 human lung lymphoblast cell line was evaluated by quantitation of the ATP present, which signals the presence of metabolically active cells using Promega's Cell Titer-GJo® Luminescent Cell Viability assay.

[0178] The U937 cells were seeded in a clear polystyrene 96-well microculture plate (Corning® Costar® 96-well flat bottom plate, Cat. #3997) in a total volume of 90 µL/well. After 24 hours of incubation in a humidified incubator at 37°C with 5% $C0_2$ and 95% air, 5 µL of 20X, serially diluted TNF-alpha in growth medium was added to each well in duplicate (10 pt dose response, highest concentration 20 ng/mL). Additionally, 5 µL of 20X, diluted staurosporine in growth medium was added to each well in duplicate (concentration 1 nM).

[0179] After 24 hours of culture in the presence of test agents, the concentration of compound required to show measurable modulation of TNF-alpha activity in the U937 cell line as evaluated by quantitation of the ATP present. Percent cell growth was calculated relative to untreated control wells. All tests were performed in duplicate at each concentration level.

[0180] The EC_{50} value for the test agents was estimated using Prism 6.05 by curve-fitting the data using the following four parameter-logistic equation:

$$Y = \frac{Top - Bottom}{1 + \left(X/IC_{50}\right)^{n}} + Bottom$$

where Top is the maximal % of control absorbance, Bottom is the minimal % of control absorbance at the highest agent concentration, Y is the % of control absorbance, X is the agent concentration, IC_{50} is the concentration of agent that inhibits cell growth by 50% compared to the control cells, and n is the slope of the curve.

[0181] Figures 12 and 13 demonstrate that TNF-alpha is cytotoxic to the U937 cells. The IC_{50} for TNF-alpha against U937 is 95.10pg/ml. The TNF-alpha curve shows a dose titration killing effect.

[0182] Figure 14 demonstrates that TNF-alpha cytotoxicity against U937 is inhibited by the three different anti-TNF-alpha VHHs. When anti-TNF-alpha VHH62 (SEQ ID NO:47) sdAb, anti-TNF-alpha VHH 66 (SEQ ID NO:45) sdAb, and anti-TNF-alpha VHH69 (SEQ ID NO:46) sdAb were incubated with a constant dose of TNF-alpha, at EC_{50}, all three anti-TNF-alpha VHHs inhibit killing of U937 by TNF-alpha. The IC_{50} of anti-TNF-alpha VHH62 (SEQ ID NO:47) sdAb was approximately 713.6ug/ml. The IC_{50} of anti-TNF-alpha VHH69 (SEQ ID NO:46) sdAb was greater than 208.055ug/ml. The IC_{50} of anti-TNF-alpha VHH66 (SEQ ID NO:45) sdAb could not be determined because it completely inhibited the cytotoxicity of TNF-alpha from concentrations of about $1X10^2$ ug/ml to 1 X 10^2 ug/ml of anti-TNF-alpha VHH66 (SEQ ID NO:45) sdAb. In this concentration range of anti-TNF-alpha VHH66 (SEQ ID

NO:45) sdAb, there is an increase in U937 cell growth, and thus complete inhibition of TNF-alpha activity.

[0183] Although the present invention has been described in considerable detail with reference to certain preferred embodiments, other embodiments are possible. The steps disclosed for the present methods, for example, are not intended to be limiting nor are they intended to indicate that each step is necessarily essential to the method, but instead are exemplary steps only. Therefore, the scope of the appended claims should not be limited to the description of preferred embodiments contained in this disclosure. All references cited herein are incorporated by reference in their entirety.

What is claimed is:

1. A single-domain antibody (sdAb) directed against an intracellular component.

2. The sdAb of claim 1, wherein the intracellular component comprises a protein, nucleic acid, lipid, carbohydrate, or combination thereof.

3. The sdAb of claim 1, wherein the intracellular component comprises STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6.

4. The sdAb of claim 1, wherein the intracellular component comprises TNF-alpha.

5. The sdAb of claim 1, wherein the intracellular component comprises KRAS.

6. The sdAb of claim 1, wherein the intracellular component comprises KRAS(G12D).

7. An anti-STAT3 sdAb.

8. A method of diagnosing a disorder mediated by STAT3 in a biological sample from a subject, the method comprising the steps of:
   a) contacting the biological sample with the sdAb according to claim 7;
   b) determining the amount of STAT3 protein in the biological sample; and
   c) comparing the amount determined in step (b) with a standard amount of STAT3 protein, wherein a difference in amount of STAT3 protein between the standard and the biological sample indicates the presence of the disorder.

9. A method of treating a disease, preventing development of a disease, or preventing recurrence of a disease in a subject using the anti-STAT3 sdAb according to claim 9, the method comprising administering an effective amount of the anti-STAT3 sdAb to a subject in need thereof.

10. The method of claim 9, wherein the subject is a mammal.

11. The method of claim 10, wherein the mammal is a human.

12. A method of claim 9, wherein administering an effective amount of the anti-STAT3 sdAb to a subject in need thereof comprises intravenous administration, intramuscular administration, oral administration, rectal administration, enteral administration, parenteral

administration, intraocular administration, subcutaneous administration, transdermal administration, administered as eye drops, administered as nasal spray, administered by inhalation or nebulization, topical administration, and administered as an implantable drug.

13. A method of treating a disease, preventing a disease or preventing the reoccurrence of a disease in a subject using the sdAb of claim 1 in combination with one or more compounds.

14. The method of claim 13, wherein the one or more compounds is a transcriptional inhibitor.

15. A method of quantifying the levels of an sdAb in sample from a subject, the method comprising the steps of:

a. generating a mouse monoclonal antibody directed against one or more domains of an sdAb;
b. obtaining a sample from the subject;
c. performing an quantitative immunoassay with the mouse monoclonal antibody and the sample to determine the amount of sdAb in a subject; and
d. quantifying the amount of sdAb in the subject.

16. The method of claim 15 wherein the quantitative immunoassay comprises an enzyme-linked immunosorbent assay (ELISA), specific analyte labeling and recapture assay (SALRA), liquid chromatography, mass spectrometry, fluorescence-activated cell sorting, or a combination thereof.

17. An anti-STAT3 single domain antibody (sdAb) comprising an amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4.

18. An isolated polypeptide, the isolated polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4.

19. A host cell, the host cell expressing a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4.

20. A pharmaceutical composition, the pharmaceutical composition comprising a polypeptide as set forth in SEQ ID NO:3 or SEQ ID NO:4.

21. A method of diagnosing a disorder mediated by STAT3 in a subject, the method comprising the steps of:

a) contacting a biological sample with a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4;

b) determining the amount of STAT3 in the biological sample; and

c) comparing the amount determined in step (b) with a standard, a difference in amount between the sample and the standard diagnostic of the disorder.

22. A method of treating a disease or disorder, or preventing recurrence of a disease or disorder mediated by STAT3, comprising administering an effective amount of a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4 to a subject in need thereof.

23. The method of claim 22, wherein the subject is a mammal.

24. The method of claim 23, wherein the mammal is a human.

25. The method of claim 22, wherein administering a polypeptide comprises intravenous administration, intramuscular administration, oral administration, rectal administration, enteral administration, intraocular administration, parenteral administration, subcutaneous administration, transdermal administration, administered as eye drops, administered as nasal spray, administered by inhalation or nebulization, topical administration, and administered as an implantable drug.

26. The method of claim 22, wherein the polypeptide is administered in combination with one or more compounds.

27. The method of claim 26, wherein the one or more compounds is a transcriptional inhibitor.

28. An antibody directed toward the polypeptide comprising SEQ ID NO:3 or SEQ ID NO:4.

29. A method of measuring the levels of an anti-STAT3 sdAb in a sample from a subject, the method comprising the steps of:

a. generating a mouse monoclonal antibody directed against one or more domains of a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4;

b. obtaining a sample from the subject;

c. performing an quantitative immunoassay with the mouse monoclonal antibody and the sample to determine the amount of sdAb in a subject; and

d. quantifying the amount of sdAb in the subject.

30. The method of claim 29 wherein the quantitative immunoassay comprises an enzyme-linked immunosorbent assay (ELISA), specific analyte labeling and recapture assay (SALRA), liquid chromatography, mass spectrometry, fluorescence-activated cell sorting, or a combination thereof.

31. An anti-TNF-alpha single domain antibody (sdAb) comprising an amino acid sequence as set forth in SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.

32. An isolated polypeptide, the isolated polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.

33. A host cell, the host cell expressing a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.

34. A pharmaceutical composition, the pharmaceutical composition comprising a polypeptide as set forth in SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.

35. A method of diagnosing a disorder mediated by TNF-alpha in a subject, the method comprising the steps of:

a) contacting a biological sample with a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47;

b) determining the amount of TNF-alpha in the biological sample; and

c) comparing the amount determined in step (b) with a standard, a difference in amount between the sample and the standard diagnostic of the disorder.

36. A method of treating a disease or disorder, or preventing recurrence of a disease or disorder mediated by TNF-alpha, comprising administering an effective amount of a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47 to a subject in need thereof.

37. The method of claim 36, wherein the subject is a mammal.

38. The method of claim 37, wherein the mammal is a human.

39. The method of claim 36, wherein administering a polypeptide comprises intravenous administration, intramuscular administration, oral administration, rectal administration, enteral administration, intraocular administration, parenteral administration, subcutaneous administration, transdermal administration, administered as eye drops, administered as nasal spray, administered by inhalation or nebulization, topical administration, and administered as an implantable drug.

40. The method of claim 36, wherein the polypeptide is administered in combination with one or more compounds.

41. The method of claim 40, wherein the one or more compounds is a transcriptional inhibitor.

42. An antibody directed toward the polypeptide comprising SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.

43. A method of measuring the levels of an anti-TNF-alpha sdAb in a sample from a subject, the method comprising the steps of:

a. generating a mouse monoclonal antibody directed against one or more domains of a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47;

b. obtaining a sample from the subject;

c. performing an quantitative immunoassay with the mouse monoclonal antibody and the sample to determine the amount of sdAb in a subject; and

d. quantifying the amount of sdAb in the subject.

44. The method of claim 43 wherein the quantitative immunoassay comprises an enzyme-linked immunosorbent assay (ELISA), specific analyte labeling and recapture assay (SALRA), liquid chromatography, mass spectrometry, fluorescence-activated cell sorting, or a combination thereof.

45. An anti-KRAS single domain antibody (sdAb) comprising an amino acid sequence as set forth in SEQ ID NO: 2.

46. An isolated polypeptide, the isolated polypeptide comprising an amino acid sequence as set forth in SEQ ID NO: 2.

47. A host cell, the host cell expressing the amino acid sequence as set forth in SEQ ID NO: 2.

48. A pharmaceutical composition, comprising an sdAb according to claim 45 or a polypeptide of claim 46, and a pharmaceutically acceptable carrier.

49. A method to diagnose a disorder mediated by KRAS in a subject, the method comprising the steps of:

a) contacting a biological sample with a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO. 2;

b) determining the amount of KRAS in the biological sample; and

c) comparing the amount determined in step (b) with a standard, a difference in amount between the sample and the standard diagnostic of the disorder.

50. A method of treating a disease or disorder mediated by an aberrant form of KRAS, preventing a disease or disorder, or recurrence of a disease or disorder mediated by an aberrant form of KRAS, comprising administering an effective amount of a polypeptide comprising SEQ ID NO: 2 to a subject in need thereof.

51. The method of claim 50, wherein the subject is a mammal.

52. The method of claim 51, wherein the mammal is a human.

53. The method of claim 50, wherein the administering comprises intravenous administration, intramuscular administration, oral administration, intraocular administration, rectal administration, enteral administration, parenteral administration, subcutaneous administration, transdermal administration, administered as eye drops, administered as nasal spray, administered by inhalation or nebulization, topical administration, and administered as an implantable drug.

54. The method of claim 50, wherein the polypeptide comprising SEQ ID NO: 2 is administered in combination with one or more compounds.

55. The method of claim 54, wherein the one or more compounds is a transcriptional inhibitor.

56. An antibody directed toward the polypeptide comprising SEQ ID NO: 2.

57. A method of measuring the levels of an anti-KRAS sdAb in a sample from a subject, the method comprising the steps of:

a. generating a mouse monoclonal antibody directed against one or more domains of a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2;

b. obtaining a sample from the subject;

c. performing an quantitative immunoassay with the mouse monoclonal antibody and the sample to determine the amount of sdAb in a subject; and

d. quantifying the amount of sdAb in the subject.

58. The method of claim 57 wherein the quantitative immunoassay comprises an enzyme-linked immunosorbent assay (ELISA), specific analyte labeling and recapture assay (SALRA), liquid chromatography, mass spectrometry, fluorescence-activated cell sorting, or a combination thereof.

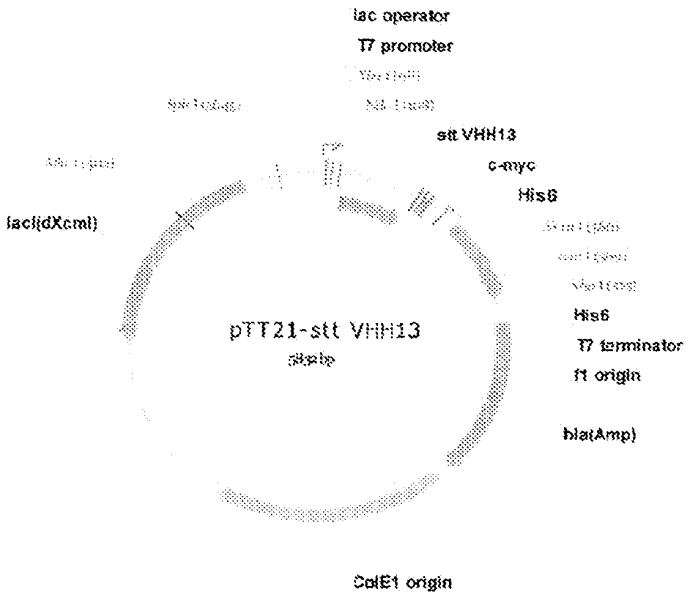

lac operator
T7 promoter
stt VHH13
c-myc
His6
lacI(dXcml)
pTT21-stt VHH13
His6
T7 terminator
f1 origin
bla(Amp)
ColE1 origin

FIG. 1

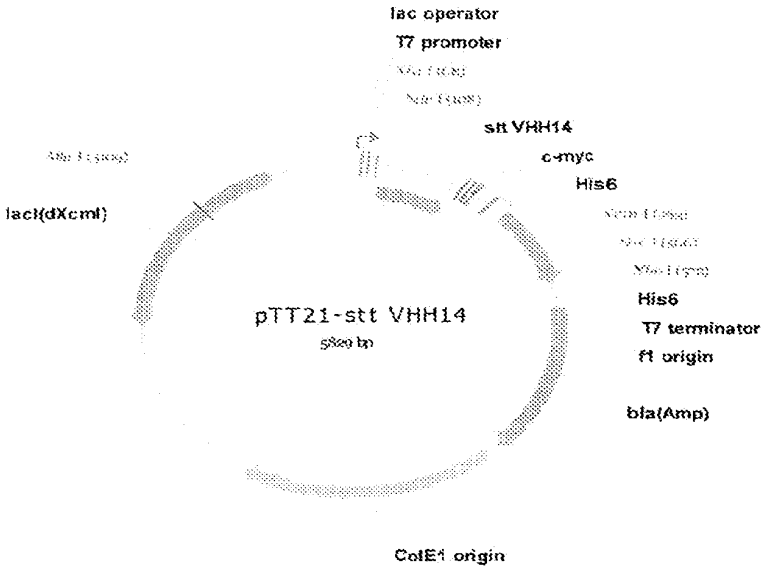
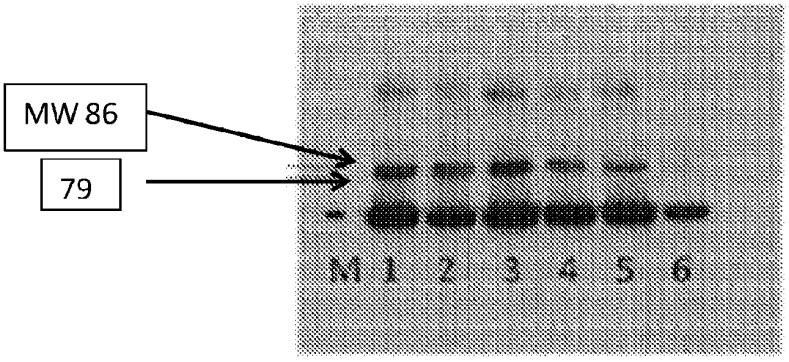
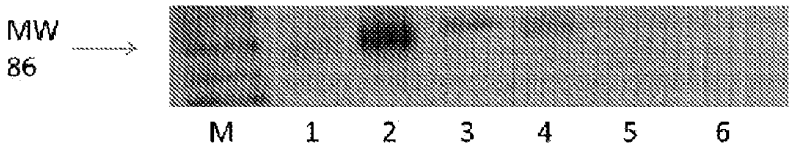

lac operator
T7 promoter
stt VHH14
c-myc
His6
lacI(dXcml)
pTT21-stt VHH14
His6
T7 terminator
f1 origin
bla(Amp)
ColE1 origin

FIG. 2



Lanes:
M- Marker
1- STAT3 VHH13 (mammalian)
2- STAT3 VHH14 (mammalian)
3- STAT3 VHH13 (bacterial)
4- STAT3- VHH14 (bacterial)
5- Positive control (STAT-3)
6- Negative control (STAT-1)

FIG. 3



Lanes:
M= Marker
1= PANC-1 with anti-STAT3 B VHH13
2= DU145 with anti-STAT3 B VHH13
3= HeLa + IFN-gamma with anti-STAT3 B VHH13
4= 4T1 with anti-STAT3 B VHH13
5= PANC-1 with anti-KRAS (G12D) VHH
6= PC-3 (negative control) with anti-STAT3 B VHH13

FIG. 4

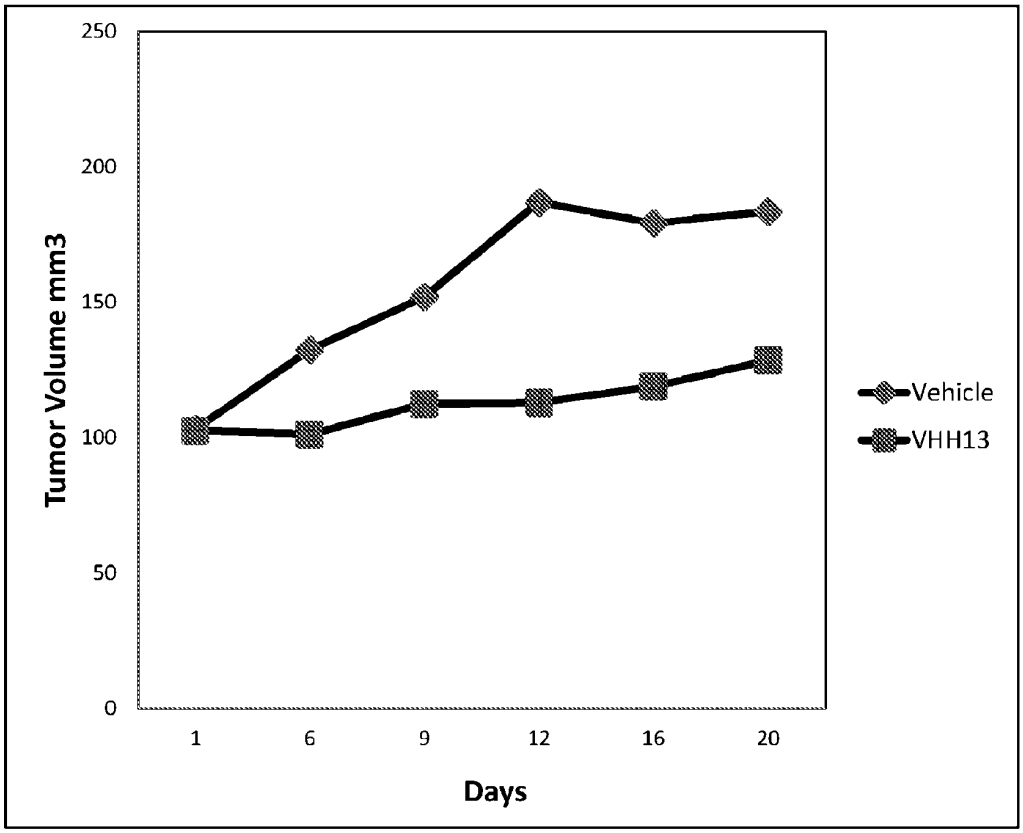
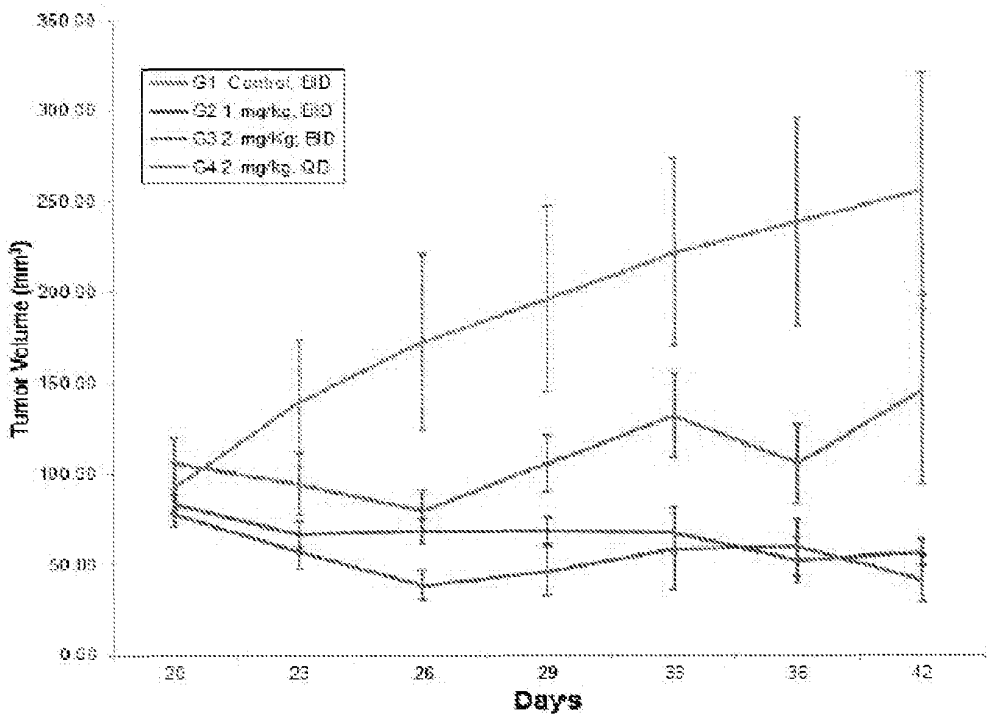

250
200
150
100
50
0
Tumor Volume mm3
1
6
9
12
16
20
Days
Vehicle
VHH13

FIG. 5



Top curve: G1 is control group with vehicle only IP; given twice daily
Second curve: G2 is given anti-STAT3 B VHH13 IP; 1mg/kg/twice daily
Third curve at the beginning: G3 is given anti-STAT3 B VHH13 IP; 2mg/kg/twice daily
Bottom curve at the beginning: G4 is given anti-STAT3 B VHH13 2mg/kg/once daily

FIG. 6

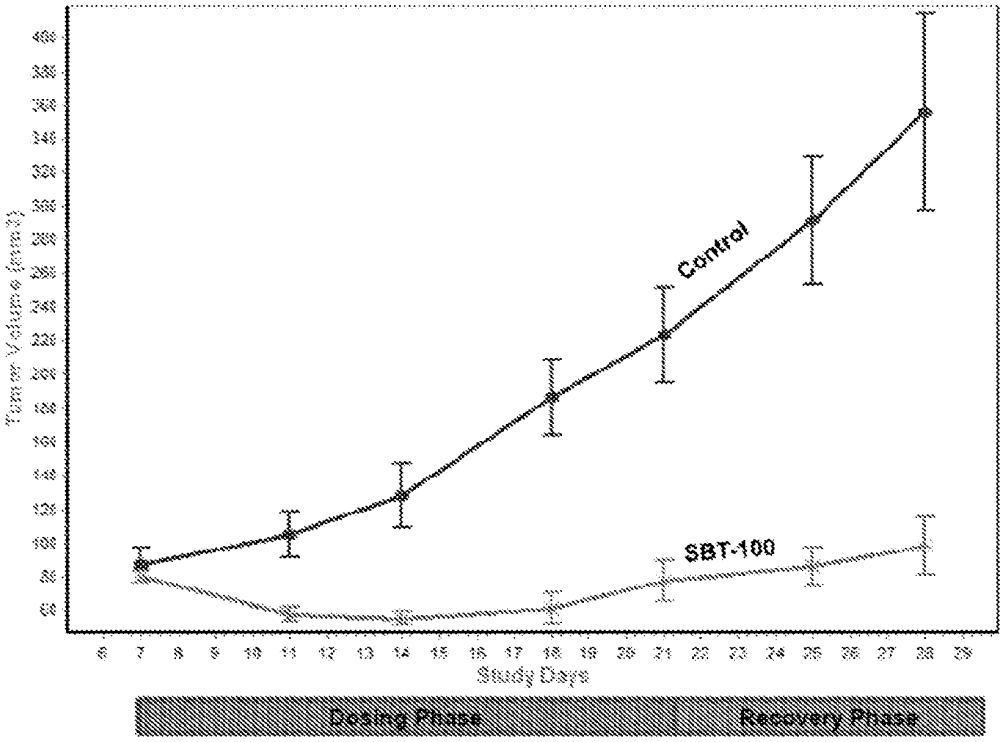

Tumor Volume (mm3)
Control
SBT-100
Study Days
Dosing Phase
Recovery Phase

FIG. 7

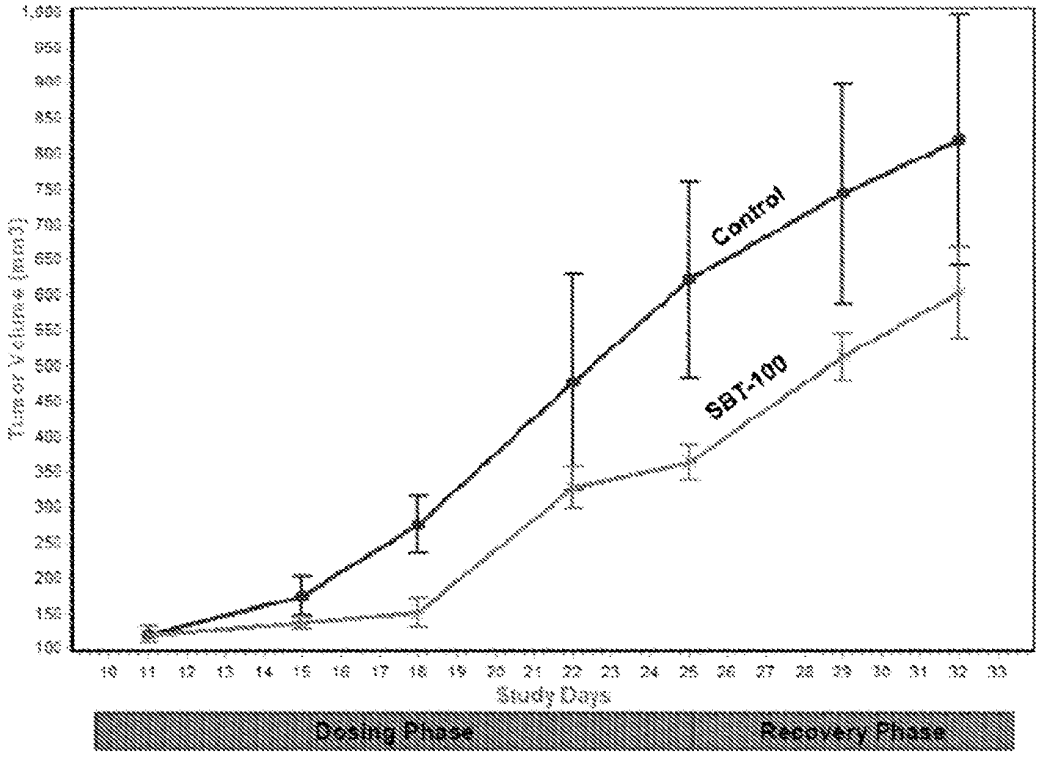

Tumor Volume (mm3)
Control
SBT-100
Study Days
Dosing Phase
Recovery Phase

FIG. 8

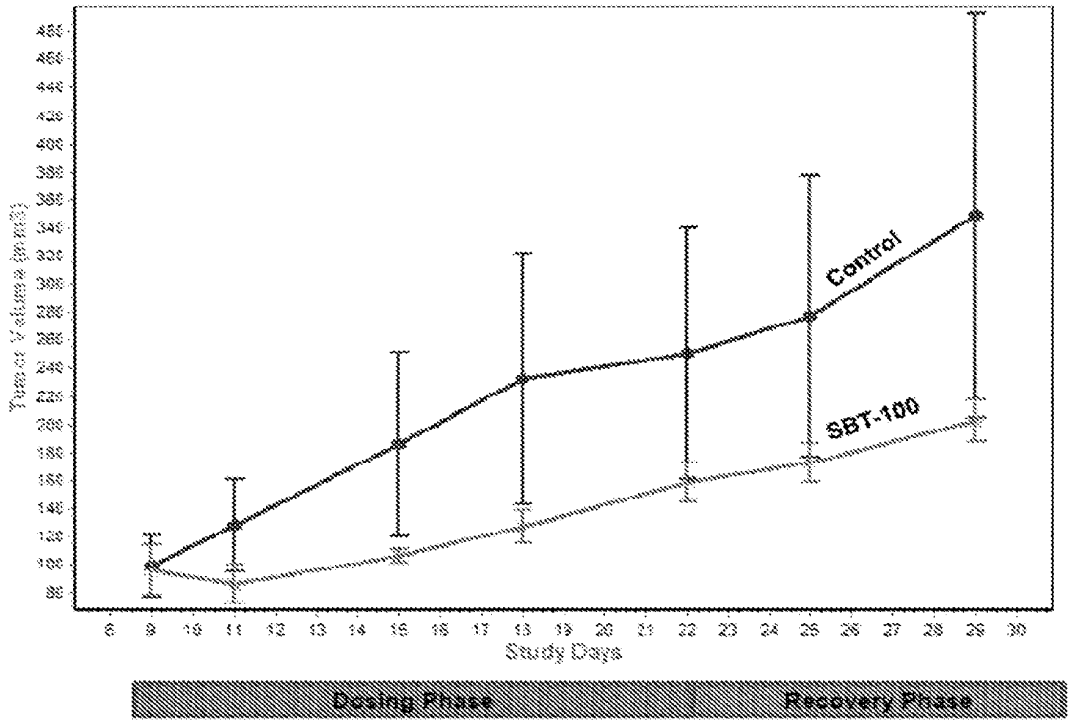


FIG. 9

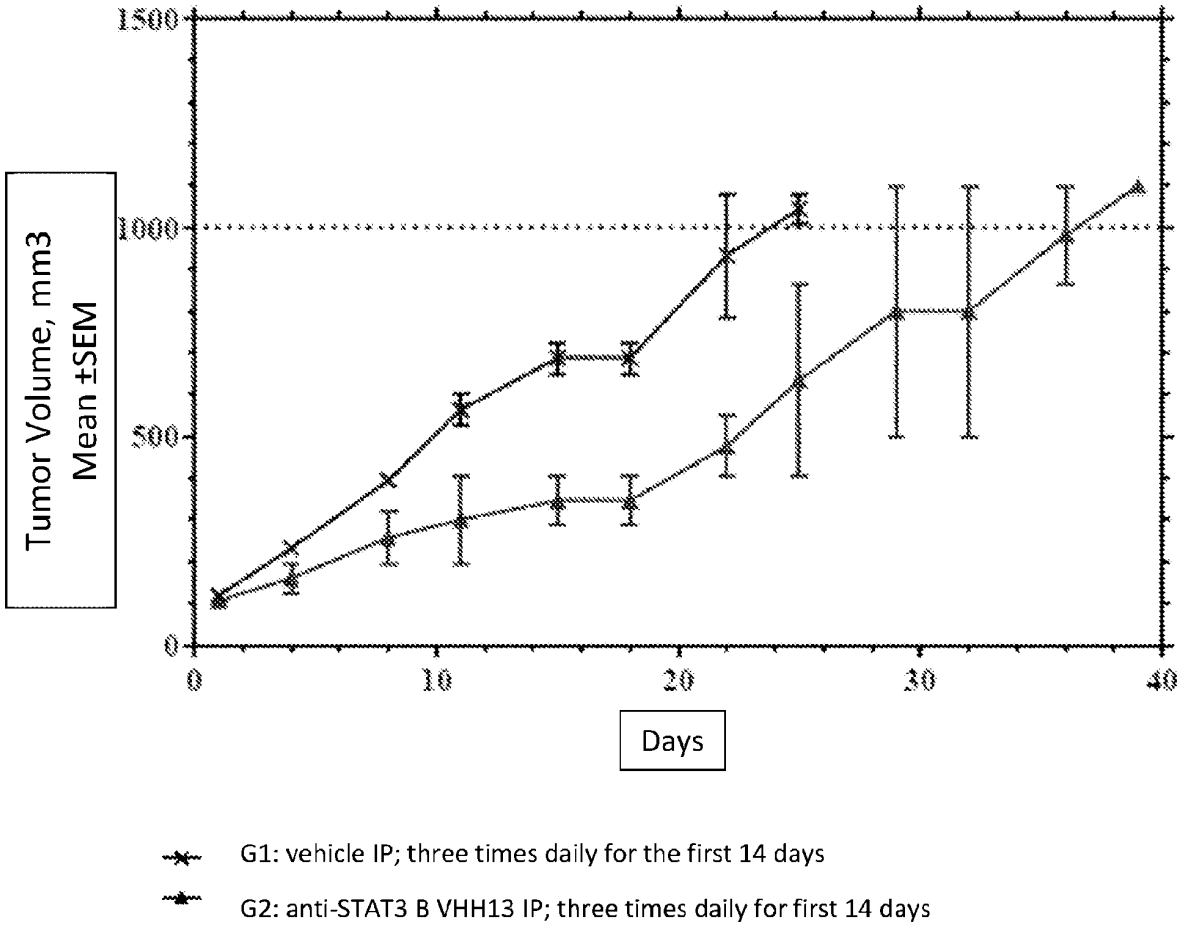

Tumor Volume, mm3
Mean ±SEM
1500
1000
500
0
0
10
20
30
40
Days
G1: vehicle IP; three times daily for the first 14 days
G2: anti-STAT3 B VHH13 IP; three times daily for first 14 days

FIG. 10

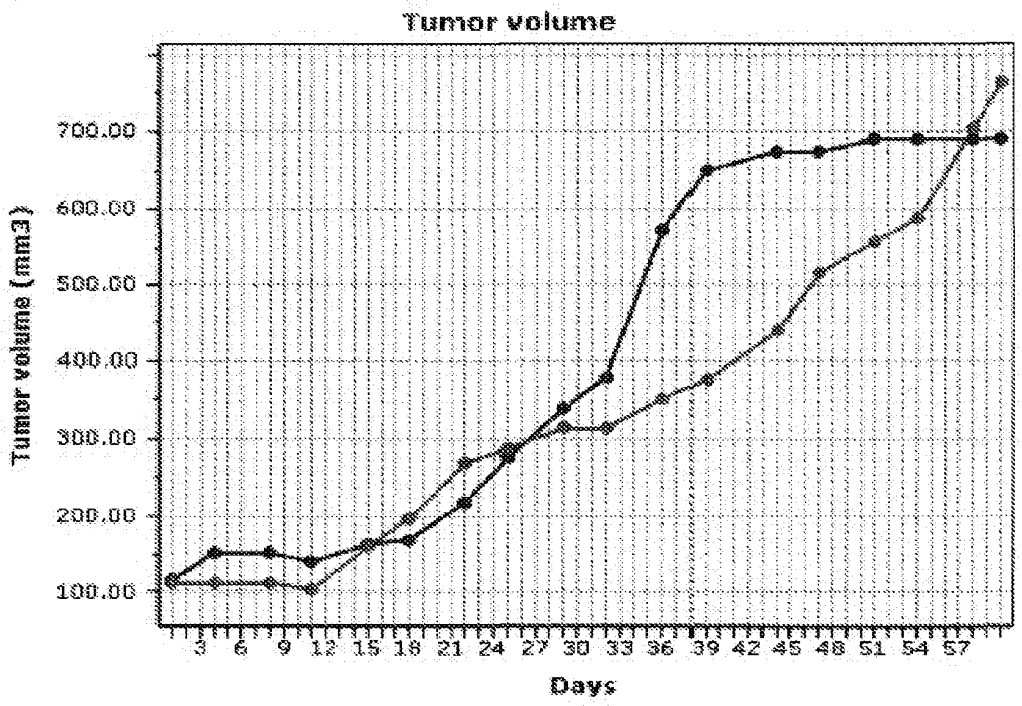
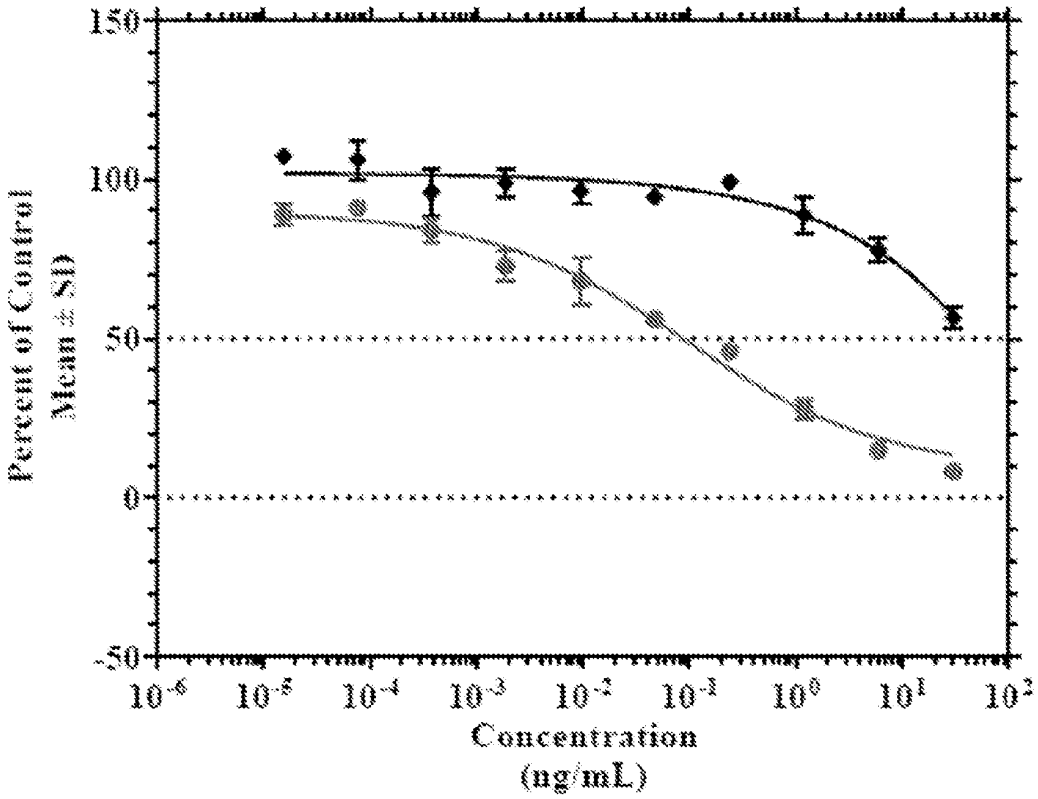

Tumor volume
Tumor volume (mm3)
700.00
600.00
500.00
400.00
300.00
200.00
100.00
3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57
Days

FIG. 11

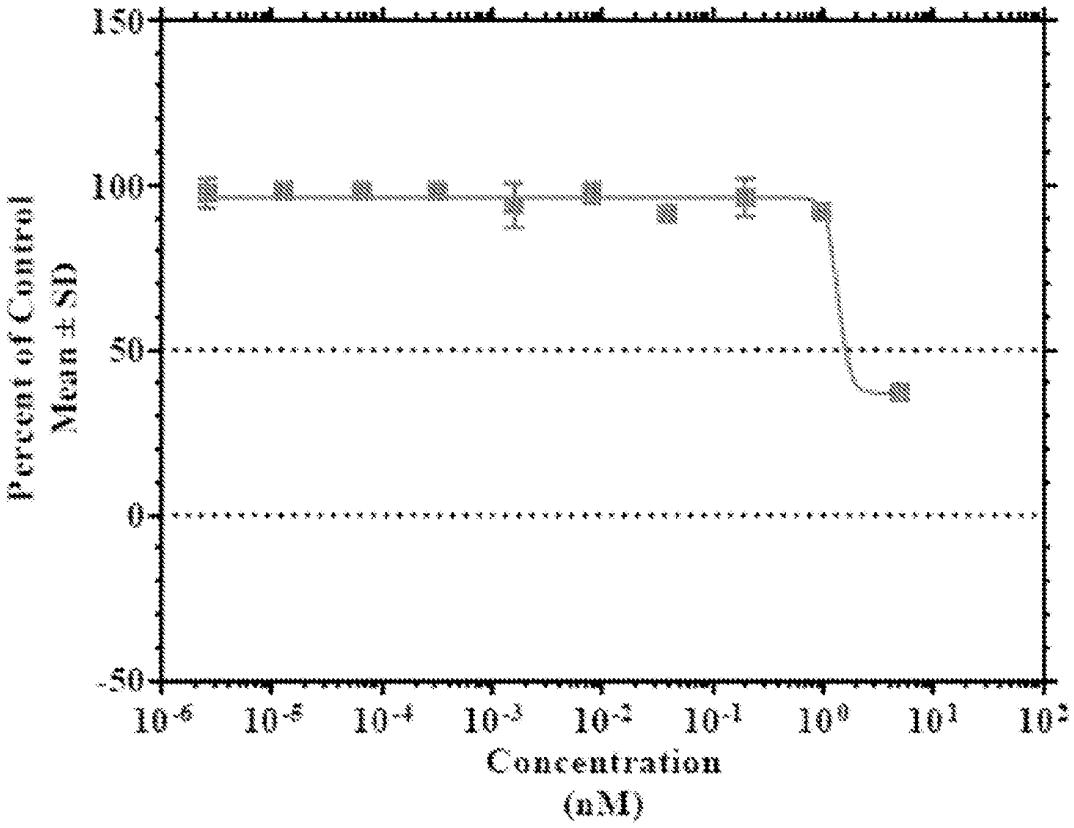


Mean IC_{50}

TNFα (IC_{50} – 95.10 pg/mL)

TNFα + constant Staurosporine (1 nM) (IC_{50} > 30 ng/mL)

FIG. 12



Mean IC_{50}

Staurosporine (IC_{50} Not determined)

FIG. 13

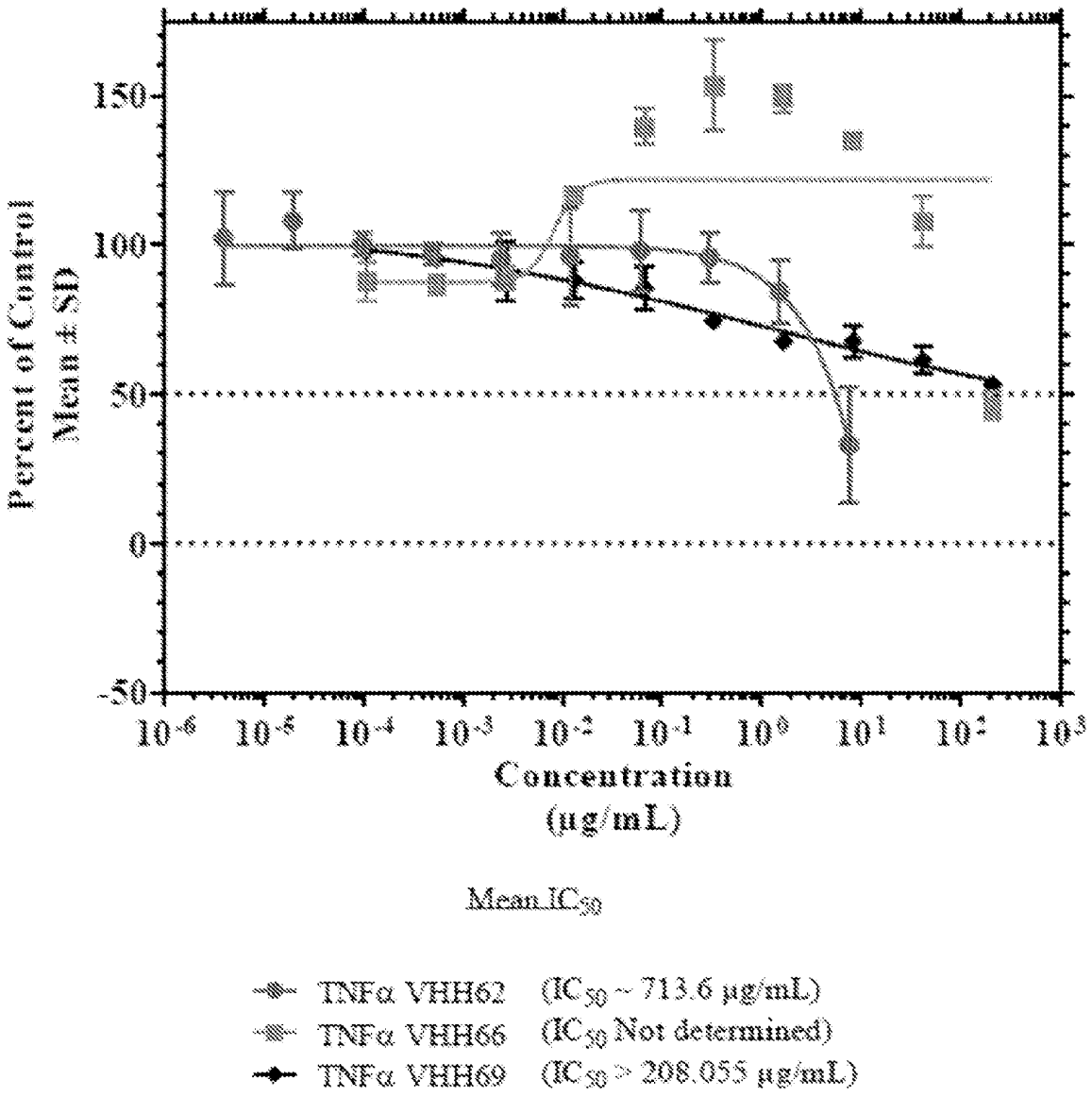

Percent of Control
Mean ± SD
150
100
50
0
-50
10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
Concentration
(µg/mL)
Mean IC_{50}
TNFα VHH62 (IC_{50} ~ 713.6 µg/mL)
TNFα VHH66 (IC_{50} Not determined)
TNFα VHH69 (IC_{50} > 208.055 µg/mL)

FIG. 14

United States Patent and Trademark Office

Office of the Chief Financial Officer

Document Code:WFEE

User :Tram Vo

Sale Accounting Date:03/23/2023

Sale Item Reference Number	Effective Date
18066420	12/15/2022

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I20233MH15026025	2202	CLAIMS IN EXCESS OF 20	$50.00	Deposit Account

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
18/066,420	12/15/2022		1480	148165.001401	23	1

CONFIRMATION NO. 1909

78442
Troutman Pepper Hamilton Sanders LLP
899 Cassatt Road
400 Berwyn Park
Berwyn, PA 19312

FILING RECEIPT

OC00000055233913

Date Mailed: 03/27/2023

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Ronnie M. Russell, Philadelphia, PA;
Philip R. Johnson, Philadelphia, PA;
James I. Andorko, Philadelphia, PA;
Mohit Sachdeva, Philadelphia, PA;
Bruce Schnepp, Havertown, PA;
Michael Marinelli, Philadelphia, PA;
Jeffrey Foss, Philadelphia, PA;
Debasish Boral, Philadelphia, PA;

Applicant(s)

INTERIUS BIOTHERAPEUTICS, INC., Philadelphia, PA;

Power of Attorney: The patent practitioners associated with Customer Number 78442

Domestic Priority data as claimed by applicant

This appln claims benefit of 63/289,888 12/15/2021
and claims benefit of 63/289,977 12/15/2021
and claims benefit of 63/266,044 12/27/2021
and claims benefit of 63/267,039 01/21/2022

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 03/23/2023
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 18/066,420**
Projected Publication Date: To Be Determined - pending completion of Omitted Items.
Non-Publication Request: No
Early Publication Request: No
**** SMALL ENTITY ****
Title

PSEUDOTYPED VIRAL PARTICLES, COMPOSITIONS COMPRISING THE SAME, AND USES THEREOF

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor

community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 18/066,420

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	80		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	350		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	400		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	23 minus 20 =	* 3	x 50 =	350	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*	x =	0		x =	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			420			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			x =	0		x =	
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	1250		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	*	=			OR		
	Independent (37 CFR 1.16(h))	*	Minus	*	=	x =	x =	OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	*	=	x =	x =	OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	*	=	x =	x =	OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
18/066,420	12/15/2022	Ronnie M. Russell	148165.001401

CONFIRMATION NO. 1909

78442
Troutman Pepper Hamilton Sanders LLP
899 Cassatt Road
400 Berwyn Park
Berwyn, PA 19312

FORMALITIES LETTER

OC000000055233915

Date Mailed: 03/27/2023

NOTICE OF OMITTED ITEM(S) IN A NONPROVISIONAL APPLICATION
FILED UNDER 37 CFR 1.53(b)

A filing date has been accorded to the above-identified nonprovisional application; however, the following item(s) appear to have been omitted from the application:

- Figure(s) **1, 2, 9, 11, 12, 13, 14, 15** described in the specification.

Applicant must reply to this Notice within the time period set forth in this Notice to avoid abandonment of the above-identified nonprovisional application. Applicant must select one of the three following options and the reply must comply with the requirements set forth in the selected option and any other requirements set forth in this Notice. **The reply should also indicate which option applicant has selected.** Petitions in accordance with option I or II should be addressed to: Mail Stop Petitions, Commissioner for Patents, P.O. Box 1450, Alexandria VA 22313-1450.

I. **Petition for date of deposit**: Should applicant contend that the above-noted item(s) was in fact deposited in the U.S. Patent and Trademark Office (USPTO) with the nonprovisional application papers, a copy of this Notice a petition, the petition fee set forth in 37 CFR 1.17(f), and evidence of such deposit must be filed within **TWO MONTHS** of the date of this Notice. See 37 CFR 1.53(e)(2). THIS TWO MONTH PERIOD IS EXTENDABLE UNDER 37 CFR 1.136(a) or (b). The petition fee will be refunded if it is determined that the above-noted item(s) was received by the USPTO with the application papers deposited on filing.

Applicant should review their application file and determine whether their file differs from USPTO's records. In some situations, there may be a mislabeling of the drawings and/or a failure to describe in the brief description each of the figures as labeled. See 37 CFR 1.74, 1.77(b)(9), and 1.84(u)(1).

The brief description of the drawings in the specification must correspond with the figures (see 37 CFR 1.74 and 1.77(b)(9)), and the figures must be labeled in accordance with 37 CFR 1.84(u)(1). For example, if the brief description describes only Figure 1 and Figure 2, but the drawings show Fig. 1 and Fig. 2A and Fig. 2B, then applicant must file a substitute specification, which adds references to Figure 2A and Figure 2B and deletes the reference to Figure 2, in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125. As another example, if a drawing is labeled Figure 2, but Figure 2 includes multiple figures labeled as A, B, and C, then applicant must file replacement drawing sheets in compliance with 37 CFR 1.84 and 1.121(d), with figure labeling in accordance with 37 CFR 1.84(u)(1) (e.g. Fig. 2A, Fig. 2B, and Fig. 2C). If the specification does not refer to the figures as relabeled, then applicant must also file a substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125 that amends the specification to refer to the relabeled figures (e.g. Fig. 2A, Fig. 2B, and Fig. 2C). **A petition for date of deposit under option I should NOT be filed in situations where there is simply a mislabeling of the drawings and/or a failure to describe in the brief description each of the figures. Instead, applicant**

should file a reply under option III(A) below (including replacement drawing sheets and/or a substitute specification as described).

II. **Petition for later filing date**: Should applicant desire to supply the omitted item(s) and accept the date that such omitted item(s) was filed in the USPTO as the filing date of the above-identified application, a copy of this Notice, the omitted item(s), and a petition under 37 CFR 1.182 with the petition fee set forth in 37 CFR 1.17(f) requesting the later filing date must be filed within **TWO MONTHS** of the date of this Notice. THIS TWO MONTH PERIOD IS EXTENDABLE UNDER 37 CFR 1.136(a) or (b).

Applicant is advised that the filing fee required for an application is generally the filing fee in effect on the filing date accorded the application and that payment of the requisite basic filing fee on a date later than the filing date of the application requires payment of a surcharge (37 CFR 1.16(f)). To avoid processing delays and payment of a surcharge, applicant should submit any balance due for the requisite filing fee based on the later filing date being requested when submitting the omitted item(s) and the petition (and petition fee) requesting the later filing date.

III. **Accept the application as deposited by filing an appropriate amendment**: Applicant may accept the application as deposited in the USPTO by filing an appropriate amendment as set forth in either (A) or (B) below within **TWO MONTHS** of the date of this Notice. THIS TWO MONTH PERIOD IS EXTENDABLE UNDER 37 CFR 1.136(a) or (b). The filing date of the application will remain the date of deposit of the application papers in the USPTO, and original application papers (i.e. the original disclosure of the invention) will include only those application papers present in the USPTO on the date of deposit. A petition is not required for this option.

(A) If applicant chooses to accept the application as deposited without adding the subject matter that was in the omitted item(s), applicant is required to submit one or more of the following items without adding any new matter (see 35 U.S.C. 132(a)):

- **To remove a missing figure of the drawings or to correct the figure labeling**:
  - replacement drawing sheets in compliance with 37 CFR 1.121(d) to renumber the drawing figures consecutively (if necessary), and/or
  - a substitute specification (excluding claims) in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125 that amends the specification to cancel any references to any omitted drawing(s) and corrects the references in the specification to the drawing figures to correspond with any relabeled drawing figures.
- A proper substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125 consists of:
  - A copy of the previously-submitted specification, with deletions shown with strikethrough or brackets and insertions shown with underlining (marked-up version) unless it is indicated above that a marked-up version is not required for each of the correction(s).
  - A copy of the amended specification without markings (clean version).
  - A statement that the substitute specification contains no new matter, in compliance with 37 CFR 1.121(b)(3) and 1.125.

(B) Alternatively, if applicant chooses to accept the application as deposited but wishes to add the subject matter in the omitted items(s) by relying on an incorporation by reference under 37 CFR 1.57 or other portions of the original disclosure, applicant is required to submit the following without adding any new matter (see 35 U.S.C. 132(a)):

- **To add a missing figure of the drawings**: new and replacement drawing sheets in compliance with 37 CFR 1.121(d);

If applicant is relying on an incorporation by reference under 37 CFR 1.57 to add the omitted subject matter, then applicant must also comply with the requirements of 37 CFR 1.57.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be addressed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

REPLY VIA ELECTRONIC FILING

A reply may be submitted via electronic filing only by registered users of the appropriate USPTO patent electronic filing system and must include:

- A separate document having the document description, "**Applicant response to Pre-Exam Formalities Notice**"; and
- the item(s) for which a reply period is set forth above.

For more information about electronic filing, contact the Patent Electronic Business Center (EBC) 1-866-217-9197 or visit https://www.uspto.gov/ebc

NOTE: If the reply is not being submitted electronically, a copy of this notice must be included.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/tle/

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82A (07-13)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	18/066,420
Filing Date	December 15, 2022
First Named Inventor	Ronnie M. Russell
Title	PSEUDOTYPED VIRAL PARTICLES, COMPOSITIONS COMPRISING THE SAME, AND USES THEREOF
Art Unit	Not yet assigned
Examiner Name	Not yet assigned
Attorney Docket Number	INH-014US

SIGNATURE of Applicant or Patent Practitioner

Signature	/Daniel M. Scolnick/	Date (Optional)	
Name	Daniel M. Scolnick/	Registration Number	52,201
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ *Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 051414

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

INTERIUS BIOTHERAPEUTICS, INC.

☐ Inventor or Joint Inventor (title not required below)

☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	Dora Mitchell	Date (Optional)	
Name	DORA MITCHELL		
Title	3/15/2023		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/066,420	**04/06/2023 10:47:10 AM ET**	**148165.001401**

Title of Invention

PSEUDOTYPED VIRAL PARTICLES, COMPOSITIONS COMPRISING THE SAME, AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	1909	FILED BY	Madison Sauer
PATENT CENTER #	61876857	FILING DATE	12/15/2022
CUSTOMER #	78442	FIRST NAMED INVENTOR	Ronnie M. Russell
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Daniel Scolnick

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
INH-014US POA.pdf	2	Power of Attorney	13390 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
INH-014US POA.pdf	769F74CCC1D89CC55D0F90C886BA448C1966CB8CC9BAEC94 9EBB89B5292CAF1354D9C371E996CEE389B481C04DABC630 EFF4CDD3D5216B82E8501335D115EC8C

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized

by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Attorney Docket No: INH-014US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Ronnie M. Russell et al.
Application No.	:	18/066,420
Filed	:	December 15, 2022
Group Art Unit	:	Not yet assigned
Confirmation No.	:	1909
Examiner	:	Not yet assigned

Title: PSEUDOTYPED VIRAL PARTICLES, COMPOSITIONS COMPRISING THE SAME, AND USES THEREOF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO PRE-EXAM FORMALITIES NOTICE

Dear Commissioner:

The enclosed amendments and response are in reply to the Notice of Omitted Items mailed March 27, 2023 in the above referenced application. A two month shortened statutory period for reply is set forth in the Notice. Accordingly, this response is timely filed.

Amendments to the Specification begin on page 2;
Listing of the Claims begin on page 3; and
Remarks begin on page 6.

AMENDMENTS TO THE SPECIFICATION

Please replace the Specification with the Substitute Specification with markings attached herewith. Additionally, a Substitute Specification without markings is included in compliance with 37 C.F.R. § 1.125(c).

LISTING OF THE CLAIMS

This listing of the claims will replace all prior versions, or listings of the claims in the present application.

1 - 70. (Cancelled)

71. (Previously Presented) A polypeptide comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 2 and comprises a mutation that corresponds to a mutation at position 182 of SEQ ID NO: 2.

72. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide comprises an amino acid sequence that is at least 96% identical to the amino acid sequence of SEQ ID NO: 2 and comprises a mutation that corresponds to a mutation at position 182 of SEQ ID NO: 2.

73. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide comprises an amino acid sequence that is at least 97% identical to the amino acid sequence of SEQ ID NO: 2 and comprises a mutation that corresponds to a mutation at position 182 of SEQ ID NO: 2.

74. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide comprises an amino acid sequence that is at least 98% identical to the amino acid sequence of SEQ ID NO: 2 and comprises a mutation that corresponds to a mutation at position 182 of SEQ ID NO: 2.

75. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide comprises an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO: 2 and comprises a mutation that corresponds to a mutation at position 182 of SEQ ID NO: 2.

76. (Previously Presented) The polypeptide of claim 1, wherein the mutation at position is a I182E mutation as compared to SEQ ID NO: 2.

77. (Previously Presented) The polypeptide of claim 1, wherein the mutation at position 182 comprises a I182D mutation as compared to SEQ ID NO: 2.

78. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide further comprises a mutation that corresponds to a mutation at position 214, a mutation at position 352, or a mutation at both position 214 and position 352 of SEQ ID NO: 2.

79. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide further comprises a T214N mutation, a T352A mutation, or a combination thereof, as compared to SEQ ID NO: 2.

80. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide further comprises a T214N mutation and a T352A mutation as compared to SEQ ID NO: 2.

81. (Previously Presented) The polypeptide of claim 1, wherein the mutation at position 182 comprises a I182E or I182D mutation as compared to SEQ ID NO: 2 and wherein the polypeptide further comprises a T214N mutation, a T352A mutation, or a combination thereof, as compared to SEQ ID NO: 2.

82. (Previously Presented) The polypeptide of claim 1, wherein the mutation at position 182 comprises a I182E mutation as compared to SEQ ID NO: 2 and wherein the polypeptide further comprises a T214N mutation, a T352A mutation, or a combination thereof, as compared to SEQ ID NO: 2.

83. (Previously Presented) The polypeptide of claim 1, wherein the mutation at position 182 comprises a I182E or I182D mutation as compared to SEQ ID NO: 2 and wherein the polypeptide further comprises a T214N mutation and a T352A mutation as compared to SEQ ID NO: 2.

84. (Previously Presented) The polypeptide of claim 1, wherein the mutation at position 182 comprises a I182E mutation as compared to SEQ ID NO: 2 and wherein the polypeptide further comprises a T214N mutation and a T352A mutation as compared to SEQ ID NO: 2.

85. (Previously Presented) The polypeptide of claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 23.

86. (Previously Presented) A nucleic acid molecule encoding the polypeptide of claim 1.

87. (Previously Presented) A vector comprising the nucleic acid molecule of claim 86.

88. (Previously Presented) A cell comprising the nucleic acid molecule of claim 86.

89. (Previously Presented) A viral particle comprising the polypeptide of claim 1.

90. (Previously Presented) The viral particle of claim 89, wherein the viral particle is a lentivirus comprising the polypeptide.

91. (Previously Presented) The viral particle of claim 89, wherein the viral particle further comprises a heterologous nucleic acid molecule encoding a heterologous molecule of interest.

92. (Previously Presented) The viral particle of claim 91, wherein the heterologous molecule of interest is an siRNA, an shRNA, a non-coding RNA, a polypeptide, a viral payload, a viral genome, or a combination thereof.

93. (Previously Presented) The viral particle of claim 91, wherein the heterologous molecule of interest is a chimeric antigen receptor ("CAR").

REMARKS

The specification has been amended to correct figure labeling in the Brief Description of the Drawings section of the application with respect to Figures 1, 2, 9, 11, 12, 13, 14, and 15. Applicant submits that all necessary figures were filed on the filing date of the above-referenced application, no figures were missing. Accordingly, no new matter has been added. Further, paragraphs [0077] and [0086] of the specification as filed contained no text. Therefore, the paragraph numbering for [0077] and [0086] has been deleted, and the remaining paragraphs have been renumbered accordingly.

For the Office's convenience, Applicant lists the pending claims here without amendment. Accordingly, claims 71-93 will be pending.

Applicant respectfully submits that all amendments are made without prejudice or disclaimer solely in order to expedite prosecution. Applicant further expressly reserves the right to file additional claims in further applications, whether such claims have been previously canceled, withdrawn, or are newly presented.

As required under the rules of Prioritized Examination, the present application does not contain more than four independent claims, more than thirty total claims, or any multiple dependent claims. Therefore, Applicant respectfully requests that the Request for Prioritized Examination under 37 C.F.R. § 1.102(e) be granted.

AUTHORIZATION

In view of the foregoing, Applicant respectfully submits that the claims are in condition for allowance. A notice of allowance is, therefore, earnestly solicited. If the Examiner believes for any reason that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned directly.

The Commissioner is hereby authorized to charge any additional fees which may be required for this submission, or credit any overpayment, to Deposit Account No. 07-1700.

Respectfully submitted,
/Christopher R. Bodle /

Christopher R. Bodle, Ph.D.
Registration No. 80,876

GOODWIN PROCTOR LLP
2929 Arch Street, Suite 1700
Philadelphia, PA 19104
Telephone 1-445-207-7829
Date: April 6, 2023

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/066,420	**04/06/2023 04:39:53 PM ET**	**148165.001401**

Title of Invention

PSEUDOTYPED VIRAL PARTICLES, COMPOSITIONS COMPRISING THE SAME, AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	1909	FILED BY	Madison Sauer
PATENT CENTER #	61879689	FILING DATE	12/15/2022
CUSTOMER #	78442	FIRST NAMED INVENTOR	Ronnie M. Russell
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Christopher Bodle

Documents

TOTAL DOCUMENTS: 6

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
INH-014US Response to Notice of Omitted Items filed April 6 2023.pdf		7	-	478 KB
INH-014US Response to Notice of Omitted Items filed April 6 2023-PEFR.pdf	(1-1)	1	Applicant Response to Pre-Exam Formalities Notice	78 KB
INH-014US Response to Notice of Omitted Items filed April 6 2023-SPEC.pdf	(2-2)	1	Specification	77 KB
INH-014US Response to Notice of Omitted Items filed	(3-5)	3	Claims	81 KB

April 6 2023-CLM.pdf				
INH-014US Response to Notice of Omitted Items filed April 6 2023-REM.pdf	(6-7)	2	Applicant Arguments/Remarks Made in an Amendment	93 KB
INH-014US CLEAN Substitute Specification filed April 6 2023.pdf		164	Specification	970 KB
INH-014US MARKED UP Substitute Specification filed April 6 2023.pdf		164	Specification	1987 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
INH-014US Response to Notice of Omitted Items filed April 6 2023.pdf	EE0E9F1FE849D488D1AF33AD8F0877A8CF328418EA29B134C CD7DB5243CC5DBFC7FA336D0E5E849F6D06431E2EFEB0E7A A1E012289EACDF2345DC63FF55B299F
INH-014US Response to Notice of Omitted Items filed April 6 2023-PEFR.pdf	EE3E0D41CDCF407E60FACA9014253E392B95167765B564D15 808C09E9C190AFD6DE8056DBE0514E9CE5AAB0BB9F77D556 BD1A14B39CFB412F57D75B0B0768A3C
INH-014US Response to Notice of Omitted Items filed April 6 2023-SPEC.pdf	0185E821CF5031740B5A32964904A67C125A71BD112625880B6 72FD2E4942E7B991700C2B65092DDBBF348A77B04E9EDB31D 5A26231124351EB884FB84BB7D37
INH-014US Response to Notice of Omitted Items filed April 6 2023-CLM.pdf	07C523F4BD8CDD432C4D41D5C754CFE4062796B9844EC088F 3E0C7EAF2F21B504D240640EF31CC218375350284F0B2D201C FA1FDE74AC0CF28BD23BF534F41F3
INH-014US Response to Notice of Omitted Items filed April 6 2023-REM.pdf	BD59F6BF5C27F9DEEF24CABE05A84FAD394BF4E0F082A4B8 C8A1883C3CD7832BD69091E2814C4EB0964DFB893B444CC4E DAD64421562B05F74AA97A8CE2E0BA0

INH-014US CLEAN Substitute Specification filed April 6 2023.pdf	E26E239DF809D4C0F592BA06BD24DBB715D0AEF9B9373D25A061B275C5E89279BFF860C5813180016B93F944A3B91808F8E0A2C758325605 4B7AFF5A1309DADF
INH-014US MARKED UP Substitute Specification filed April 6 2023.pdf	F0937B9563A008FEFA4723F9DDD0BD11FE4F0FE350B7472657E3F3C184DF1CF856CBDA1630C2B72007C483BE3A6E34A28E883BD94DE603E222D3F92BB0FC03E7

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PSEUDOTYPED VIRAL PARTICLES, COMPOSITIONS COMPRISING THE SAME, AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Ser. No. 63/289,888 filed 15 December 2021, and U.S. Provisional Application Ser. No. 63/289,977 filed 15 December 2021, and U.S. Provisional Application Ser. No. 63/266,044 filed 27 December 2021, and U.S. Provisional Application Ser. No. 63/267,039 filed 21 January 2022, each of which is hereby incorporated by reference in their entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0002] The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on 28 November 2022, is named "148165.001401_SL.xml" and is 86.3 kilobytes in size.

BACKGROUND

[0003] Vesicular stomatitis virus (VSV) is an enveloped, negative-strand RNA virus that belongs to the Vesiculovirus genus of the Rhabdovirus family. It is an arbovirus which can infect insects, cattle, horses and pigs. VSV genome encodes five structural proteins among which include a single transmembrane glycoprotein (G). The glycoprotein is a classic type I membrane glycoprotein with an amino-terminal signal peptide, an ectodomain of about 450 amino acids, a single alpha helical transmembrane segment and a small intraviral carboxy-terminal domain. The signal peptide is cleaved in the lumen of the endoplasmic reticulum and the native glycoprotein consists in the ectodomain, the transmembrane domain and the intraviral domain.

[0004] G plays a critical role during the initial steps of virus infection (Molecular and Cellular Aspects of Rhabdovirus Entry. Viruses 4, 117-139.), which is hereby incorporated by reference in its entirety. First, it is responsible for virus attachment to specific receptors. After binding, virions enter the cell by a clathrin-mediated endocytic pathway. In the acidic environment of the endocytic vesicle, G triggers the fusion between the viral and endosomal membranes, which releases the genome in the cytosol for the subsequent steps of infection. Fusion is catalyzed by a

low-pH-induced large structural transition from a pre-toward a post-fusion conformation which are both trimeric (Roche, S., Bressanelli, S., Rey, F. A., and Gaudin, Y. (2006). Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science 313, 187-191. Roche, S., Rey, F. A., Gaudin, Y., and Bressanelli, S. (2007). Structure of the prefusion form of the vesicular stomatitis virus glycoprotein g. Science 315, 843-848), each of which is hereby incorporated by reference in its entirety).

[0005] The polypeptide chain of G ectodomain folds into three distinct domains which are the fusion domain (FD), the pleckstrin homology domain (PHD), and the trimerization domain (TrD). During the structural transition, the FD, the PHD and the TrD retain their tertiary structure. Nevertheless, they undergo large rearrangements in their relative orientation due to secondary changes in hinge segments (S1 to S5) which refold during the low-pH induced conformational change (Roche et al., 2006; Roche et al., 2007).

[0006] It has been shown that low-density lipoprotein receptor (LDL-R) and other members of this receptor family serve as VSV receptors (Finkelshtein, D., Werman, A., Novick, D., Barak, S., and Rubinstein, M. (2013). LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America 110, 7306-7311, which is hereby incorporated by reference in its entirety). VSV-G can be used for pseudotyping other viruses and VSV-G-pseudotyped lentiviruses (VSV-G-LVs) exhibit the same broad tropism as VSV. However, this broad tropism can inhibit the selective targeting of specific cell types. Therefore, there is a need, for modified (mutated or mutant) VSV-G proteins that can be used to pseudotype viruses that abrogate its binding to the LDL receptor. The present embodiments, fulfill these needs as well as others.

[0007] The human CD7 molecule is a cell surface glycoprotein with a molecular weight of approximately 40 kDa belonging to the immunoglobulin superfamily. The CD7 molecule is mainly expressed on the surface of most thymocytes, more than 85% of the surface of peripheral blood T lymphocytes and the surface of natural killer cells. The embodiments disclosed herein provide for polypeptide and antibodies against CD7, compositions comprising the same, and uses thereof.

[0008] CD8 (cluster of differentiation 8) is a transmembrane glycoprotein which is a specific marker for a subclass of T-cells (which includes cytotoxic T-cells). Without wishing to be bound to a particular theory, CD8 assembles as either a heterodimer of the CD8 alpha and CD8

beta subunits or a CD8 alpha homodimer. The assembled dimeric CD8 complex acts as a co-receptor together with the T cell receptor (TCR) to recognize antigen presentation by MHC class I cells. CD8 plays a role in the development of T-cells and activation of mature T-cells. Changes in T-cell localization can reflect the progression of an immune response and can occur over time. The embodiments disclosed herein provide for polypeptide and antibodies against CD8, compositions comprising the same, and uses thereof.

BRIEF SUMMARY

[0009] In some embodiments, a VSV-G polypeptide is provided. In some embodiments, the VSV-G polypeptide comprises a mutation at position 182 of SEQ ID NO: 2. In some embodiments, the polypeptide comprises an I182E or I182D mutation as compared to SEQ ID NO: 2. In some embodiments, the VSV-G polypeptide comprises the sequence of SEQ ID NO: 4. In some embodiments, the VSV-G polypeptide comprises the sequence of SEQ ID NO: 5.

[0010] In some embodiments, the VSV-G polypeptide further comprises a mutation in the VSV-G protein that corresponds to a position of 8, 10, 47, 209, and/or 354 as compared to SEQ ID NO: 2. In some embodiments, the VSV-G polypeptide comprises a H8A and/or a K47Q substitution. In some embodiments, the VSV-G polypeptide comprises a substitution at position 10 as compared to SEQ ID NO: 2. In some embodiments, the substitution at position 10 is Q10A, Q10R, or Q10K.

[0011] In some embodiments, a VSV-G polypeptide is provided. In some embodiments, the VSV-G polypeptide comprises a substitution at position I182 and at least one of T214, and T352 of SEQ ID NO: 2. In some embodiments, the VSV-G polypeptide comprises a substitution at position I182, T214, and T352 of SEQ ID NO: 2. In some embodiments, the VSV-G polypeptide comprises the sequence of SEQ ID NO: 22. In some embodiments, the VSV-G polypeptide comprises the sequence of SEQ ID NO: 23. In some embodiments, the VSV-G polypeptide comprises the sequence of SEQ ID NO: 24. In some embodiments, the VSV-G polypeptide comprises the sequence of SEQ ID NO: 25.

[0012] In some embodiments, a nucleic acid molecule is provided. In some embodiments, the nucleic acid molecule encodes for the VSV-G polypeptide as provided for herein.

[0013] In some embodiments, a vector is provided comprising a nucleic acid molecule as

provided for herein.

[0014] In some embodiments, a plasmid is provided comprising a nucleic acid molecule as provided for herein.

[0015] In some embodiments, a viral particle is provided. In some embodiments, the viral particle comprises a VSV-G polypeptide as provided for herein. In some embodiments, the viral particle further comprises a targeting moiety. In some embodiments, the viral particle further comprises a nucleic acid molecule encoding for a heterologous molecule of interest.

[0016] In some embodiments, a method of delivering a heterologous molecule of interest to a target cell is provided. In some embodiments, the method comprises contacting the cell with a viral vector comprising a VSV-G protein as provided for herein, a targeting moiety that binds to the target cell, and a nucleic acid molecule encoding a heterologous molecule of interest. In some embodiments, the heterologous molecule of interest is a chimeric antigen receptor.

[0017] In some embodiments, a method of delivering a heterologous molecule of interest to a target cell in a subject is provided. In some embodiments, the method comprises contacting the cell with a viral vector comprising a VSV-G protein as provided for herein, a targeting moiety that binds to the target cell, and a nucleic acid molecule encoding a heterologous molecule of interest. In some embodiments, the heterologous molecule of interest is a chimeric antigen receptor.

[0018] In some embodiments, a method of treating cancer in a subject is provided. In some embodiments, the method comprises contacting the cell with a viral vector comprising a VSV-G protein as provided for herein, a targeting moiety that binds to the target cell, and a nucleic acid molecule encoding a heterologous molecule of interest. In some embodiments, the heterologous molecule of interest is a chimeric antigen receptor.

[0019] In some embodiments, the targeting moiety of the viral particle binds to CD7. In some embodiments, the targeting moiety comprises a polypeptide comprising a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 35, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 36, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 37, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 38, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 39, a light chain CDR3 having the amino acid sequence of SEQ ID NO: 40. In some embodiments, the targeting moiety comprises a

polypeptide comprising a heavy chain variable region having the amino acid sequence of SEQ ID NO: 47 and a light chain variable region having the amino acid sequence of SEQ ID NO: 48.

[0020] In some embodiments, the targeting moiety that binds to CD7 comprises a polypeptide comprising a sequence having at least 90% sequence identity to SEQ ID NO: 51, at least 95% sequence identity to SEQ ID NO: 51, at least 99% sequence identity to SEQ ID NO: 51, or a sequence as set forth in SEQ ID NO: 51. In some embodiments, the targeting moiety that binds to CD7 comprises a polypeptide comprising the sequence of SEQ ID NO: 51.

[0021] In some embodiments, the targeting moiety that binds to CD7 comprises a polypeptide comprising a sequence having at least 90% sequence identity to SEQ ID NO: 52, at least 95% sequence identity to SEQ ID NO: 52, at least 99% sequence identity to SEQ ID NO: 52, or a sequence as set forth in SEQ ID NO: 52. In some embodiments, the targeting moiety that binds to CD7 comprises a polypeptide comprising the sequence of SEQ ID NO: 52.

[0022] In some embodiments, the targeting moiety of the viral particle binds to CD8. In some embodiments, the targeting moiety comprises a polypeptide comprising a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 55, a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 56, a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 57, a light chain CDR1 having the amino acid sequence of SEQ ID NO: 58, a light chain CDR2 having the amino acid sequence of SEQ ID NO: 59, a light chain CDR3 having the amino acid sequence of SEQ ID NO: 60. In some embodiments, the targeting moiety comprises a polypeptide comprising a heavy chain variable region having the amino acid sequence of SEQ ID NO: 67 and a light chain variable region having the amino acid sequence of SEQ ID NO: 68.

[0023] In some embodiments, the targeting moiety that binds to CD8 comprises a polypeptide comprising a sequence having at least 90% sequence identity to SEQ ID NO: 69, at least 95% sequence identity to SEQ ID NO: 69, at least 99% sequence identity to SEQ ID NO: 69, or a sequence as set forth in SEQ ID NO: 69. In some embodiments, the targeting moiety that binds to CD7 comprises a polypeptide comprising the sequence of SEQ ID NO: 69.

[0024] In some embodiments, the targeting moiety that binds to CD7 comprises a polypeptide comprising a sequence having at least 90% sequence identity to SEQ ID NO: 70, at least 95% sequence identity to SEQ ID NO: 70, at least 99% sequence identity to SEQ ID NO: 70, or a sequence as set forth in SEQ ID NO: 70. In some embodiments, the targeting moiety that

binds to CD7 comprises a polypeptide comprising the sequence of SEQ ID NO: 70.

[0025] In some embodiments, a method of delivering a heterologous molecule of interest to a target cell is provided. In some embodiments, the method comprises contacting the cell with a viral vector comprising a VSV-G protein as provided for herein, a targeting moiety as provided for herein that binds to the target cell, and a nucleic acid molecule encoding a heterologous molecule of interest. In some embodiments the targeting moiety binds to CD7. In some embodiments, the targeting moiety binds to CB8. In some embodiments, the heterologous molecule of interest is a chimeric antigen receptor.

[0026] In some embodiments, a method of delivering a heterologous molecule of interest to a target cell in a subject is provided. In some embodiments, the method comprises contacting the cell with a viral vector comprising a VSV-G protein as provided for herein, a targeting moiety as provided for herein that binds to the target cell, and a nucleic acid molecule encoding a heterologous molecule of interest. In some embodiments the targeting moiety binds to CD7. In some embodiments, the targeting moiety binds to CB8. In some embodiments, the heterologous molecule of interest is a chimeric antigen receptor.

[0027] In some embodiments, a method of treating cancer in a subject is provided. In some embodiments, the method comprises contacting the cell with a viral vector comprising a VSV-G protein as provided for herein, a targeting moiety as provided for herein that binds to the target cell, and a nucleic acid molecule encoding a heterologous molecule of interest. In some embodiments the targeting moiety binds to CD7. In some embodiments, the targeting moiety binds to CB8. In some embodiments, the heterologous molecule of interest is a chimeric antigen receptor.

BRIEF DESCRIPTION OF FIGURES

[0028] FIG. 1A and FIG. 1B illustrate[[s]] crystal structures of VSV-G bound to LDL-R. FIG. 1A illustrates the crystal structure of VSV-G bound to CR3 of the LDL-R. FIG. 1B illustrates the crystal structure of VSV-G bound to CR2 of the LDL-R.

[0029] FIG. 2A and FIG. 2B illustrate[[s]] the effect of adding negatively charged amino acids to the VSV-G:LDL-R binding interface on native tropism and fusogenicity. FIG.2A illustrates the titration of VSV-G constructs on SupT1 cells. FIG. 2B illustrates functional titer of each construct calculated from the titration in FIG. 2A.

[0030] FIG. 3 illustrates an alignment of the ectodomains of different VSV-G proteins from different strains.

[0031] FIG. 4 illustrates the effect of various VSV-G mutations on the serum stability of viral constructs in combination with a CD7 binder.

[0032] FIG. 5 illustrates the effect of various VSV-G mutations on the serum stability of viral constructs in combination with a CD7 binder.

[0033] FIG. 6A-L show flow cytometry data of human PBMCs transduced with exemplary vectors comprising CD7 binders as disclosed herein.

[0034] FIG. 6M shows flow cytometry data of human PBMCs transduced with exemplary vectors comprising CD7 binders as disclosed herein.

[0035] FIG. 7A-L shows flow cytometry data of non-human primate PBMCs transduced with exemplary vectors comprising CD7 binders as disclosed herein.

[0036] FIG. 7M shows flow cytometry data of non-human primate PBMCs transduced with exemplary vectors comprising CD7 binders as disclosed herein.

[0037] FIG. 8A shows flow cytometry data of human PBMCs transduced with exemplary vectors comprising CD8 binders as disclosed herein.

[0038] FIG. 8B shows flow cytometry data of non-human primate PBMCs transduced with exemplary vectors comprising CD8 binders as disclosed herein.

[0039] FIG. 9A and FIG. 9B illustrate[[s]] GFP or CAR transduction of SupT1 cells utilizing VSV-G pseudotyped lentiviral particles harboring CD7 or CD8 binders as provided for herein. FIG. 9A illustrates GFP transduction. FIG. 9B illustrates CAR transduction.

[0040] FIG. 10A illustrates the ability of VSV-G* pseudotyped lentiviral particles harboring a CD7 binder to transduce SupT1 cells as well as human and non-human primate PBMCs. FIG. 10B illustrates the transduction of cells in the absence of the CD7 binder. FIG. 10C illustrates the transduction of human and non-human primate PBMCs in terms of MOI calculated from SupT1 titration. VSV-G* denotes VSV-G polypeptide comprising I182E, T214N, T352A, which corresponds to a mature polypeptide of SEQ ID NO: 23 or SEQ ID NO: 25 (with I196E, T230N and T368A mutations (with leader sequence and adjusted numbering).

[0041] FIG. 11A and FIG. 11B illustrate[[s]] the off target transduction of VSV-G* pseudotyped lentiviral particles harboring a CD7 binder in a panel of B-cell cell lines as compared

to control SupT1 cells. FIG. 11A illustrates the data for GFP transduction. FIG. 11B illustrates the data for CAR20-T2A-GFP transduction. VSV-G* denotes a VSV-G polypeptide comprising I182E, T214N, and T352A mutations as provided for herein.

[0042] FIG. 12A and FIG. 12B illustrate[[s]] the ability of VSV-G* pseudotyped lentiviral particles utilizing CD7 binders and a CD20-CAR transgene as provided for herein to kill Duadi lymphoma cells (FIG. 12A) or Raji lymphoma cells (FIG. 12B). CAR20 constructs utilized antigen binding domains comprising rituximab, or CD20AB1 SEQ ID NOs 75 or 76 as provided for herein.

[0043] FIG. 13A and FIG. 13B illustrate[[s]] the ability of VSV-G* pseudotyped lentiviral particles utilizing CD7 binders and a CD20-CAR transgene as provided for herein to deplete B-cells *in vivo* in huCD34 NSG mice. B-cells were detected via assessment for CD20 (FIG. 13A) or CD19 (FIG. 13B).

[0044] FIG. 14A and FIG. 14B illustrate[[s]] the ability of VSV-G* pseudotyped lentiviral particles utilizing CD7 binders and a CD20-CAR transgene as provided for herein to prevent tumor formation *in vivo*. FIG. 14A depicts the experimental design. FIG. 14B illustrates that mice receiving i.v. lentiviral particles as provided for herein prior to Raji tumor infusion had significantly lower tumor burden than mice receiving control (GFP) vector or untreated mice. Tumor burden measured via IVIS imaging.

[0045] FIG. 15A and FIG. 15B illustrate[[s]] the ability of VSV-G* pseudotyped lentiviral particles utilizing CD7 binders and a CD20-CAR transgene as provided for herein to eliminate estabilished Raji tumors *in vivo*. FIG. 15A depicts the experimental design. FIG. 15B illustrates that mice receiving i.v. lentiviral particles as provided for herein after 6 days after Raji tumor infusion had their tumor burdens diminished below the limit of detection. Tumor burden measured via IVIS imaging.

DETAILED DESCRIPTION

[0046] Provided for herein are mutant VSV-G proteins that can be used, for example, to pseudotype a virus, such as a lentivirus. In some embodiments, the pseudotyped viral-like particles are pseudotyped using viral glycoproteins of a vesicular stomatitis New Jersey virus strain, a vesicular stomatitis Indiana virus strain, a vesicular stomatitis Alagoas virus strain, a vesicular

stromatitis Maraba virus strain, or a vesicular stomatitis Carajas virus strain.

[0047] The pseudotyped viruses comprising the mutant VSV-G proteins can be used in conjunction with a targeting moiety to facilitate the fusion of the pseudotyped virus with a specific cell or tissue based on the expression of the target on the cell or the tissue.

[0048] Unless defined otherwise, all technical and scientific terms have the same meaning as is commonly understood by one of ordinary skill in the art to which the embodiments disclosed belongs.

[0049] As used herein, the terms "a" or "an" means that "at least one" or "one or more" unless the context clearly indicates otherwise.

[0050] As used herein, the term "about" means that the numerical value is approximate and small variations would not significantly affect the practice of the disclosed embodiments. Where a numerical limitation is used, unless indicated otherwise by the context, "about" means the numerical value can vary by ±10% and remain within the scope of the disclosed embodiments. Additionally, where a phrase recites "about x to y," the term "about" modifies both x and y and can be used interchangeably with the phrase "about x to about y" unless context dictates differently.

[0051] "Activation," as used herein in reference to a T cell, refers to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation. Activation can also be associated with induced cytokine production, and detectable effector functions. The term "activated T cells" refers to, among other things, T cells that are undergoing cell division.

[0052] As used herein, to "alleviate" a disease means reducing the severity of one or more symptoms of the disease.

[0053] The term "antigen" as used herein is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. The term "antigen" can also refer to a molecule that an antibody or antibody-like molecule can bind to or is recognized by the antibody or antibody-like molecule.

[0054] The term "antibody molecule," "antibody" or antigen binding domain, as that term is used herein, refers to a polypeptide, e.g., an immunoglobulin chain or fragment thereof,

comprising at least one functional immunoglobulin variable domain sequence. An antibody molecule encompasses antibodies (e.g., full-length antibodies) and antibody fragments. In some embodiments, an antibody molecule comprises an antigen binding or functional fragment of a full-length antibody, or a full-length immunoglobulin chain. For example, a full-length antibody is an immunoglobulin (Ig) molecule (e.g., an IgG antibody) that is naturally occurring or formed by normal immunoglobulin gene fragment recombinatorial processes. In embodiments, an antibody molecule refers to an immunologically active, antigen binding portion of an immunoglobulin molecule, such as an antibody fragment. An antibody fragment, e.g., functional fragment, comprises a portion of an antibody, e.g., Fab, Fab', F(ab')2, F(ab)2, variable fragment (Fv), domain antibody (dAb), or single chain variable fragment (scFv). A functional antibody fragment binds to the same antigen as that recognized by the intact (e.g., full-length) antibody. The terms "antibody fragment" or "functional fragment" also include isolated fragments consisting of the variable regions, such as the "Fv" fragments consisting of the variable regions of the heavy and light chains or recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins"). In some embodiments, an antibody fragment does not include portions of antibodies without antigen binding activity, such as Fc fragments or single amino acid residues. Exemplary antibody molecules include full-length antibodies and antibody fragments, e.g., dAb (domain antibody), single chain, Fab, Fab', and F(ab')2 fragments, and single chain variable fragments (scFvs).

[0055] The term "antibody molecule" also encompasses whole or antigen binding fragments of domain, or single domain, antibodies, which can also be referred to as "sdAb" or "VHH." Domain antibodies comprise either VH or VL that can act as stand-alone, antibody fragments. Additionally, domain antibodies include heavy-chain-only antibodies (HCAbs). Domain antibodies also include a CH2 domain of an IgG as the base scaffold into which CDR loops are grafted. It can also be generally defined as a polypeptide or protein comprising an amino acid sequence that is comprised of four framework regions interrupted by three complementarity determining regions. This is represented as FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. sdAbs can be produced in camelids such as llamas, but can also be synthetically generated using techniques that are well known in the art. The numbering of the amino acid residues of a sdAb or polypeptide is according to the general numbering for VH domains given by Kabat et al. ("Sequence of proteins

of immunological interest," US Public Health Services, NIH Bethesda, MD, Publication No. 91, which is hereby incorporated by reference). According to this numbering, FR1 of a sdAb comprises the amino acid residues at positions 1-30, CDR1 of a sdAb comprises the amino acid residues at positions 31-36, FR2 of a sdAb comprises the amino acids at positions 36-49, CDR2 of a sdAb comprises the amino acid residues at positions 50-65, FR3 of a sdAb comprises the amino acid residues at positions 66-94, CDR3 of a sdAb comprises the amino acid residues at positions 95-102, and FR4 of a sdAb comprises the amino acid residues at positions 103-113. Domain antibodies are also described in WO2004041862 and WO2016065323, each of which is hereby incorporated by reference. The domain antibodies can be a targeting moiety as described herein.

[0056] As used herein, unless otherwise indicated, "antibody fragment" or "antigen binding fragment" refers to antigen binding fragments of antibodies, *i.e.* antibody fragments that retain the ability to bind specifically to the antigen bound by the full-length antibody, *e.g.* fragments that retain one or more CDR regions. Examples of antibody binding fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules, *e.g.*, sc-Fv; nanobodies (single domain antibody) and multispecific antibodies formed from antibody fragments.

[0057] A "Fab fragment" is comprised of one light chain and the C_H1 and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule.

[0058] An "Fc" region contains two heavy chain fragments comprising the C_H2 and C_H3 domains of an antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the C_H3 domains.

[0059] A "Fab' fragment" contains one light chain and a portion or fragment of one heavy chain that contains the V_H domain and the C_H1 domain and also the region between the C_H1 and C_H2 domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form a $F(ab')_2$ molecule.

[0060] A "$F(ab')_2$ fragment" contains two light chains and two heavy chains containing a portion of the constant region between the C_H1 and C_H2 domains, such that an interchain disulfide bond is formed between the two heavy chains. A $F(ab')_2$ fragment thus is composed of two Fab'

fragments that are held together by a disulfide bond between the two heavy chains.

[0061] The "Fv region" comprises the variable regions from both the heavy and light chains, but lacks the constant regions.

[0062] The term "single-chain Fv" or "scFv" antibody refers to antibody fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see Pluckthun (1994) THE PHARMACOLOGY OF MONOCLONAL ANTIBODIES, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315. See also, International Patent Application Publication No. WO 88/01649 and U.S. Pat. Nos. 4,946, 778 and 5,260,203.

[0063] Antibody molecules can be monospecific (e.g., monovalent or bivalent), bispecific (e.g., bivalent, trivalent, tetravalent, pentavalent, or hexavalent), trispecific (e.g., trivalent, tetravalent, pentavalent, or hexavalent), or with higher orders of specificity (e.g, tetraspecific) and/or higher orders of valency beyond hexavalency. An antibody molecule can comprise a functional fragment of a light chain variable region and a functional fragment of a heavy chain variable region, or heavy and light chains may be fused together into a single polypeptide.

[0064] Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an "antigen" as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. Moreover, a skilled artisan will understand that an antigen need not be encoded by a "gene" at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

[0065] In certain embodiments, monoclonal antibodies herein also include camelized single domain antibodies. *See, e.g.*, Muyldermans *et al.* (2001) *Trends Biochem. Sci.* 26:230; Reichmann *et al.* (1999) *J. Immunol. Methods* 231:25; WO 94/04678; WO 94/25591; U.S. Pat. No. 6,005,079). In one embodiment, the present invention provides single domain antibodies

comprising two V_H domains with modifications such that single domain antibodies are formed.

[0066] As used herein, the term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L or V_L-V_H). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, *e.g.*, EP 404,097; WO 93/11161; and Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 6444-6448. For a review of engineered antibody variants generally see Holliger and Hudson (2005) *Nat. Biotechnol.* 23:1126-1136.

[0067] "Isolated antibody" refers to the purification status of a binding compound and in such context means the molecule is substantially free of other biological molecules such as nucleic acids, proteins, lipids, carbohydrates, or other material such as cellular debris and growth media. Generally, the term "isolated" is not intended to refer to a complete absence of such material or to an absence of water, buffers, or salts, unless they are present in amounts that substantially interfere with experimental or therapeutic use of the binding compound as described herein.

[0068] The term "monoclonal antibody", as used herein, refers to population of substantially homogeneous antibodies, *i.e.*, the antibody molecules comprising the population are identical in amino acid sequence except for possible naturally occurring mutations and/or post-translational modifications that may be present in minor amounts. In contrast, conventional (polyclonal) antibody preparations typically include a multitude of different antibodies having different amino acid sequences in their variable domains, particularly their CDRs, that are often specific for different epitopes. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.* (1975) *Nature* 256: 495, or may be made by recombinant DNA methods (*see, e.g.*, U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.* (1991) *Nature* 352: 624-628 and Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597, for example. *See also* Presta (2005) *J. Allergy Clin. Immunol.* 116:731.

[0069] As used herein, the term "humanized antibody" refers to forms of antibodies that contain sequences from both human and non-human (*e.g.*, murine, rat) antibodies. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all of the framework (FR) regions are those of a human immunoglobulin sequence. The humanized antibody may optionally comprise at least a portion of a human immunoglobulin constant region (Fc).

[0070] The term "fully human antibody" refers to an antibody that comprises human immunoglobulin protein sequences only. A fully human antibody may contain murine carbohydrate chains if produced in a mouse, in a mouse cell, or in a hybridoma derived from a mouse cell. Similarly, "mouse antibody" refers to an antibody that comprises mouse immunoglobulin sequences only. Alternatively, a fully human antibody may contain rat carbohydrate chains if produced in a rat, in a rat cell, or in a hybridoma derived from a rat cell. Similarly, "rat antibody" refers to an antibody that comprises rat immunoglobulin sequences only.

[0071] In some embodiments, the basic antibody structural unit comprises a tetramer. Each tetramer includes two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of the heavy chain may define a constant region primarily responsible for effector function. Typically, human light chains are classified as kappa and lambda light chains. Furthermore, human heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See generally, *Fundamental Immunology* Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989).

[0072] The variable regions of each light/heavy chain pair form the antibody binding site. Thus, in general, an intact antibody has two binding sites. However, in bifunctional or bispecific antibodies, the two binding sites are, in general, not the same.

[0073] Typically, the variable domains of both the heavy and light chains comprise three

hypervariable regions, also called complementarity determining regions (CDRs), located within relatively conserved framework regions (FR). The CDRs are usually aligned by the framework regions, enabling binding to a specific epitope. In general, from N-terminal to C-terminal, both light and heavy chains variable domains comprise FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is, generally, in accordance with the definitions of *Sequences of Proteins of Immunological Interest*, Kabat, *et al.*; National Institutes of Health, Bethesda, Md. ; 5th ed.; NIH Publ. No. 91-3242 (1991); Kabat (1978) Adv. Prot. Chem. 32:1-75; Kabat, *et al.*, (1977) J. Biol. Chem. 252:6609-6616; Chothia, *et al.*, (1987) J Mol. Biol. 196:901-917 or Chothia, *et al.*, (1989) Nature 342:878-883.

[0074] As used herein, the term "hypervariable region" refers to the amino acid residues of an antibody that are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (CDRL1), 50-56 (CDRL2) and 89-97 (CDRL3) in the light chain variable domain and residues 31-35 (CDRH1), 50-65 (CDRH2) and 95-102 (CDRH3) in the heavy chain variable domain; Kabat *et al.* (1991) Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md.) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (CDRL1), 50-52 (CDRL2) and 91-96 (CDRL3) in the light chain variable domain and 26-32 (CDRH1), 53-55 (CDRH2) and 96-101 (CDRH3) in the heavy chain variable domain; Chothia and Lesk (1987) *J. Mol. Biol.* 196: 901-917). The CDRs can also be referenced according to the IMGT system for the identification of CDRs, which is described in Lefranc MP. Unique database numbering system for immunogenetic analysis. Immunol Today (1997) 18:509. As used herein, the term "framework" or "FR" residues refers to those variable domain residues other than the hypervariable region residues defined herein as CDR residues. CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest can be derived from donor antibody variable heavy and light chain sequences, and include analogs of the naturally occurring CDRs, which analogs also share or retain the same antigen binding specificity and/or neutralizing ability as the donor antibody from which they were derived.

[0075] As used herein, "specific binding" or "immunospecific binding" or "binds immunospecifically" refer to antibody binding to a predetermined antigen at a much higher affinity than for another antigen(s). In some embodiments, the antibody binds the predetermined antigen

with a dissociation constant (K_D) of 10^{-7} M or less, and such K_D is at least two-fold less than its K_D for binding to a non-specific antigen (*e.g.*, BSA, casein, or another non-specific polypeptide).

[0076] Methods for determining mAb specificity and affinity by competitive inhibition can be found in Harlow, et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988), Colligan et al., eds., Current Protocols in Immunology, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), and Muller, Meth. Enzymol. 92:589 601 (1983), which references are entirely incorporated herein by reference.

~~[0077]~~

~~[0078]~~[0077] As used herein, the term "individual" or "subject," or "patient" used interchangeably, means any animal, including mammals, such as mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, or primates, such as humans. In some embodiments, the subject is a human. A subject that is "in need thereof" refers to a subject that has been identified as requiring treatment for the condition that is to be treated and is treated with the specific intent of treating such condition. The conditions can be, for example, any of the conditions described herein.

~~[0079]~~[0078] As used herein, the terms "comprising" (and any form of comprising, such as "comprise", "comprises", and "comprised"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include"), or "containing" (and any form of containing, such as "contains" and "contain"), are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. Any step or composition that uses the transitional phrase of "comprise" or "comprising" can also be said to describe the same with the transitional phase of "consisting of" or "consists."

~~[0080]~~[0079] As used herein, the term "contacting" means bringing together of two elements in an in vitro system or an in vivo system. For example, "contacting" virus or vector described herein with an individual or patient or cell includes the administration of the virus to an individual or patient, such as a human, as well as, for example, introducing a compound into a sample containing a cellular or purified preparation containing the cell.

~~[0081]~~[0080] As used herein, the term "fused" or "linked" when used in reference to a protein having different domains or heterologous sequences means that the protein domains are part of the same peptide chain that are connected to one another with either peptide bonds or other

covalent bonding. The domains or section can be linked or fused directly to one another or another domain or peptide sequence can be between the two domains or sequences and such sequences would still be considered to be fused or linked to one another. In some embodiments, the various domains or proteins provided for herein are linked or fused directly to one another or a linker sequences, such as the glycine/serine sequences described herein link the two domains together.

~~[0082]~~[0081] A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

~~[0083]~~[0082] "Effective amount" or "therapeutically effective amount" are used interchangeably herein, and refer to an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result or provides a therapeutic or prophylactic benefit. Such results may include, but are not limited to an amount that when administered to a mammal, causes a detectable level of immune cell activation compared to the immune cell activation detected in the absence of the composition. The immune response can be readily assessed by a plethora of art-recognized methods. The skilled artisan would understand that the amount of the composition administered herein varies and can be readily determined based on a number of factors such as the disease or condition being treated, the age and health and physical condition of the mammal being treated, the severity of the disease, the particular compound being administered, and the like.

~~[0084]~~[0083] "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the

template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

~~[0085]~~[0084] "Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., Sendai viruses, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

~~[0086]~~

~~[0087]~~[0085] As used herein, the phrase "ex vivo" in reference to a cell being transduced, transfected or transformed ex vivo, refers to a cell being transduced, transfected or transformed outside of the subject, that is with the cells being removed from the subject before such cells are transduced, transfected or transformed.

~~[0088]~~[0086] "Identity" as used herein refers to the subunit sequence identity between two polymeric molecules such as between two nucleic acid or amino acid molecules, such as, between two polynucleotide or polypeptide molecules. When two amino acid sequences have the same residues at the same positions; e.g., if a position in each of two polypeptide molecules is occupied by an Arginine, then they are identical at that position. The identity or extent to which two amino acid or two nucleic acid sequences have the same residues at the same positions in an alignment is often expressed as a percentage. The identity between two amino acid or two nucleic acid sequences is a direct function of the number of matching or identical positions; e.g., if half of the positions in two sequences are identical, the two sequences are 50% identical; if 90% of the positions (e.g., 9 of 10), are matched or identical, the two amino acids sequences are 90% identical.

~~[0089]~~[0087] By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). In some embodiments, such a sequence is at least 60%, 80% or 85%, or 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the

sequence used for comparison. Other percentages of identity in reference to specific sequences are described herein.

~~[0090]~~[0088] Sequence identity can be measured/determined using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e3 and e100 indicating a closely related sequence. In some embodiments, sequence identity is determined by using BLAST with the default settings.

~~[0091]~~[0089] To the extent embodiments provided for herein, includes composition comprising various proteins, these proteins may, in some instances, comprise amino acid sequences that have sequence identity to the amino acid sequences disclosed herein. Therefore, in certain embodiments, depending on the particular sequence, the degree of sequence identity is preferably greater than 50% (e.g. 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) to the SEQ ID NOs disclosed herein. In addition to these percentages, other percentages of identity are provided for herein. Identity between polypeptides can be determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters gap open penalty – 12 and gap extension penalty = 1.

~~[0092]~~[0090] These proteins may, compared to the disclosed proteins, include one or more (e.g. 1, 2, 3,4, 5, 6, 7, 8, 9, 10, etc.) conservative amino acid replacements i.e. replacements of one amino acid with another which has a related side chain. Genetically-encoded amino acids are generally divided into four families: (1) acidic i.e. aspartate, glutamate; (2) basic i.e. lysine, arginine, histidine; (3) non polar i.e. alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar i.e. glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified

jointly as aromatic amino acids. In general, Substitution of single amino acids within these families does not have a major effect on the biological activity. The proteins may have one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, etc.) single amino acid deletions relative to the disclosed protein sequences. The proteins may also include one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, etc.) insertions (e.g. each of 1, 2, 3, 4 or 5 amino acids) relative to the disclosed protein sequences.

~~[0093]~~[0091] As used herein, the phrase "in vivo" in reference to a cell being transduced, transfected or transformed in vivo, refers to a cell being transduced, transfected or transformed in the subject without the cells being removed from the subject before such cells are transduced, transfected or transformed.

~~[0094]~~[0092] "Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

~~[0095]~~[0093] A "lentivirus" as used herein refers to a genus of the Retroviridae family that is able to infect non-dividing cells. Non-limiting examples of lentiviruses are HIV, SIV, and FIV. Vectors or viral-like particles derived from lentiviruses can be used to transduce cells and deliver genes or other molecules and have them expressed in a cell either in vitro (ex-vivo) or in vivo.

~~[0096]~~[0094] By the term "modified" as used herein, is meant a changed state or structure of a molecule or cell as provided herein. Molecules may be modified in many ways, including chemically, structurally, and functionally, such as mutations, substitutions, insertions, or deletions (e.g. internal deletions truncations). Cells may be modified through the introduction of nucleic acids or the expression of heterologous proteins.

~~[0097]~~[0095] By the term "modulating," as used herein, is meant mediating an increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, such as, a human.

~~[0098]~~[0096] As used herein, the following abbreviations for the commonly occurring nucleic acid bases are used: "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

~~[0099]~~[0097] Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

~~[0100]~~[0098] The "Nipah virus" (NiV) is member of the family Paramyxoviridae, genus Henipavirus. Nipah virus is an enveloped virus with negative-stranded polarity and a non-segmented RNA genome consisting of helical nucleocapsids. Two strains of Nipah virus include, but are not limited to, the Malaysian (MY) and the Bangladesh (BD) strains.

~~[0101]~~[0099] The term "oligonucleotide" typically refers to short polynucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, C, G), this also provides the corresponding RNA sequence (i.e., A, U, C, G) in which "U" replaces "T."

~~[0102]~~[0100] "Parenteral" administration of a composition includes, e.g., subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

~~[0103]~~[0101] The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, the terms "nucleic acids" and "polynucleotides" as used herein are interchangeable. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any methods available in the art, including, without limitation, recombinant methods, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using cloning technology and PCR, and the like, and by synthetic means.

~~[0104]~~[0102] As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of a plurality of amino acid residues covalently linked by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many

types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

~~[0105]~~[0103] The term "pseudotyped" or "pseudotyped viral particle", as used herein, refers to a viral particle bearing glycoproteins derived from other viruses having envelopes or a viral vector encoding envelope glycoproteins from a virus that is different from the parental virus. The host range of the vector particles can thus be expanded or altered depending on the type of cell surface receptor used by the glycoprotein. For example, a virus can be pseudotyped with a VSV-G mutant protein as provided for herein or other virus glycoproteins, such as those provided for herein.

~~[0106]~~[0104] By the term "specifically binds," as used herein with respect to an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody. In some embodiments, the targeting moieties described herein that can be used to target the viral particles comprising the mutant VSV-G protein can specifically bind to their target.

~~[0107]~~[0105] The term "subject" includes living organisms, including those in which an immune response can be elicited (e.g., mammals). A "subject" or "patient," as used therein, may be a human or non-human mammal. Non-human mammals include, for example, livestock and

pets, such as ovine, bovine, porcine, canine, non-human primates, feline and murine mammals. In some embodiments, the subject is human.

~~[0108]~~[0106] The term "therapeutic" as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

~~[0109]~~[0107] The term "transfected" or "transformed" or "transduced" as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into a cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny. In some embodiments, the transfection, transformation, or transduction is performed or occurs in vivo.

~~[0110]~~[0108] To "treat" a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject. As used herein, "inhibit" or "treat" or "treatment" also includes a postponement of development of the symptoms associated with a disorder and/or a reduction in the severity of the symptoms of such disorder. The terms further include ameliorating existing uncontrolled or unwanted symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result has been conferred on a vertebrate subject with a disorder, disease or symptom, or with the potential to develop such a disorder, disease or symptom.

~~[0111]~~[0109] A "vector" is a composition of matter which comprises an isolated nucleic acid encoding a protein or a peptide. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, plasmids, DNA, and RNA. Examples of viral vectors include, but are not limited to, Sendai viral vectors, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like.

~~[0112]~~[0110] A "carrier" or "delivery vehicle" includes viral particles, viruses, polylysine compounds, and liposomes, which facilitate transfer of nucleic acid into cells. A carrier or delivery vehicle can also be used to deliver a protein or peptide to a cell.

~~[0113]~~[0111] Ranges: throughout this disclosure, various aspects of the embodiments can be presented in a range format. It should be understood that the description in range format is

merely for convenience and brevity and should not be construed as an inflexible limitation. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range. Unless otherwise explicitly stated to the contrary, a range that is disclosed also includes the endpoints of the range.

~~[0114]~~[0112] Without being bound to any particular theory, the embodiments provided for herein have been found to show that a mutant VSV-G protein comprising a mutation at position 182 can be used to pseudotype a virus and transduce a cell when the virus comprises a targeting moiety. This mutation inhibits or reduces the VSV-G affinity to its natural co-receptor, the LDL-R. The mutant VSV-G proteins as provided can be used, in some embodiments, to transduce a target cell and deliver a heterologous molecule to the targeted cells.

~~[0115]~~[0113] In some embodiments, a VSV-G protein is provided that comprises a mutation at position 198 as compared to SEQ ID NO: 1 or at position 182 as compared to SEQ ID NO: 2. SEQ ID NO: 1 is the full length protein and SEQ ID NO: 2 is the ectodomain of the VSV-G protein. The 16-mer signal peptide of MKCLLYLAFLFIGVNC (SEQ ID NO: 26) as shown at the N-terminus of SEQ ID NO: 1 is cleaved leaving a protein of SEQ ID NO: 2. Thus, although a mutation may be referred to in the context of SEQ ID NO: 2, it should be understood to also be made in the context of SEQ ID NO: 1, which contains the leader sequence, and thus would be a position number that is 16 more than the position recited for SEQ ID NO: 2. In some embodiments, the mutation inhibits or decreases the binding of the VSV-G protein to the LDL receptor (LDL-R). In some embodiments, the mutation is a I182D mutation as compared to SEQ ID NO: 2. In some embodiments, the mutation is a I182E mutation as compared to SEQ ID NO: 2.

~~[0116]~~[0114] In some embodiments, a VSV-G protein is provided that comprises a mutation at position 198 as compared to SEQ ID NO: 10 or at position 182 as compared to SEQ ID NO: 11. SEQ ID NO: 10 is the full length protein and SEQ ID NO: 11 is the ectodomain of the VSV-G protein. The 16-mer signal peptide of MLSYLIFALVVSPILG (SEQ ID NO: 27) as shown at the N-terminus of SEQ ID NO: 10 is cleaved leaving a protein of SEQ ID NO: 11. Thus,

although a mutation may be referred to in the context of SEQ ID NO: 11, it should be understood to also be made in the context of SEQ ID NO: 10, which contains the leader sequence, and thus would be a position number that is 16 more than the position recited for SEQ ID NO: 11. In some embodiments, the mutation inhibits or decreases the binding of the VSV-G protein to the LDL receptor (LDL-R). In some embodiments, the mutation is a T182D mutation as compared to SEQ ID NO: 11. In some embodiments, the mutation is a T182E mutation as compared to SEQ ID NO: 11.

~~[0117]~~[0115] In some embodiments, a VSV-G protein is provided that comprises a mutation at position 198 as compared to SEQ ID NO: 12 or at position 182 as compared to SEQ ID NO: 13. SEQ ID NO: 12 is the full length protein and SEQ ID NO: 13 is the ectodomain of the VSV-G protein. The 16-mer signal peptide of MLRLFLFCFLALGAHS (SEQ ID NO: 28) as shown at the N-terminus of SEQ ID NO: 12 is cleaved leaving a protein of SEQ ID NO: 13. Thus, although a mutation may be referred to in the context of SEQ ID NO: 13, it should be understood to also be made in the context of SEQ ID NO: 12, which contains the leader sequence, and thus would be a position number that is 16 more than the position recited for SEQ ID NO: 13. In some embodiments, the mutation inhibits or decreases the binding of the VSV-G protein to the LDL receptor (LDL-R). In some embodiments, the mutation is a A182D mutation as compared to SEQ ID NO: 13. In some embodiments, the mutation is a A182E mutation as compared to SEQ ID NO: 13.

~~[0118]~~[0116] In some embodiments, a VSV-G protein is provided that comprises a mutation at position 203 as compared to SEQ ID NO: 14 or at position 182 as compared to SEQ ID NO: 15. SEQ ID NO: 14 is the full length protein and SEQ ID NO: 15 is the ectodomain of the VSV-G protein. The 21-mer signal peptide of MKMKMVIAGLILCIGILPAIG (SEQ ID NO: 29) as shown at the N-terminus of SEQ ID NO: 14 is cleaved leaving a protein of SEQ ID NO: 15. Thus, although a mutation may be referred to in the context of SEQ ID NO: 15, it should be understood to also be made in the context of SEQ ID NO: 14, which contains the leader sequence, and thus would be a position number that is 21 more than the position recited for SEQ ID NO: 15. In some embodiments, the mutation inhibits or decreases the binding of the VSV-G protein to the LDL receptor (LDL-R). In some embodiments, the mutation is a V182D mutation as compared to SEQ ID NO: 15. In some embodiments, the mutation is a V182E mutation as compared to SEQ

ID NO: 15.

~~[0119]~~[0117] In some embodiments, a VSV-G protein is provided that comprises a mutation at position 199 as compared to SEQ ID NO: 16 or at position 182 as compared to SEQ ID NO: 17. SEQ ID NO: 16 is the full length protein and SEQ ID NO: 17 is the ectodomain of the VSV-G protein. The 17-mer signal peptide of MTPAFILCMLLAGSSWA (SEQ ID NO: 30) as shown at the N-terminus of SEQ ID NO: 16 is cleaved leaving a protein of SEQ ID NO: 17. Thus, although a mutation may be referred to in the context of SEQ ID NO: 17, it should be understood to also be made in the context of SEQ ID NO: 16, which contains the leader sequence, and thus would be a position number that is 17 more than the position recited for SEQ ID NO: 17. In some embodiments, the mutation inhibits or decreases the binding of the VSV-G protein to the LDL receptor (LDL-R). In some embodiments, the mutation is a V182D mutation as compared to SEQ ID NO: 17. In some embodiments, the mutation is a V182E mutation as compared to SEQ ID NO: 17.

~~[0120]~~[0118] In some embodiments, a VSV-G protein is provided that comprises a mutation at position 199 as compared to SEQ ID NO: 18 or at position 182 as compared to SEQ ID NO: 19. SEQ ID NO: 18 is the full length protein and SEQ ID NO: 19 is the ectodomain of the VSV-G protein. The 17-mer signal peptide of MNFLLLTFIVLPLCSHA (SEQ ID NO: 31) as shown at the N-terminus of SEQ ID NO: 18 is cleaved leaving a protein of SEQ ID NO: 19. Thus, although a mutation may be referred to in the context of SEQ ID NO: 19, it should be understood to also be made in the context of SEQ ID NO: 18, which contains the leader sequence, and thus would be a position number that is 17 more than the position recited for SEQ ID NO: 19. In some embodiments, the mutation inhibits or decreases the binding of the VSV-G protein to the LDL receptor (LDL-R). In some embodiments, the mutation is a V182D mutation as compared to SEQ ID NO: 19. In some embodiments, the mutation is a V182E mutation as compared to SEQ ID NO: 19.

~~[0121]~~[0119] In some embodiments, a VSV-G protein is provided that comprises a mutation at position 199 as compared to SEQ ID NO: 20 or at position 182 as compared to SEQ ID NO: 21. SEQ ID NO: 20 is the full length protein and SEQ ID NO: 21 is the ectodomain of the VSV-G protein. The 17-mer signal peptide of MLVLYLLLSLLALGAQC (SEQ ID NO: 32) as shown at the N-terminus of SEQ ID NO: 20 is cleaved leaving a protein of SEQ ID NO: 21.

Thus, although a mutation may be referred to in the context of SEQ ID NO: 21, it should be understood to also be made in the context of SEQ ID NO: 20, which contains the leader sequence, and thus would be a position number that is 17 more than the position recited for SEQ ID NO: 21. In some embodiments, the mutation inhibits or decreases the binding of the VSV-G protein to the LDL receptor (LDL-R). In some embodiments, the mutation is a I182D mutation as compared to SEQ ID NO: 21. In some embodiments, the mutation is a I182E mutation as compared to SEQ ID NO: 21.

~~[0122]~~[0120] As used herein, when a polypeptide is said to have a mutation as compared to a reference sequence, such comparison is based on an alignment such as using BlastP or ClustalW or ClutalOmega alignment software using default parameters. For example, position 182 can be found in SEQ ID NO: 2 and also as compared to the other strains as illustrated in FIG. 3. FIG. 3 illustrates a clustal alignment of the wild-type sequences of the ectodomains of the various strains of the VSV-G protein. The residue that is bolded and underlined are the residues that align to position 182 of SEQ ID NO: 2 of the various strains. SEQ ID NO: 2 refers to ectodomain of the VSV-G protein of the Indiana strain. SEQ ID NO: 11 refers to ectodomain of the VSV-G protein of the New Jersey strain. SEQ ID NO: 13 refers to ectodomain of the VSV-G protein of the Marraba strain. SEQ ID NO: 15 refers to ectodomain of the VSV-G protein of the Carajas strain. SEQ ID NO: 17 refers to ectodomain of the VSV-G protein of the Alagoa strain. SEQ ID NO: 19 refers to ectodomain of the VSV-G protein of the Cocal strain. SEQ ID NO: 21 refers to ectodomain of the VSV-G protein of the Morreton strain. Accordingly, the residue that aligns to residues 182 as compared to SEQ ID NO: 2 can also be mutated as provided for herein.

~~[0123]~~[0121] In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 2 is not an alanine. In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 2 is not a valine.

~~[0124]~~[0122] In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 2 is I182S, I182H, I182T, I182Q, or I182N. In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 11 is T182S, T182H, T182Q, or T182N. In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 13 is A182S, A182H, A182T, A182Q, or A182N. In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 15 is V182S, V182H, V182T, V182Q, or V182N. In some embodiments, the mutation at position 182

as compared to SEQ ID NO: 17 is V182S, V182H, V182T, V182Q, or V182N. In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 19 is V182S, V182H, V182T, V182Q, or V182N. In some embodiments, the mutation at position 182 as compared to SEQ ID NO: 21 is I182S, I182H, I182T, I182Q, or I182N. In some embodiments, the mutation at position 182 is not a hydrophobic residue. In some embodiments, the mutation at position 182 is a charged residue. In some embodiments, the mutation at position 182 is a negatively charged residue.

~~[0125]~~[0123] Although, the mutations may be described in reference to SEQ ID NO: 1 or SEQ ID NO: 2, which is the VSV-G protein from the Indiana strain, the mutation can also be used in other strains of the VSV-G protein. For example, the mutation can be made in the New Jersey Strain of VSV-G, the Marraba strain of VSV-G, the Carajas strain of VSV-G, the Alagoa strain of VSV-G, the Cocal strain of VSV-G, or the Morreton strain of VSV-G. In some embodiments, the sequences of each are as provided herein. Examples of these can be found, for example in U.S. Patent Application Publication No. 20200216502, which is hereby incorporated by reference. For example, the wild-type full length or ectodomain of the New Jersey Strain of VSV-G are SEQ ID NO: 10 and SEQ ID NO: 11, respectively, the wild-type full length or ectodomain of Marraba strain of VSV-G are SEQ ID NO: 12 and SEQ ID NO: 13, respectively, the wild-type full length or ectodomain of Carajas strain of VSV-G are SEQ ID NO: 14 and SEQ ID NO: 15, respectively, the wild-type full length or ectodomain of Alagoa strain of VSV-G are SEQ ID NO: 16 and SEQ ID NO: 17, respectively, the wild-type full length or ectodomain of Cocal strain of VSV-G are SEQ ID NO: 18 and SEQ ID NO: 19, respectively, or the wild-type full length or ectodomain of Morreton strain of VSV-G are SEQ ID NO: 20 and SEQ ID NO: 21, respectively.

~~[0126]~~[0124] A VSV-G protein comprising a mutation at position 182 as compared to SEQ ID NO: 2 can also comprise other mutations, such as those described in U.S. Patent Application Publication No. 2020/0216502, which is hereby incorporated by reference in its entirety. For example, the VSV-G protein can comprise a mutation at a position that corresponds to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2.

~~[0127]~~[0125] In some embodiments, the substitution at position 8 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except Y. In some embodiments, the substitution at position 8 is by any amino acid different from the amino

acid indicated at that position in the sequence SEQ ID NO: 2, except H. In some embodiments, the substitution at position 8 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except Q. In some embodiments, the substitution at position 8 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except Y or H or Q. In some embodiments, the substitution at position 8 is selected from alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, isoleucine, valine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, or tryptophan. In some embodiments, the substitution at position 8 is selected from 8A, 8I, 8V, 8L, and the like. In some embodiments, the substitution at position 8 is selected from H8A, H8I, H8V, H8L, and the like.

~~[0128]~~[0126] In some embodiments, the substitution at position 209 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except H. In some embodiments, the substitution at position 209 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except Y. In some embodiments, the substitution at position 209 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except H or Y. In some embodiments, the substitution at position 209 is selected from alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, isoleucine, valine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, or tryptophan.

~~[0129]~~[0127] In some embodiments, the substitution at position 47 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except K. In some embodiments, the substitution at position 47 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except R. In some embodiments, the substitution at position 47 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except K or R. In some embodiments, the substitution at position 47 is selected from alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, valine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, or tyrosine. In some embodiments, the substitution at position 47 is selected from A, G, F, N, or Q. In some embodiments, the substitution at position 47 is selected from Q or N.

~~[0130]~~[0128] In some embodiments, the substitution at position 354 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except K. In some embodiments, the substitution at position 354 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except R. In some embodiments, the substitution at position 354 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except K or R. In some embodiments, the substitution at position 354 is selected from alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, valine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, or tyrosine. In some embodiments, the substitution at position 354 is selected from A, G, F, N, or Q.

~~[0131]~~[0129] In some embodiments, the substitution is at position 47 or at position 354, or at both positions 47 and 354 are, independently, substituted by A, G, F, N or Q. In some embodiments, the substitution at position 47 or at position 354, or at both positions 47 and 354 is, independently, A or Q or N.

~~[0132]~~[0130] In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to positions of 8, 47, 209 and/or 354. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 8 as provided for herein, at position 47 as provided for herein, at position 209 as provided for herein, and/or position 354 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 8 as provided for herein and at position 47 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 8 as provided for herein and at position 209 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 8 as provided for herein and at position 354 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 47 as provided for herein and at position 209 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 47 as provided for herein and at position 354 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 209 as provided for herein and at position 354 as provided for herein. In some embodiments, the VSV-G protein can comprise a

mutation at a position that corresponds to position 8 as provided for herein, at position 47 as provided for herein, and position 209 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 8 as provided for herein, at position 47 as provided for herein, and position 354 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 8 as provided for herein, at position 209 as provided for herein, and position 354 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 47 as provided for herein, at position 209 as provided for herein, and position 354 as provided for herein. In some embodiments, the VSV-G protein can comprise a mutation at a position that corresponds to position 8 as provided for herein, at position 47 as provided for herein, at position 209 as provided for herein, and at position 354 as provided for herein.

~~[0133]~~[0131] In some embodiments, the substitution at position 8 is an alanine, i.e., H8A.

~~[0134]~~[0132] In some embodiments, the substitution at position 47 is Q or N or A, i.e., K47Q or K47N or K47A.

~~[0135]~~[0133] In some embodiments, the protein comprises a substitution at position 8 and/or a substitution at position 47. In some embodiments, the protein comprises a substitution at position 8 and a substitution at position 47. In some embodiments, the protein comprises a substitution at position 8 or a substitution at position 47. In some embodiments, the substitution at position 8 and/or a substitution at position 47 comprises a H8A and/or K47Q mutation.

~~[0136]~~[0134] In some embodiments, the protein comprises a mutation (substitution) at position 10. In some embodiments, the substitution/mutation is Q10A, Q10R, or Q10K.

~~[0137]~~[0135] In some non-limiting embodiments, the substitution at position 209 is A, i.e., Y209A.

~~[0138]~~[0136] In some non-limiting embodiments, the substitution at position 354 is A or Q, i.e. R354A or R354Q.

~~[0139]~~[0137] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 11 can also comprise other mutations, such as those described in U.S. Patent Application Publication No. 2020/0216502. For example, the VSV-G protein can comprise a mutation at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2. In some

embodiments, the mutation in SEQ ID NO: 11 at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2 are as provided for herein.

~~[0140]~~[0138] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 13 can also comprise other mutations, such as those described in U.S. Patent Application Publication No. 2020/0216502. For example, the VSV-G protein can comprise a mutation at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2. In some embodiments, the mutation in SEQ ID NO: 13 at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2 are as provided for herein.

~~[0141]~~[0139] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 15 can also comprise other mutations, such as those described in U.S. Patent Application Publication No. 2020/0216502. For example, the VSV-G protein can comprise a mutation at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2. In some embodiments, the mutation in SEQ ID NO: 15 at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2 are as provided for herein.

~~[0142]~~[0140] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 17 can also comprise other mutations, such as those described in U.S. Patent Application Publication No. 2020/0216502. For example, the VSV-G protein can comprise a mutation at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2. In some embodiments, the mutation in SEQ ID NO: 17 at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2 are as provided for herein.

~~[0143]~~[0141] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 19 can also comprise other mutations, such as those described in U.S. Patent Application Publication No. 2020/0216502. For example, the VSV-G protein can comprise a mutation at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2. In some embodiments, the mutation in SEQ ID NO: 19 at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2 are as provided for herein.

~~[0144]~~[0142] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 21 can also comprise other mutations, such as those described in U.S. Patent Application Publication No. 2020/0216502. For example, the VSV-G protein can comprise a mutation at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2. In some

embodiments, the mutation in SEQ ID NO: 21 at a position analogous to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2 are as provided for herein.

~~[0145]~~[0143] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 2 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 2 (or SEQ ID NO: 1 if using the full length protein). In some embodiments, the protein is at least, or about, 80% identical to SEQ ID NO: 2. In some embodiments, the protein is at least, or about, 85% identical to SEQ ID NO: 2. In some embodiments, the protein is at least, or about, 90% identical to SEQ ID NO: 2. In some embodiments, the protein is at least, or about, 95% identical to SEQ ID NO: 2. In some embodiments, the mutation at position 182 is I182D or I182E mutation. In some embodiments, the mutation is I182D. In some embodiments, the mutation is I182E mutation. In some embodiments, the mutation is I182S, I182H, I182T, I182Q, or I182N mutation.

~~[0146]~~[0144] In some embodiments, a VSV-G is provided wherein the protein comprises the amino acid sequence of SEQ ID NO: 2 with a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 2.

~~[0147]~~[0145] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 11 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 11 (or SEQ ID NO: 10 if using the full length protein). In some embodiments, the polypeptide comprises a T182D or T182E mutation. In some embodiments, the VSV-G protein comprises a T182S, T182H, T182Q, or T182N mutation.

~~[0148]~~[0146] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 13 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as

compared to SEQ ID NO: 13 (or SEQ ID NO: 12 if using the full length protein). In some embodiments, the polypeptide comprises a A182D or A182E mutation. In some embodiments, the VSV-G protein comprises a A182S, A182H, A182T, A182Q, or A182N mutation.

~~[0149]~~[0147] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 15 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 15 (or SEQ ID NO: 14 if using the full length protein). In some embodiments, the polypeptide comprises a V182D or V182E mutation. In some embodiments, the VSV-G protein comprises a V182S, V182H, V182T, V182Q, or V182N mutation.

~~[0150]~~[0148] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 17 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 17 (or SEQ ID NO: 16 if using the full length protein). In some embodiments, the polypeptide comprises a V182D or V182E mutation. In some embodiments, the VSV-G protein comprises a V182S, V182H, V182T, V182Q, or V182N mutation.

~~[0151]~~[0149] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 19 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 19 (or SEQ ID NO: 18 if using the full length protein). In some embodiments, the polypeptide comprises a V182D or V182E mutation. In some embodiments, the VSV-G protein comprises a V182S, V182H, V182T, V182Q, or V182N mutation.

~~[0152]~~[0150] In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 21 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 21 (or SEQ ID NO: 20 if using the full length protein). In some embodiments, the polypeptide comprises a I182D or I182E mutation. In some embodiments, the

VSV-G protein comprises a I182S, I182H, I182T, I182Q, or I182N mutation.

Viral Particles

~~[0153]~~[0151] The mutant VSV-G proteins can be used, for example, to pseudotype a virus, such as, but not limited to a lentivirus. Accordingly, in some embodiments, a viral particle comprising a mutant VSV-G protein as provided herein are provided. In some embodiments, the viral particle comprises a VSV-G protein comprising a mutation at position 198 as compared to SEQ ID NO: 1. In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 2 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 2 (or SEQ ID NO: 1 if using the full length protein). In some embodiments, the polypeptide comprises a I182D or I182E mutation as compared to SEQ ID NO: 2. In some embodiments, the VSV-G protein comprises a I182S, I182H, I182T, I182Q, or I182N mutation.

~~[0154]~~[0152] In some embodiments, the viral particle comprises a VSV-G protein comprising a mutation at position 198 as compared to SEQ ID NO: 10. In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 11 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 11 (or SEQ ID NO: 10 if using the full length protein). In some embodiments, the polypeptide comprises a T182D or T182E mutation as compared to SEQ ID NO: 11. In some embodiments, the VSV-G protein comprises a T182S, T182H, T182Q, or T182N mutation.

~~[0155]~~[0153] In some embodiments, the viral particle comprises a VSV-G protein comprising a mutation at position 198 as compared to SEQ ID NO: 12. In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 13 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 13 (or SEQ ID NO: 12 if using the full length protein). In some embodiments, the polypeptide comprises a A182D or A182E mutation as compared to SEQ ID NO: 13. In some embodiments, the VSV-G protein

comprises a A182S, A182H, A182T, A182Q, or A182N mutation.

~~[0156]~~[0154] In some embodiments, the viral particle comprises a VSV-G protein comprising a mutation at position 203 as compared to SEQ ID NO: 14. In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 15 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 15 (or SEQ ID NO: 14 if using the full length protein). In some embodiments, the polypeptide comprises a V182D or V182E mutation as compared to SEQ ID NO: 15. In some embodiments, the VSV-G protein comprises a V182S, V182H, V182T, V182Q, or V182N mutation.

~~[0157]~~[0155] In some embodiments, the viral particle comprises a VSV-G protein comprising a mutation at position 199 as compared to SEQ ID NO: 16. In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 17 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 17 (or SEQ ID NO: 16 if using the full length protein). In some embodiments, the polypeptide comprises a V182D or V182E mutation as compared to SEQ ID NO: 17. In some embodiments, the VSV-G protein comprises a V182S, V182H, V182T, V182Q, or V182N mutation.

~~[0158]~~[0156] In some embodiments, the viral particle comprises a VSV-G protein comprising a mutation at position 199 as compared to SEQ ID NO: 18. In some embodiments, a protein comprising a mutation at position 182 as compared to SEQ ID NO: 19 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 19 (or SEQ ID NO: 18 if using the full length protein). In some embodiments, the polypeptide comprises a V182D or V182E mutation as compared to SEQ ID NO: 19. In some embodiments, the VSV-G protein comprises a V182S, V182H, V182T, V182Q, or V182N mutation.

~~[0159]~~[0157] In some embodiments, the viral particle comprises a VSV-G protein comprising a mutation at position 199 as compared to SEQ ID NO: 20. In some embodiments, a

protein comprising a mutation at position 182 as compared to SEQ ID NO: 21 comprises a mutation at position 182 and at least, or about, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical as compared to SEQ ID NO: 21 (or SEQ ID NO: 20 if using the full length protein). In some embodiments, the polypeptide comprises a I182D or I182E mutation as compared to SEQ ID NO: 21. In some embodiments, the VSV-G protein comprises a I182S, I182H, I182T, I182Q, or I182N mutation.

~~[0160]~~[0158] In some embodiments, the VSV-G protein further comprises a mutation at position that corresponds to positions 214 and/or 352 of SEQ ID NO: 2. In some embodiments, the residue that corresponds to position 214 of SEQ ID NO: 2 is T214. In some embodiments, the residue that corresponds to position 352 of SEQ ID NO: is T352. In some embodiments, the VSV-G protein comprises mutation that corresponds to T214N mutation as compared to SEQ ID NO: 2. In some embodiments, the VSV-G protein comprises mutation that corresponds to T352A mutation as compared to SEQ ID NO: 2. In some embodiments, the VSV-G protein comprises a T214N and T352A mutations as compared to SEQ ID NO: 2. These mutations can be combined with any other mutations as provided for herein. In some embodiments, the T214N and/or T352A mutations are combined with the I182E or I182D mutations. In some embodiments, a VSV-G protein comprises an amino acid sequence of SEQ ID NO: 22 and SEQ ID NO: 23, which combines the I182D or I182E, respectively, with the T214N and T352A mutations. The sequences are also illustrated below with the leader sequences, which are removed during protein processing.

VSV-G Protein_ I196D, T230N and T368A mutations (with leader sequence and adjusted numbering)

MKCLLYLAFLFIGVNCKFTIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHNDLIG
TALQVKMPKSHKAIQADGWMCHASKWVTTCDFRWYGPKYITHSIRSFTPSVEQ
CKESIEQTKQGTWLNPGFPPQSCGYATVTDAEAVIVQVTPHHVLVDEYTGEWVD
SQFINGKCSNYICPTVHNSTTWHSDYKVKGLCDSNLDSMDITFFSEDGELSSLGK
EGTGFRSNYFAYENGGKACKMQYCKHWGVRLPSGVWFEMADKDLFAAARFPE
CPEGSSISAPSQTSVDVSLIQDVERILDYSLCQETWSKIRAGLPISPVDLSYLAPKN
PGTGPAFTIINGTLKYFETRYIRVDIAAPILSRMVGMISGTTAERELWDDWAPYED

VEIGPNGVLRTSSGYKFPLYMIGHGMLDSDLHLSSKAQVFEHPHIQDAASQLPDD
ESLFFGDTGLSKNPIELVEGWFSSWKSSIASFFFIIGLIIGLFLVLRVGIHLCIKLKHT
KKRQIYTDIEMNRLGK (SEQ ID NO: 24)

VSV-G Protein_I182D, T214N and T352A mutations (without leader sequence)

KFTIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHNDLIGTALQVKMPKSHKAIQ
ADGWMCHASKWVTTCDFRWYGPKYITHSIRSFTPSVEQCKESIEQTKQGTWLNP
GFPPQSCGYATVTDAEAVIVQVTPHHVLVDEYTGEWVDSQFINGKCSNYICPTV
HNSTTWHSDYKVKGLCDSNLDSMDITFFSEDGELSSLGKEGTGFRSNYFAYENG
GKACKMQYCKHWGVRLPSGVWFEMADKDLFAAARFPECPEGSSISAPSQTSVD
VSLIQDVERILDYSLCQETWSKIRAGLPISPVDLSYLAPKNPGTGPAFTIINGTLKY
FETRYIRVDIAAPILSRMVGMISGTTAERELWDDWAPYEDVEIGPNGVLRTSSGY
KFPLYMIGHGMLDSDLHLSSKAQVFEHPHIQDAASQLPDDESLFFGDTGLSKNPI
ELVEGWFSSWKSSIASFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRL
GK (SEQ ID NO: 22)

VSV-G Protein with I196E, T230N and T368A mutations (with leader sequence and adjusted numbering)

MKCLLYLAFLFIGVNCKFTIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHNDLIG
TALQVKMPKSHKAIQADGWMCHASKWVTTCDFRWYGPKYITHSIRSFTPSVEQ
CKESIEQTKQGTWLNPGFPPQSCGYATVTDAEAVIVQVTPHHVLVDEYTGEWVD
SQFINGKCSNYICPTVHNSTTWHSDYKVKGLCDSNLESMDITFFSEDGELSSLGK
EGTGFRSNYFAYENGGKACKMQYCKHWGVRLPSGVWFEMADKDLFAAARFPE
CPEGSSISAPSQTSVDVSLIQDVERILDYSLCQETWSKIRAGLPISPVDLSYLAPKN
PGTGPAFTIINGTLKYFETRYIRVDIAAPILSRMVGMISGTTAERELWDDWAPYED
VEIGPNGVLRTSSGYKFPLYMIGHGMLDSDLHLSSKAQVFEHPHIQDAASQLPDD
ESLFFGDTGLSKNPIELVEGWFSSWKSSIASFFFIIGLIIGLFLVLRVGIHLCIKLKHT
KKRQIYTDIEMNRLGK (SEQ ID NO: 25)

VSV-G Protein with I182E, T214N and T352A mutations (without leader sequences)

KFTIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHNDLIGTALQVKMPKSHKAIQ
ADGWMCHASKWVTTCDFRWYGPKYITHSIRSFTPSVEQCKESIEQTKQGTWLNP
GFPPQSCGYATVTDAEAVIVQVTPHHVLVDEYTGEWVDSQFINGKCSNYICPTV
HNSTTWHSDYKVKGLCDSNLESMDITFFSEDGELSSLGKEGTGFRSNYFAYENG
GKACKMQYCKHWGVRLPSGVWFEMADKDLFAAARFPECPEGSSISAPSQTSVD
VSLIQDVERILDYSLCQETWSKIRAGLPISPVDLSYLAPKNPGTGPAFTIINGTLKY
FETRYIRVDIAAPILSRMVGMISGTTAERELWDDWAPYEDVEIGPNGVLRTSSGY
KFPLYMIGHGMLDSDLHLSSKAQVFEHPHIQDAASQLPDDESLFFGDTGLSKNPI
ELVEGWFSSWKSSIASFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRL
GK (SEQ ID NO: 23)

The other strains of the VSV-G protein as described herein can also comprises the mutations that correspond to T214N and/or T352A in SEQ ID NO: 2 and as illustrated in SEQ ID NO: 22 and SEQ ID NO: 23.

~~[0161]~~[0159] In some embodiments, the composition comprises a mutation as described in Hwang et al., Gene Ther 2013 Aug;20(8):807-15. (Epub 2013 Jan 31), which is hereby incorporated by reference in its entirety. For example, the mutations can be. at positions 230, 368, 66, and/or 162 that corresponds to SEQ ID NO: 1. The positions will be 16 positions less as compared to SEQ ID NO: 2, when the leader sequence is removed. In some embodiments, the mutations at those positions are, for example, T230N, T368A, K66T, S162T, or any combination thereof. In some embodiments, the VSV-G protein comprises a T230N and a T368A mutation. In some embodiments, the VSV-G polypeptide comprises a K66T, S162T, T230N, and a T368A. These positions are those that correspond to the positions in the full length protein (SEQ ID NO: 1). In some embodiments, the VSV-G protein comprises T230N mutation, a T368A mutation, a K66T mutation, a S162T mutation, or any combination thereof. In some embodiments, the VSV-G protein further comprises one or more mutations in addition to the mutation that corresponds to position 182 of SEQ ID NO: 2, such as those described in U.S. Patent Application Publication No. 20200216502, which is hereby incorporated by reference in its entirety. For example, the VSV-G protein can further comprise a mutation at a position that corresponds to positions of 8, 47, 209 and/or 354 of SEQ ID NO: 2.

~~[0162]~~[0160] In some embodiments, the substitution at position 8 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except Y. In some embodiments, the substitution at position 209 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except H. In some embodiments, the substitution at position 47 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except K or R. In some embodiments, the substitution at position 354 is by any amino acid different from the amino acid indicated at that position in the sequence SEQ ID NO: 2, except K or R. In some embodiments, the substitution is at position 47 or at position 354, or at both positions 47 and 354 are substituted by A, G, F or Q. In some embodiments, the substitution is A or Q. In some embodiments, the substitution at position 8 is an alanine, i.e., H8A. In some embodiments, the substitution at position 47 is Q or N, i.e., K47Q or K47N. In some embodiments, the protein comprises a mutation (substitution) at position 10. In some embodiments, the substitution/mutation is Q10A, Q10R, or Q10K.

~~[0163]~~[0161] In some embodiments, the viral particle comprises a targeting moiety. The targeting moiety can be used to target the viral particle comprising the mutant VSV-G protein to a cell that expresses the target to which the targeting moiety binds to. In some embodiments, the targeting moiety is an antibody, a scFv antibody, an antigen binding domain, an ankyrin repeat (e.g., DARPIN), a VHH domain antibody, a nanobody, single domain antibody, a FN3 domain, or any combination thereof. The targeting moiety can be attached to the viral surface through an IgG Fc stalk. In some embodiments, the stalk comprises a transmembrane domain. In some embodiments, the transmembrane domain comprises a CD8 transmembrane domain. In some embodiments, the transmembrane domain comprises a CD28 transmembrane domain. In some embodiments, the targeting moiety is attached (fused or linked) an envelope glycoprotein G or H of a virus of the Paramyxoviridae family, such as a morbillivirus, such as Measles virus, or a henipavirus, such as Nipah virus, Cedar virus, or Hendra virus. In some embodiments, the targeting moiety can be attached (fused or linked) to a glycoprotein of a virus of the Rhabdoviridae family, such as a vesicular stomatitis New Jersey virus, a vesicular stomatitis Indiana virus, a vesicular stomatitis Alagoas virus, a vesicular stromatitis Maraba virus, a vesicular stomatitis Carajas virus, Parainfluenza virus, Spodoptera frugiperda rhabdovirus isolate Sf G, Drosophila obscura sigmavirus 10A, Wuhan insect virus 7, Perch virus, or Spring viremia of carp virus. In

some embodiments, the VSV protein is the mutated proteins, such as those provided for herein. In some embodiments, the targeting moiety is attached to a glycoprotein of a virus of the Filoviridae family, such as Ebola virus or a glycoprotein of a virus of the Arenaviridae family, such as Machupo virus.

~~[0164]~~[0162] In some embodiments, the targeting moiety is a scFv. In some embodiments, the targeting moiety is a single domain antibody. In some embodiments, the targeting moiety is a VHH.

~~[0165]~~[0163] In some embodiments, the targeting moiety binds to CD7, CD8, cKit (CD117), CD4, CD3, CD5, CD6, CD2, TCR alpha, TCR beta, TCR gamma, TCR delta, CD10, CD34, CD110, CD33, CD14, CD68, CCR7, CD62L, CD25, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, or CXCR3, A glycosylated CD43 epitope expressed on acute leukemia or lymphoma but not on hematopoietic progenitors; A glycosylated CD43 epitope expressed on non-hematopoietic cancers; A kinase anchor protein 4 (AKAP-4); Adrenoceptor beta 3 (ADRB3); AFP; Anaplastic lymphoma kinase (ALK); Androgen receptor; Angiopoietin-binding cell surface receptor 2 (Tie 2); Auto antibody to desmoglein 1 (Dsgl); Auto antibody to desmoglein 3 (Dsg3); B7H3 (CD276); Biotin; Bone marrow stromal cell antigen 2 (BST2); BST1/CD157; Cancer/testis antigen 1 (NY-ESO-1); Cancer/testis antigen 2 (LAGE-la); Carbonic anhydrase IX (CA1X); Carcinoembryonic antigen (CEA); CCCTC-Binding Factor (Zinc Finger Protein)-Like (BORIS or Brother of the Regulator of Imprinted Sites); CCR4; CD5; CD19; CD20; CD22; CD24; CD30; CD32 (FCGR2A); CD33; CD34; CD38; CD44v6; CD72; CD79a; CD79b; CD97; CD99; CD123; CD171; CD179a; CD179b-IGLll; CD200R; CD276/B7H3; CD300 molecule-like family member f (CD300LF); CDH1-CD324; CDH6; CDH17; CDH19; Chromosome X open reading frame 61 (CXORF61); Claudin 6 (CLDN6); Claudinl8.2 (CLD18A2 or CLDN18A.2); CMV pp65; C-MYC epitope Tag; Cripto; CS1 (also referred to as CD2 subset 1 or CRACC or SLAMF7 or CD319 or 19A24); CSF2RA (GM-CSFR-alpha); C-type lectin domain family 12 member A (CLEC12A); C-type lectin-like molecule-1 (CLL-1 or CLECL1); Cyclin Bl; Cytochrome P450 IB 1 (CYP1B 1); DLL3; EBV-EBNA3c; EGF-bke module- containing mucin-like hormone receptor-like 2 (EMR2); Elongation factor 2 mutated (ELF2M); Ephrin B2; Ephrin type-A receptor 2 (EphA2); Epidermal growth factor receptor (EGFR); Epidermal growth factor receptor variant III (EGFRviii); Epithelial cell adhesion molecule (EPCAM); ERG; ETS translocation-variant gene 6 located on

chromosome 12p (ETV6-AML); Fc fragment of IgA receptor (FCAR or CD89); Fc receptor-like 5 (FCRL5); Fibroblast activation protein alpha (FAP); FITC; Fms Like Tyrosine Kinase 3 (FLT3); Folate receptor alpha (FRa or FR1); Folate receptor beta (FRb); Follicle stimulating hormone receptor (FSHR); Fos-related antigen 1; Fucosyl-GMl; G protein coupled receptor class C group 5 member D (GPRC5D); G protein-coupled receptor 20 (GPR20); GAD; Ganglioside G2 (GD2) ; Ganglioside GD3 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(l-4)bDGlcp(l-l)Cer); Ganglioside GM3 (aNeu5Ac(2-3)bDClalp(l- 4)bDGlcp(l-l)Cer); GD3; GFRalpha4; Glycoprotein 100 (gplOO); Glypican-3 (GPC3); Gonadotropin Hormone receptor (CGHR or GR); GpA33; GpNMB; GPRC5D; Guanylyl cyclase C (GCC); Heat shock protein 70-2 mutated (mut hsp70-2); Hepatitis A virus cellular receptor 1 (HAVCR1); Hexasaccharide portion of globoH glycoceramide (GloboH); High molecular weight-melanoma associated antigen (HMWMAA); HIV1 envelope glycoprotein; HLA; HLA-DOA; HLA-A; HLA-A2; HLA-B; HLA-C; HLA-DM; HLA-DOB; HLA-DP; HLA-DQ; HLA-DR; HLA-G; HTLVl-Tax; Human papilloma virus E6 (HPV E6); Human papilloma virus E7 (HPV E7); Human Telomerase reverse transcriptase (hTERT); IgE; IL13Ra2; IL l lRa; Immunoglobulin lambda-like polypeptide 1 (IGLL1); Influenza A hemagglutinin (HA); Insulin-like growth factor 1 receptor (IGF-I receptor); Interleukin 11 receptor alpha (IL-llRa); Interleukin- 13 receptor subunit alpha-2 (IL-13Ra2 or CD213A2); Intestinal carboxyl esterase; KIT (CD117); KSHV K8.1; KSHV-gH; LAMP1 ; Legumain; Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1); Leutenizing hormone receptor (LHR); Lewis(Y) antigen; Lews Ag; Livl; Locus K 9 (LY6K); Low conductance chloride channel; Lymphocyte antigen 6 complex; Lymphocyte antigen 75 (LY75); Lymphocyte-specific protein tyrosine kinase (LCK); Mammary gland differentiation antigen (NY-BR-1); Melanoma antigen recognized by T cells 1 (MelanA or MARTI); Melanoma- associated antigen 1 (MAGE-A1); Melanoma cancer testis antigen-1 (MAD-CT-1); Melanoma cancer testis antigen-2 (MAD-CT-2); Melanoma inhibitor of apoptosis (ML-IAP); Mesothelin; MPL; Mucin 1 cell surface associated (MUC1); N-Acetyl glucosaminyl-transferase V (NA17); Nectin-4; Neural cell adhesion molecule (NCAM); NKG2D; NYBR1; O-acetyl-GD2 ganglioside (OAcGD2); Olfactory receptor 51E2 (OR51E2); Oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl); P53 mutant; Paired box protein Pax-3

(PAX3); Paired box protein Pax-5 (PAX5); Pannexin 3 (PANX3); PDL1; P-glycoprotein; Placenta-specific 1 (PLAC1); Platelet-derived growth factor receptor beta (PDGFR-beta); Polysialic acid; Proacrosin binding protein sp32 (OY-TES1); Prostase; Prostate carcinoma tumor antigen-1 (PCT A-1 or Galectin 8); Prostate stem cell antigen (PSCA); Prostate-specific membrane antigen (PSMA); Prostatic acid phosphatase (PAP); Prostein; Protease Serine 21 (Testisin or PRSS21); Proteasome (Prosome Macropain) Subunit Beta Type 9 (LMP2); PTK7; Ras G12V; Ras Homolog Family Member C (RhoC); Rat sarcoma (Ras) mutant; Receptor for Advanced Gly cation Endproducts (RAGE-1); Receptor tyrosine kinase-like orphan receptor 1 (ROR1); Receptor tyrosine-protein kinase ERBB2 or Her-22/neu; Renal ubiquitous 1 (RU1); Renal ubiquitous 2 (RU2); Sarcoma translocation breakpoints; Serine 2 (TMPRSS2) ETS fusion gene; Sialyl Lewis adhesion molecule (sLe); SLAMF4; SLAMF6; Slea (CA19.9 or Sialyl Lewis Antigen); Sperm protein 17 (SPA17); Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Stage-specific embryonic antigen-4 (SSEA-4); STEAP1; Survivin; Synovial sarcoma X breakpoint 2 (SSX2); TCR Gamma Alternate Reading Frame Protein (TARP); TCR-beta1 chain; TCR-beta2 chain; TCR-delta chain; TCR-gamma chain; TCRgamma-delta; Telomerase; TGFbetaR2; The antigen recognized by TNT antibody; Thyroid stimulating hormone receptor (TSHR); Timl-/HVCR1; Tissue Factor 1 (TF1); Tn ag; Tn antigen ((Tn Ag) or (GalNAca-Ser/Thr)); TNF receptor family member B cell maturation (BCMA); Transglutaminase 5 (TGS5); Transmembrane protease; TROP2; Tumor endothelial marker 1 (TEM1/CD248); Tumor endothelial marker 7-related (TEM7R); Tumor protein p53 (p53); Tumor-associated glycoprotein 72 (TAG72); Tyrosinase; Tyrosinase-related protein 2 (TRP-2); Uroplakin 2 (UPK2); Vascular endothelial growth factor receptor 2 (VEGFR2); V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Wilms tumor protein (WT1); or X Antigen Family Member 1A (XAGE1). In some embodiments, the targeting moiety binds to CD7. In some embodiments, the targeting moiety binds to CD8.

~~[0166]~~[0164] In some embodiments, the targeting moiety binds to a target that is present on a cell, such as an immune cell. In some embodiments, the cell is an immune cell, such as, but not limited to, T cell, B cell; NK cell, dendritic cell, neutrophils, macrophages, a cancer cell; or, for example, CD3+ T cell; CD4+ T cell; CD7+ T cell, CD8+ T cell; CD19+ B cell; CD19+ cancer cell; CD20+ B cell; CD20+ cancer cell; CD30+ lung epithelial cell; CD34+ haematopoietic stem

cell; CD105+ endothelial cell; CD105+ haematopoietic stem cell; CD117+ haematopoietic stem cell; CD133+ cancer cell; EpCAM+ cancer cell; GluA2+ neuron; GluA4+ neuron; Haematopoietic stem cell; Hepatocyte; Her2/Neu+ cancer cell; NKG2D+ natural killer cell; SLC1A3+ astrocyte; SLC7A10+ adipocyte. In some embodiments, the cell is a T cell. In some embodiments, the cell is a B cell. In some embodiments, the cell is a CD7+ T cell and/or CD8+ T cell.

~~[0167]~~[0165] In some embodiments, the targeting moiety (a polypeptide) can bind to CD7.

~~[0168]~~[0166] In some embodiments, the polypeptide binds to CD7. In some embodiments, the polypeptide that binds to CD7 is an antibody which binds to non-human primate CD7. In some embodiments, the polypeptide that binds to CD7 is an antibody which binds to human CD7. The sequence of human CD7 (UniProtKB P09564) is as follows (SEQ ID NO: 33):

MAGPPRLLLLPLLLALARGLPGALAAQEVQQSPHCTTVPVGASVNITCSTSGGLR
GIYLRQLGPQPQDIIYYEDGVVPTTDRRFRGRIDFSGSQDNLTITMHRLQLSDTGT
YTCQAITEVNVYGSGTLVLVTEEQSQGWHRCSDAPPRASALPAPPTGSALPDPQT
ASALPDPPAASALPAALAVISFLLGLGLGVACVLARTQIKKLCSWRDKNSAACV
VYEDMSHSRCNTLSSPNQYQ (SEQ ID NO: 33).

~~[0169]~~[0167] In some embodiments, the CD7 that the polypeptide binds to is expressed on the surface of a cell. In some embodiments, the cell is an immune cell. In some embodiments, the immune cell is a CD7+ T cell, CD4+ T cell, CD8+ T cell, NK cell, alpha-beta T cell, gamma-delta T cell, lymphoid progenitor cell, hematopoietic stem cell, myeloid cell, monocyte, macrophage, central memory T cell, effector memory T cell, stem-cell like memory T cells, naïve T cell, activated T cell, regulatory T cell (TReg), terminally differentiated effector memory T cell (TEMRA), resident memory T cell (TRM) or a T-cellCD8+CCR7+.

~~[0170]~~[0168] In some embodiments, the antibody comprises a Fc region. The Fc region can be linked to the heavy or light chain of the antibody. In some embodiments, the Fc region is an IgG Fc. In some embodiments, the IgG is selected from IgG1, IgG2, IgG3, or IgG4. In some embodiments, the IgG fc is IgG1 Fc. In some embodiments, the antibody comprises an Fc constant region of SEQ ID NO: 34 as set forth below.

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT

CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK (SEQ ID NO: 34)

~~[0171]~~[0169] In some embodiments, the IgG fc is IgG2 Fc. In some embodiments, the antibody comprises an Fc constant region of SEQ ID NO: 71 as set forth below.

STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPA
PPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHN
AKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO: 71)

~~[0172]~~[0170] In some embodiments, the IgG fc is IgG4 Fc. In some embodiments, the antibody comprises an Fc constant region of SEQ ID NO: 72 as set forth below.

STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPSCPA
PEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVH
NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKA
KGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
(SEQ ID NO: 72)

~~[0173]~~[0171] In some embodiments, polypeptides (e.g. CD7-binding polypeptide) are provided herein. In some embodiments, antibodies (*e.g.* an anti-CD7 antibody) are provided herein. In some embodiments, the antibody is a recombinant antibody that binds to CD7. In some embodiments, the CD7 protein is a human CD7 protein. In some embodiments, the CD7 protein is a non-human CD7 protein (e.g., mouse, rat, pig, dog, non-human primate). As used herein, the term "recombinant antibody" refers to an antibody that is not naturally occurring. In some embodiments, the term "recombinant antibody" refers to an antibody that is not isolated from a

human subject.

~~[0174]~~[0172] In some embodiments, an antibody, or antigen binding fragment thereof is provided, wherein the antibody or antibody fragment comprises a peptide selected from the following table, which illustrate the CDRs based on Chothia numbering.

Chothia CDRs						
Ab ID No	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	HCDR3
CD7AB1	GYPFTSY (SEQ ID NO: 35)	DPNSGD (SEQ ID NO: 36)	SPYYSNDNSMDY (SEQ ID NO: 37)	RASQSIGTSIH (SEQ ID NO: 38)	YASESIS (SEQ ID NO: 39)	QQSNSWPTT (SEQ ID NO: 40)

~~[0175]~~[0173] In some embodiments, an antibody, or antigen binding fragment thereof is provided, wherein the antibody or antibody fragment comprises a peptide selected from the following table, which illustrate the CDRs based on Kabat numbering.

Kabat CDRs						
Ab ID No	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	LCDR3
CD7AB1	SYWIH (SEQ ID NO: 41)	RIDPNSGDTKYNEKFKN (SEQ ID NO: 42)	SPYYSNDNSMDY (SEQ ID NO: 37)	RASQSIGTSIH (SEQ ID NO: 38)	YASESIS (SEQ ID NO: 39)	QQSNSWPTT (SEQ ID NO: 40)

~~[0176]~~[0174] In some embodiments, an antibody, or antigen binding fragment thereof is provided, wherein the antibody or antibody fragment comprises a peptide selected from the following table, which illustrate the CDRs based on IMGT numbering.

IMGT CDRs						
Ab ID No	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	HCDR3
CD7AB1	GYPFTSYW (SEQ ID NO: 43)	IDPNSGDT (SEQ ID NO: 44)	ARSPYYSNDNSMDY (SEQ ID NO: 45)	QSIGTS (SEQ ID NO: 46)	YA	QQSNSWPTT (SEQ ID NO: 40)

~~[0177]~~[0175] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy or light chain CDR as provided in the tables above. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy or light chain CDR as provided in the tables above and binds to non-human primate CD7. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy

or light chain CDR as provided in the tables above and binds to human CD7. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain CDR having a sequence selected from SEQ ID NO: 38-40. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain CDR having a sequence of SEQ ID NO: 38. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain CDR having a sequence of SEQ ID NO: 39. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain CDR having a sequence of SEQ ID NO: 40. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence selected from SEQ ID NO: 35-37. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence of SEQ ID NO: 35. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence of SEQ ID NO: 36. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence of SEQ ID NO: 37. The CDRs referenced in the embodiments throughout the present specification can be interchanged with the CDRs that are characterized by different formats, such as Kabat and IMGT.

~~[0178]~~[0176] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain variable region having a LCDR1, a LCDR2, and a LCDR3, wherein the LCDR1 has a sequence of SEQ ID NO: 38, the LCDR2 has a sequence of SEQ ID NO: 39, and the LCDR3 has a sequence of SEQ ID NO: 40.

~~[0179]~~[0177] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain variable region having a LCDR1, a LCDR2, and a LCDR3, wherein the LCDR1 has a sequence of SEQ ID NO: 46, the LCDR2 has a sequence of YA, and the LCDR3 has a sequence of SEQ ID NO: 40.

~~[0180]~~[0178] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain variable region having a HCDR1, a HCDR2, and a HCDR3, wherein the HCDR1 has a sequence of SEQ ID NO: 35, the HCDR2 has a sequence of SEQ ID NO: 36, and the HCDR3 has a sequence of SEQ ID NO: 37.

~~[0181]~~[0179] In some embodiments, a polypeptide, an antibody, or antibody binding

fragment thereof, comprises a heavy chain variable region having a HCDR1, a HCDR2, and a HCDR3, wherein the HCDR1 has a sequence of SEQ ID NO: 41, the HCDR2 has a sequence of SEQ ID NO: 42, and the HCDR3 has a sequence of SEQ ID NO: 37.

~~[0182]~~[0180] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain variable region having a HCDR1, a HCDR2, and a HCDR3, wherein the HCDR1 has a sequence of SEQ ID NO: 43, the HCDR2 has a sequence of SEQ ID NO: 44, and the HCDR3 has a sequence of SEQ ID NO: 45.

~~[0183]~~[0181] In some embodiments, a polypeptide, an antibody or antibody binding fragment thereof, comprises: (i) a light chain having any one of the foregoing recited combinations of LCDR1, LCDR2, and LCDR3 sequences; and (ii) a heavy chain having any one of the foregoing recited combinations of HCDR1, HCDR2, and HCDR3 sequences.

~~[0184]~~[0182] The different CDR motifs can be combined in any combination including those not depicted in the table above. For example, the following embodiments are provided as non-limiting examples of such combinations.

~~[0185]~~[0183] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises: (i) a light chain variable region comprising light chain CDRl, CDR2, and CDR3 sequences, wherein the light chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 38; the light chain CDR2 has the amino acid sequence of SEQ ID NO: 39; and the light chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 40; and (ii) a heavy chain variable region comprising heavy chain CDR1, CDR2, and CDR3 sequences, wherein the heavy chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 35; the heavy chain CDR2 sequence has the amino acid sequence of SEQ ID NO: 36; and the heavy chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 37; or variants of any of the foregoing.

~~[0186]~~[0184] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises: (i) a light chain variable region comprising light chain CDRl, CDR2, and CDR3 sequences, wherein the light chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 38; the light chain CDR2 has the amino acid sequence of SEQ ID NO: 39; and the light chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 40; and (ii) a heavy chain variable region comprising heavy chain CDR1, CDR2, and CDR3 sequences, wherein the heavy chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 41; the heavy chain CDR2

sequence has the amino acid sequence of SEQ ID NO: 42; and the heavy chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 37; or variants of any of the foregoing.

~~[0187]~~[0185] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises: (i) a light chain variable region comprising light chain CDRl, CDR2, and CDR3 sequences, wherein the light chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 46; the light chain CDR2 has the amino acid sequence of YA; and the light chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 40; and (ii) a heavy chain variable region comprising heavy chain CDR1, CDR2, and CDR3 sequences, wherein the heavy chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 43; the heavy chain CDR2 sequence has the amino acid sequence of SEQ ID NO: 44; and the heavy chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 45; or variants of any of the foregoing.

~~[0188]~~[0186] In some embodiments, the light chain variable region CDR1 is replaced with any of the other light chain CDR1 sequences. In some embodiments, the light chain variable region CDR2 is replaced with any of the other light chain CDR2 sequences. In some embodiments, the light chain variable region CDR3 is replaced with any of the other light chain CDR3 sequences. In some embodiments, the heavy chain variable region CDR1 is replaced with any of the other heavy chain CDR1 sequences. In some embodiments, the heavy chain variable region CDR2 is replaced with any of the other heavy chain CDR2 sequences. In some embodiments, the heavy chain variable region CDR3 is replaced with any of the other heavy chain CDR3 sequences.

~~[0189]~~[0187] In some embodiments, the polypeptide comprises a heavy chain variable region peptide having one of the following sequences, or a variant thereof:

SEQ ID NO:	AB ID NO.	Sequence
47	CD7AB1	QVQLQQPGAELVKPGASVKLSCKASGYPFTSYWIHWVKQRPGRGL EWLGRIDPNSGDTKYNEKFKNKATLTVDKSSTTAYMQLSSLTSED SAVYYCARSPYYSNDNSMDYWGQGTSVTVSS

~~[0190]~~[0188] In some embodiments, the polypeptide comprises a light chain variable region peptide having one of the following sequences, or a variant thereof:

SEQ ID NO:	AB ID NO.	Sequence
48	CD7AB1	DILLTQSPAILSVSPGERVSFSCRASQSIGTSIHWYQQRTNDSPR LLIKYASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQ SNSWPTTFGGGTKLEIKR

~~[0191]~~[0189] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide of SEQ ID NO: 47. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_L peptide of SEQ ID NO: 48. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide and a V_L peptide, wherein the wherein the V_H peptide comprises a sequence of SEQ ID NO: 47, or a variant thereof; and the V_L peptide comprises a sequence of SEQ ID NO: 48, or a variant thereof. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide and a V_L peptide, wherein the wherein the V_H peptide comprises a sequence of SEQ ID NO: 47, or a variant thereof; and the V_L peptide comprises a sequence of SEQ ID NO: 48, or a variant thereof, and the polypeptide, the antibody, or antigen binding fragment thereof, binds to non-human primate CD7. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide and a V_L peptide, wherein the wherein the V_H peptide comprises a sequence of SEQ ID NO: 47, or a variant thereof; and the V_L peptide comprises a sequence of SEQ ID NO: 48, or a variant thereof, and the polypeptide, the antibody, or antigen binding fragment thereof, binds to human CD7. In some embodiments, the V_H peptide comprises a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence of SEQ ID NO: 48.

~~[0192]~~[0190] The VH and the VL sequences can be in any format, including, but not limited to an scFv format where the VH and VL regions are linked with a peptide linker. Examples of peptide linkers that can be used to link various peptides provided for herein include, but are not limited to: $(GGGGS)_n$ (SEQ ID NO: 49), wherein each n is independently 1-5. In some embodiments, n is 1. In some embodiments, n is 2. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. In some embodiments, the variable regions are not linked with a peptide linker. In some embodiments, the polypeptide comprises SEQ ID NO: 47 and SEQ ID NO: 48.

~~[0193]~~[0191] In some embodiments, the VH and VL polypeptides are linked to a Fc region. In some embodiments, the Fc region is as provided for herein. In some embodiments, the Fc region comprises an amino acid sequence of SEQ ID NO: 34, SEQ ID NO: 71, or SEQ ID NO: 72 as provided for herein. As provided for herein, the heavy chain can be linked to a Fc region. Non-limiting mutations in the Fc region are provided for herein. In some embodiments, the Fc

region further comprises a transmembrane domain. Examples of transmembrane domains include, but are not limited to, a CD8 or CD28 ("CD8/CD28") transmembrane domain. In some embodiments, the Fc region further comprises a CD8 transmembrane domain. In some embodiments, the Fc region further comprises a CD28 transmembrane domain. In some embodiments, the Fc region comprising a transmembrane domain further comprises an Env incorporation motif. In some embodiments, the Fc region comprising a CD8/CD28 transmembrane domain further comprises an Env incorporation motif. In some embodiments, the VH and VL polypeptides provided herein are linked to an Fc region comprising a transmembrane domain. In some embodiments, the VH and VL polypeptides provided herein are linked to an Fc region comprising a CD8/CD28 transmembrane domain. In some embodiments, the VH and VL polypeptides provided herein are linked to an Fc region comprising a CD8/CD28 transmembrane domain and an Env incorporation motif. In some embodiments, the VH and VL polypeptides provided herein linked to an Fc region comprising a CD8/CD28 transmembrane domain are anchored to the plasma membrane on the surface of a cell. In some embodiments, the cell is an immune cell, such as those provided herein. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 47 and VL having a sequence as set forth in SEQ ID NO: 48 are linked to an Fc region comprising a transmembrane domain. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 47 and VL having a sequence as set forth in SEQ ID NO: 48 are linked to an Fc region comprising a CD8/CD28 transmembrane domain. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 47 and VL having a sequence as set forth in SEQ ID NO: 48 are linked to an Fc region comprising a CD8/CD28 transmembrane domain and an Env incorporation motif. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 47 and VL having a sequence as set forth in SEQ ID NO: 48 linked to an Fc region comprising a CD8/CD28 transmembrane domain are anchored to the plasma membrane on the surface of a cell. In some embodiments, the cell is an immune cell, such as those provided herein.

~~[0194]~~[0192] In some embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48 are linked to an Fc region comprising a transmembrane domain. In

some embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48 are linked to an Fc region comprising a CD8/CD28 transmembrane domain. In some embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48 are linked to an Fc region comprising a CD8/CD28 transmembrane domain and an Env incorporation motif. In some embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48 linked to an Fc region comprising a CD8/CD28 transmembrane domain are anchored to the plasma membrane on the surface of a cell. In some embodiments, the cell is an immune cell, such as those provided herein.

~~[0195]~~[0193] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48.

~~[0196]~~[0194] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_H peptide and a V_L peptide comprises a light chain CDR having a sequence of SEQ ID NO: 38-40; and/or a heavy chain CDR having a sequence of SEQ ID NO: 35-37. In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide

comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_H peptide and a V_L peptide comprise a light chain CDR1 having a sequence of SEQ ID NO: 38; a light chain CDR2 having a sequence of SEQ ID NO: 39; a light chain CDR3 having a sequence of SEQ ID NO: 40; and/or a heavy chain CDR1 having a sequence of SEQ ID NO: 35; a heavy chain CDR2 having a sequence of SEQ ID NO: 36; and a heavy chain CDR3 having a sequence of SEQ ID NO: 37. In some embodiments, the CDRs in the V_H or V_L chain are as set forth in the combinations provided for herein.

~~[0197]~~[0195] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 38; a LCDR2 having a sequence of SEQ ID NO: 39; and a LCDR3 having a sequence of SEQ ID NO: 40; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 35; a HCDR2 having a sequence of SEQ ID NO: 36; and a HCDR3 having a sequence of SEQ ID NO: 37.

~~[0198]~~[0196] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 38, wherein the LCDR1 comprises at most 1 conservative amino acid substitution, a LCDR2 having a sequence of SEQ ID NO: 39, wherein the LCDR2 comprises at most 1 conservative amino acid substitution, and a LCDR3 having a sequence of SEQ ID NO: 40, wherein the LCDR3 comprises at most 1 conservative amino acid substitution; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 35, wherein the HCDR1 comprises at most 1 conservative amino acid substitution,

a HCDR2 having a sequence of SEQ ID NO: 36, wherein the HCDR2 comprises at most 1 conservative amino acid substitution, and a HCDR3 having a sequence of SEQ ID NO: 37, wherein the HCDR3 comprises at most 1 conservative amino acid substitution.

~~[0199]~~[0197] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_H peptide and a V_L peptide comprise a light chain CDR1 having a sequence of SEQ ID NO: 38; a light chain CDR2 having a sequence of SEQ ID NO: 39; a light chain CDR1 having a sequence of SEQ ID NO: 40; and/or a heavy chain CDR1 having a sequence of SEQ ID NO: 41; a heavy chain CDR2 having a sequence of SEQ ID NO: 42; and a heavy chain CDR3 having a sequence of SEQ ID NO: 37. In some embodiments, the CDRs in the V_H or V_L chain are as set forth in the combinations provided for herein.

~~[0200]~~[0198] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 38; a LCDR2 having a sequence of SEQ ID NO: 39; and a LCDR3 having a sequence of SEQ ID NO: 40; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 41; a HCDR2 having a sequence of SEQ ID NO: 42; and a HCDR3 having a sequence of SEQ ID NO: 37.

~~[0201]~~[0199] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 38, wherein the LCDR1 comprises at most 1 conservative amino acid substitution, a LCDR2 having a sequence

of SEQ ID NO: 39, wherein the LCDR2 comprises at most 1 conservative amino acid substitution, and a LCDR3 having a sequence of SEQ ID NO: 40, wherein the LCDR3 comprises at most 1 conservative amino acid substitution; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 41, wherein the HCDR1 comprises at most 1 conservative amino acid substitution, a HCDR2 having a sequence of SEQ ID NO: 42, wherein the HCDR2 comprises at most 1 conservative amino acid substitution, and a HCDR3 having a sequence of SEQ ID NO: 37, wherein the HCDR3 comprises at most 1 conservative amino acid substitution.

~~[0202]~~[0200] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_H peptide and a V_L peptide comprise a light chain CDR1 having a sequence of SEQ ID NO: 46; a light chain CDR2 having a sequence of YA; a light chain CDR1 having a sequence of SEQ ID NO: 40; and/or a heavy chain CDR1 having a sequence of SEQ ID NO: 43; a heavy chain CDR2 having a sequence of SEQ ID NO: 44; and a heavy chain CDR3 having a sequence of SEQ ID NO: 45. In some embodiments, the CDRs in the V_H or V_L chain are as set forth in the combinations provided for herein.

~~[0203]~~[0201] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 46, a LCDR2 having a sequence of YA; and a LCDR3 having a sequence of SEQ ID NO: 40; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 43; a HCDR2 having a sequence of SEQ ID NO: 44; and a HCDR3 having a sequence of SEQ ID NO: 45.

~~[0204]~~[0202] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID

NO: 47; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 48; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 46, wherein the LCDR1 comprises at most 1 conservative amino acid substitution, a LCDR2 having a sequence of YA, wherein the LCDR2 comprises at most 1 conservative amino acid substitution, and a LCDR3 having a sequence of SEQ ID NO: 40, wherein the LCDR3 comprises at most 1 conservative amino acid substitution; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 43, wherein the HCDR1 comprises at most 1 conservative amino acid substitution, a HCDR2 having a sequence of SEQ ID NO: 44, wherein the HCDR2 comprises at most 1 conservative amino acid substitution, and a HCDR3 having a sequence of SEQ ID NO: 45, wherein the HCDR3 comprises at most 1 conservative amino acid substitution.

~~[0205]~~[0203] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence of SEQ ID NO: 47 and the V_L peptide comprises a sequence of SEQ ID NO: 48.

~~[0206]~~[0204] In some embodiments, a polypeptide as provided herein binds to non-human primate CD7. In some embodiments, a polypeptide as provided herein binds to human CD7.

~~[0207]~~[0205] As provided for herein, the different polypeptides (V_H or V_L) described herein can be linked with a peptide linker or not linked with a peptide linker and instead for a continuous sequence. In some embodiments, the peptide linker comprises a sequence of $(GGGGS)_n$ (SEQ ID NO: 49), wherein each n is independently 1-5. In some embodiments, n is 1. In some embodiments, n is 2. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. The linked peptide format can be represented by a formula of V_H-Z-V_L or V_L-Z-V_H, wherein Z is the peptide linker. In some embodiments, Z is $(GGGGS)_n$ (SEQ ID NO: 49), wherein each n is independently 1-5.

~~[0208]~~[0206] In some embodiments, a polypeptide comprising the linked peptide represented by a formula of V_L-Z-V_H comprises a heavy chain variable region as set forth in SEQ ID NO: 47 linked via a linker sequence GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 50) to a light chain variable region as set forth in SEQ ID NO: 48. In some embodiments, a polypeptide comprising a V_L linked via a peptide linker to a V_H has the sequence as set forth below,

DILLTQSPAILSVSPGERVSFSCRASQSIGTSIHWYQQRTNDSPRLLIKYASESISGIP
SRFSGSGSGTDFTLSINSVESEDIADYYCQQSNSWPTTFGGGTKLEIKRGGGGSGG
GGSGGGGSGGGGSQVQLQQPGAELVKPGASVKLSCKASGYPFTSYWIHWVKQR
PGRGLEWLGRIDPNSGDTKYNEKFKNKATLTVDKSSTTAYMQLSSLTS
EDSAVYYCARSPYYSNDNSMDYWGQGTSVTVSS (SEQ ID NO: 51).

~~[0209]~~[0207] In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 51. In some embodiments, a polypeptide comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 51. In some embodiments, a polypeptide comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 51. In some embodiments, a polypeptide comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 51. In some embodiments, a polypeptide comprises a sequence as set forth in SEQ ID NO: 51. In some embodiments, the polypeptide as set forth in SEQ ID NO: 51 is an antibody, or an antigen binding fragment thereof. In some embodiments, the antibody is an anti-CD7 antibody.

~~[0210]~~[0208] In some embodiments, a polypeptide comprising the linked peptide represented by a formula of V_H-Z-V_L comprises a light chain variable region as set forth in SEQ ID NO: 48 linked via a linker sequence GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 50) to a heavy chain variable region as set forth in SEQ ID NO: 49. In some embodiments, a polypeptide comprising a V_H linked via a peptide linker to a V_L has the sequence as set forth below,

QVQLQQPGAELVKPGASVKLSCKASGYPFTSYWIHWVKQRPGRGLEWLGRIDP
NSGDTKYNEKFKNKATLTVDKSSTTAYMQLSSLTSEDSAVYYCARSPYYSNDNS
MDYWGQGTSVTVSSGGGGSGGGGSGGGGSGGGGSDILLTQSPAILSVSPGERVS
FSCRASQSIGTSIHWYQQRTNDSPRLLIKYASESISGIPSRFSGSGSGTDFTLSINSV
ESEDIADYYCQQSNSWPTTFGGGTKLEIKR (SEQ ID NO: 52)

~~[0211]~~[0209] In some embodiments, a polypeptide comprises a sequence that is at least

85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 52. In some embodiments, a polypeptide comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 52. In some embodiments, a polypeptide comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 52. In some embodiments, a polypeptide comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 52. In some embodiments, a polypeptide comprises a sequence as set forth in SEQ ID NO: 52. In some embodiments, the polypeptide as set forth in SEQ ID NO: 52 is an antibody, or an antigen binding fragment thereof. In some embodiments, the antibody is an anti-CD7 antibody. In some embodiments, the anti-CD7 antibody binds to non-human primate CD7. In some embodiments, the anti-CD7 antibody binds to human CD7.

~~[0212]~~[0210] In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 51 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 51 and comprises an Fc region, such as those provided herein, and a transmembrane domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 51 and comprises an Fc region, such as those provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 51 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0213]~~[0211] In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 52 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 52 and comprises an Fc region, such as those provided herein, and a transmembrane

domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 52 and comprises an Fc region, such as those provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 52 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0214]~~[0212] As provided for herein, the polypeptide, antibodies, or antigen binding fragments thereof can be variants of the sequences.

~~[0215]~~[0213] The sequences of the polypeptides or antibodies can be modified to yield human IgG antibodies. The conversion of the sequences provided herein can be modified to yield other types of antibodies. The CDRs can also be linked to other antibodies, proteins, or molecules to create antibody fragments that bind CD7.

~~[0216]~~[0214] In some embodiments, a polypeptide or an antibody as provided herein is a targeting moiety on the surface of an engineered viral particle. In some embodiments, the targeting moiety allows for binding to a target cell. In some embodiments, the targeting moiety is a CD7 binding moiety, such as a polypeptide or an antibody as provided herein. In some embodiments, the targeting moiety comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 51. In some embodiments, the targeting moiety comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 51. In some embodiments, the targeting moiety comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 51. In some embodiments, the targeting moiety comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 51. In some embodiments, the targeting moiety comprises a sequence as set forth in SEQ ID NO: 51. In some embodiments, the targeting moiety as set forth in SEQ ID NO: 51 is an antibody, or an antigen binding fragment thereof. In some embodiments, the targeting moiety is an anti-CD7 antibody.

~~[0217]~~[0215] In some embodiments, a polypeptide or an antibody as provided for herein is a targeting moiety on the surface of an engineered viral particle. In some embodiments, the

engineered viral particle is a pseudotyped viral-like particle. In some embodiments, the targeting moiety allows for binding to a target cell. In some embodiments, the targeting moiety is a CD7 binding moiety, such as a polypeptide or an antibody as provided herein. In some embodiments, the targeting moiety comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 52. In some embodiments, the targeting moiety comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 52. In some embodiments, the targeting moiety comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 52. In some embodiments, the targeting moiety comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 52. In some embodiments, the targeting moiety comprises a sequence as set forth in SEQ ID NO: 52. In some embodiments, the targeting moiety as set forth in SEQ ID NO: 52 is an antibody, or an antigen binding fragment thereof. In some embodiments, the targeting moiety is an anti-CD7 antibody. In some embodiments, the anti-CD7 antibody binds to non-human primate CD7. In some embodiments, the anti-CD7 antibody binds to human CD7.

~~[0218]~~[0216] In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 51 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 51 and comprises an Fc region, such as those provided herein, and a transmembrane domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 51 and comprises an Fc region, such as those provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 51 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0219]~~[0217] In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 52 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 52 and comprises an Fc region, such as those provided herein, and a transmembrane domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 52 and comprises an Fc region, such as those

provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 52 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0220]~~[0218] In some embodiments, the targeting moiety (a polypeptide) can bind to CD8.

~~[0221]~~[0219] In some embodiments, the polypeptide binds to CD8. In some embodiments, the polypeptide binds to CD8-alpha. In some embodiments, the polypeptide binds to CD8-beta. In some embodiments, the polypeptide binds to CD8 heterodimer. In some embodiments, the CD8 heterodimer comprises CD8-alpha and CD8-beta subunits. In some embodiments, the polypeptide binds to CD8-alpha homodimer. In some embodiments, the polypeptide that binds to CD8 is an antibody which binds to non-human primate CD8. In some embodiments, the antibody that binds to non-human primate CD8 is an antibody which binds to non-human primate CD8-alpha. In some embodiments, the antibody that binds to non-human primate CD8 is an antibody which binds to non-human primate CD8-beta. In some embodiments, the antibody that binds to non-human primate CD8 is an antibody which binds to non-human primate CD8-alpha homodimer. In some embodiments, the antibody that binds to non-human primate CD8 is an antibody which binds to non-human primate CD8 heterodimer. In some embodiments, the polypeptide that binds to CD8 is an antibody which binds to human CD8. In some embodiments, the antibody that binds to human CD8 is an antibody which binds to human CD8-alpha. In some embodiments, the antibody that binds to human CD8 is an antibody which binds to human CD8-beta. In some embodiments, the antibody that binds to human CD8 is an antibody which binds to human CD8-alpha homodimer. In some embodiments, the antibody that binds to human CD8 is an antibody which binds to human CD8 heterodimer. The sequence of human CD8-alpha (UniProtKB Q8TAW8) is as follows (SEQ ID NO: 53):

MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCS
WLFQPRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRREN
EGCYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRP
AAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRP
VVKSGDKPSLSARYV (SEQ ID NO: 53).

~~[0222]~~[0220] The sequence of human CD8-beta (UniProtKB Q8TD28) is as follows (SEQ ID NO: 54):

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMRIY WLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNLTSVKP EDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVCRLPRPETQK GPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQLYK (SEQ ID NO: 54).

~~[0223]~~[0221] In some embodiments, the CD8 that the polypeptide binds to is expressed on the surface of a cell. In some embodiments, the cell is an immune cell. In some embodiments, the immune cell is a CD7+ T cell, CD4+ T cell, CD8+ T cell, NK cell, alpha-beta T cell, gamma-delta T cell, lymphoid progenitor cell, hematopoietic stem cell, myeloid cell, monocyte, macrophage, central memory T cell, effector memory T cell, stem-cell like memory T cells, naïve T cell, activated T cell, regulatory T cell (TReg), terminally differentiated effector memory T cell (TEMRA), resident memory T cell (TRM) or a T-cellCD8+CCR7+. In some embodiments, the cell is a CD8+ T cell. In some embodiments, the cell is a CD8+ cell.

~~[0224]~~[0222] In some embodiments, the antibody comprises a Fc region. The Fc region can be linked to the heavy or light chain of the antibody. In some embodiments, the Fc region is an IgG Fc. In some embodiments, the IgG is selected from IgG1, IgG2, IgG3, or IgG4. In some embodiments, the IgG Fc is IgG1 Fc. In some embodiments, the antibody comprises an Fc constant region as set forth below.

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK (SEQ ID NO: 34)

~~[0225]~~[0223] In some embodiments, the IgG fc is IgG2 Fc. In some embodiments, the antibody comprises an Fc constant region of SEQ ID NO: 71 as set forth below.

STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPA
PPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHN
AKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO: 71)

~~[0226]~~[0224] In some embodiments, the IgG fc is IgG4 Fc. In some embodiments, the antibody comprises an Fc constant region of SEQ ID NO: 72 as set forth below.

STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPSCPA
PEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVH
NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKA
KGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
(SEQ ID NO: 72)

~~[0227]~~[0225] In some embodiments, polypeptides (e.g. CD8-binding polypeptide) are provided herein. In some embodiments, antibodies (*e.g.* an anti-CD8 antibody) are provided herein. In some embodiments, the antibody is a recombinant antibody that binds to CD8. In some embodiments, the CD8 protein is a human CD8 protein. In some embodiments, the CD8 protein is a non-human CD8 protein (e.g., mouse, rat, pig, dog, non-human primate). As used herein, the term "recombinant antibody" refers to an antibody that is not naturally occurring. In some embodiments, the term "recombinant antibody" refers to an antibody that is not isolated from a human subject.

~~[0228]~~[0226] In some embodiments, an antibody, or antigen binding fragment thereof is provided, wherein the antibody or antibody fragment comprises a peptide selected from the following table, which illustrate the CDRs based on Chothia numbering.

Chothia CDRs

Ab ID No	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	LCDR3
CD8AB1	RYTFTDY (SEQ ID NO: 55)	YPYNGG (SEQ ID NO: 56)	DHRYNEGVSFDY (SEQ ID NO: 57)	RASESVDGFGNSFMN (SEQ ID NO: 58)	LASNLES (SEQ ID NO: 59)	QQNNEDPYT (SEQ ID NO: 60)

~~[0229]~~[0227] In some embodiments, an antibody, or antigen binding fragment thereof is provided, wherein the antibody or antibody fragment comprises a peptide selected from the following table, which illustrate the CDRs based on Kabat numbering.

Kabat CDRs						
Ab ID No	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	LCDR3
CD8AB1	DYNLH (SEQ ID NO: 61)	FIYPYNGGTGYNQKFKN (SEQ ID NO: 62)	DHRYNEGVSFDY (SEQ ID NO: 57)	RASESVDGFGNSFMN (SEQ ID NO: 58)	LASNLES (SEQ ID NO: 59)	QQNNEDPYT (SEQ ID NO: 60)

~~[0230]~~[0228] In some embodiments, an antibody, or antigen binding fragment thereof is provided, wherein the antibody or antibody fragment comprises a peptide selected from the following table, which illustrate the CDRs based on IMGT numbering.

IMGT CDRs						
Ab ID No	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	LCDR3
CD8AB1	RYTFTDYN(SEQ ID NO: 63)	IYPYNGGT (SEQ ID NO: 64)	ARDHRYNEGVSFDY (SEQ ID NO: 65)	ESVDGFGNSF (SEQ ID NO: 66)	LA	QQNNEDPYT (SEQ ID NO: 60)

~~[0231]~~[0229] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy or light chain CDR as provided in the tables above. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy or light chain CDR as provided in the tables above and binds to non-human primate CD8. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy or light chain CDR as provided in the tables above and binds to human CD8. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain CDR having a sequence selected from SEQ ID NO: 58-60. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain CDR having a sequence of SEQ ID NO: 58. In some embodiments, a polypeptide, an antibody, or

antibody binding fragment thereof, comprises a light chain CDR having a sequence of SEQ ID NO: 59. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain CDR having a sequence of SEQ ID NO: 60. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence selected from SEQ ID NO: 55-57. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence of SEQ ID NO: 55. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence of SEQ ID NO: 56. In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain CDR having a sequence of SEQ ID NO: 57. The CDRs referenced in the embodiments throughout the present specification can be interchanged with the CDRs that are characterized by different formats, such as Kabat and IMGT.

~~[0232]~~[0230] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain variable region having a LCDR1, a LCDR2, and a LCDR3, wherein the LCDR1 has a sequence of SEQ ID NO: 58, the LCDR2 has a sequence of SEQ ID NO: 59, and the LCDR3 has a sequence of SEQ ID NO: 60.

~~[0233]~~[0231] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a light chain variable region having a LCDR1, a LCDR2, and a LCDR3, wherein the LCDR1 has a sequence of SEQ ID NO: 66, the LCDR2 has a sequence of LA, and the LCDR3 has a sequence of SEQ ID NO: 60.

~~[0234]~~[0232] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain variable region having a HCDR1, a HCDR2, and a HCDR3, wherein the HCDR1 has a sequence of SEQ ID NO: 55, the HCDR2 has a sequence of SEQ ID NO: 56, and the HCDR3 has a sequence of SEQ ID NO: 57.

~~[0235]~~[0233] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain variable region having a HCDR1, a HCDR2, and a HCDR3, wherein the HCDR1 has a sequence of SEQ ID NO: 61, the HCDR2 has a sequence of SEQ ID NO: 62, and the HCDR3 has a sequence of SEQ ID NO: 57.

~~[0236]~~[0234] In some embodiments, a polypeptide, an antibody, or antibody binding fragment thereof, comprises a heavy chain variable region having a HCDR1, a HCDR2, and a

HCDR3, wherein the HCDR1 has a sequence of SEQ ID NO: 63, the HCDR2 has a sequence of SEQ ID NO: 64, and the HCDR3 has a sequence of SEQ ID NO: 65.

~~[0237]~~[0235] In some embodiments, a polypeptide, an antibody or antibody binding fragment thereof, comprises: (i) a light chain having any one of the foregoing recited combinations of LCDR1, LCDR2, and LCDR3 sequences; and (ii) a heavy chain having any one of the foregoing recited combinations of HCDR1, HCDR2, and HCDR3 sequences.

~~[0238]~~[0236] The different CDR motifs can be combined in any combination including those not depicted in the table above. For example, the following embodiments are provided as non-limiting examples of such combinations.

~~[0239]~~[0237] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises: (i) a light chain variable region comprising light chain CDR1, CDR2, and CDR3 sequences, wherein the light chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 58; the light chain CDR2 has the amino acid sequence of SEQ ID NO: 59; and the light chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 60; and (ii) a heavy chain variable region comprising heavy chain CDR1, CDR2, and CDR3 sequences, wherein the heavy chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 55; the heavy chain CDR2 sequence has the amino acid sequence of SEQ ID NO: 56; and the heavy chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 57; or variants of any of the foregoing.

~~[0240]~~[0238] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises: (i) a light chain variable region comprising light chain CDR1, CDR2, and CDR3 sequences, wherein the light chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 58; the light chain CDR2 has the amino acid sequence of SEQ ID NO: 59; and the light chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 60; and (ii) a heavy chain variable region comprising heavy chain CDR1, CDR2, and CDR3 sequences, wherein the heavy chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 61; the heavy chain CDR2 sequence has the amino acid sequence of SEQ ID NO: 62; and the heavy chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 57; or variants of any of the foregoing.

~~[0241]~~[0239] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises: (i) a light chain variable region comprising light chain CDR1, CDR2, and CDR3 sequences, wherein the light chain CDR1 sequence has the amino acid sequence of

SEQ ID NO: 66; the light chain CDR2 has the amino acid sequence of LA; and the light chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 60; and (ii) a heavy chain variable region comprising heavy chain CDR1, CDR2, and CDR3 sequences, wherein the heavy chain CDR1 sequence has the amino acid sequence of SEQ ID NO: 63; the heavy chain CDR2 sequence has the amino acid sequence of SEQ ID NO: 64; and the heavy chain CDR3 sequence has the amino acid sequence of SEQ ID NO: 65; or variants of any of the foregoing.

~~[0242]~~[0240] In some embodiments, the light chain variable region CDR1 is replaced with any of the other light chain CDR1 sequences. In some embodiments, the light chain variable region CDR2 is replaced with any of the other light chain CDR2 sequences. In some embodiments, the light chain variable region CDR3 is replaced with any of the other light chain CDR3 sequences. In some embodiments, the heavy chain variable region CDR1 is replaced with any of the other heavy chain CDR1 sequences. In some embodiments, the heavy chain variable region CDR2 is replaced with any of the other heavy chain CDR2 sequences. In some embodiments, the heavy chain variable region CDR3 is replaced with any of the other heavy chain CDR3 sequences.

~~[0243]~~[0241] In some embodiments, the polypeptide comprises a heavy chain variable region peptide having one of the following sequences, or a variant thereof:

SEQ ID NO:	AB ID NO.	Sequence
67	CD8AB1	EVQLQQSGPELVKPGASVKISCKASRYTFTDYNLHWVKLSHEKSL EWIGFIYPYNGGTGYNQKFKNKAKLTVDYSSSTAYMELRSLTSVD AAVYYCARDHRYNEGVSFDYWGQGTTLTVSS

~~[0244]~~[0242] In some embodiments, the polypeptide comprises a light chain variable region peptide having one of the following sequences, or a variant thereof:

SEQ ID NO:	AB ID NO.	Sequence
68	CD8AB1	NIVLTQSPASLAVSLGQRATISCRASESVDGFGNSFMNWYQQKPG QSPKLLIYLASNLESGVPARFSGSGSRTDFTLTIDPVEADDAATY YCQQNNEDPYTFGGGTKLEIKR

~~[0245]~~[0243] In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide of SEQ ID NO: 67. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_L peptide of SEQ ID NO: 68. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide and a V_L peptide, wherein the wherein the V_H peptide comprises a sequence of SEQ

ID NO: 67, or a variant thereof; and the V_L peptide comprises a sequence of SEQ ID NO: 68, or a variant thereof. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide and a V_L peptide, wherein the wherein the V_H peptide comprises a sequence of SEQ ID NO: 67, or a variant thereof; and the V_L peptide comprises a sequence of SEQ ID NO: 68, or a variant thereof, and the polypeptide, the antibody, or antigen binding fragment thereof, binds to non-human primate CD8. In some embodiments, a polypeptide, an antibody, or antigen binding fragment thereof, comprises a V_H peptide and a V_L peptide, wherein the wherein the V_H peptide comprises a sequence of SEQ ID NO: 67, or a variant thereof; and the V_L peptide comprises a sequence of SEQ ID NO: 68, or a variant thereof, and the polypeptide, the antibody, or antigen binding fragment thereof, binds to human CD8. In some embodiments, the V_H peptide comprises a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence of SEQ ID NO: 68.

~~[0246]~~[0244] The VH and the VL sequences can be in any format, including, but not limited to an scFv format where the VH and VL regions are linked with a peptide linker. Examples of peptide linkers that can be used to link various peptides provided for herein include, but are not limited to: $(GGGGS)_n$ (SEQ ID NO: 49), wherein each n is independently 1-5. In some embodiments, n is 1. In some embodiments, n is 2. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. In some embodiments, the variable regions are not linked with a peptide linker. In some embodiments, the polypeptide comprises SEQ ID NO: 67 and SEQ ID NO: 68.

~~[0247]~~[0245] In some embodiments, the VH and VL polypeptides are linked to a Fc region. In some embodiments, the Fc region is as provided for herein. As provided for herein, the heavy chain can be linked to a Fc region. Non-limiting mutations in the Fc region are provided for herein. In some embodiments, the Fc region further comprises a transmembrane domain. Examples of transmembrane domains include, but are not limited to, a CD8/CD28 transmembrane domain. In some embodiments, the Fc region further comprises a CD8 or CD28 ("CD8/CD28") transmembrane domain. In some embodiments, the Fc region further comprises a CD8 transmembrane domain. In some embodiments, the Fc region further comprises a CD28 transmembrane domain. In some embodiments, the Fc region comprising a transmembrane domain further comprises an Env incorporation motif. In some embodiments, the Fc region

comprising a CD8/CD28 transmembrane domain further comprises an Env incorporation motif. In some embodiments, the VH and VL polypeptides provided herein are linked to an Fc region comprising a transmembrane domain. In some embodiments, the VH and VL polypeptides provided herein are linked to an Fc region comprising a CD8/CD28 transmembrane domain. In some embodiments, the VH and VL polypeptides provided herein are linked to an Fc region comprising a CD8/CD28 transmembrane domain and an Env incorporation motif. In some embodiments, the VH and VL polypeptides provided herein linked to an Fc region comprising a CD8/CD28 transmembrane domain are anchored to the plasma membrane on the surface of a cell. In some embodiments, the cell is an immune cell, such as those provided herein. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 67 and VL having a sequence as set forth in SEQ ID NO: 68 are linked to an Fc region comprising a transmembrane domain. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 67 and VL having a sequence as set forth in SEQ ID NO: 68 are linked to an Fc region comprising a CD8/CD28 transmembrane domain. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 67 and VL having a sequence as set forth in SEQ ID NO: 68 are linked to an Fc region comprising a CD8/CD28 transmembrane domain and an Env incorporation motif. In some embodiments, the VH having a sequence as set forth in SEQ ID NO: 67 and VL having a sequence as set forth in SEQ ID NO: 68 linked to an Fc region comprising a CD8/CD28 transmembrane domain are anchored to the plasma membrane on the surface of a cell. In some embodiments, the cell is an immune cell, such as those provided herein.

~~[0248]~~[0246] In some embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68 are linked to an Fc region comprising a transmembrane domain. In some embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68 are linked to an Fc region comprising a CD8/CD28 transmembrane domain. In some

embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68 are linked to an Fc region comprising a CD8/CD28 transmembrane domain and an Env incorporation motif. In some embodiments, the V_H peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprising a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68 linked to an Fc region comprising a CD8/CD28 transmembrane domain are anchored to the plasma membrane on the surface of a cell. In some embodiments, the cell is an immune cell, such as those provided herein.

~~[0249]~~[0247] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68.

~~[0250]~~[0248] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_H peptide and a V_L peptide comprises a light chain CDR having a sequence of SEQ ID NO: 58-60; and/or a heavy chain CDR having a sequence of SEQ ID NO: 55-57. In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_H peptide and a V_L peptide comprise a light chain CDR1 having a sequence of SEQ ID NO: 58; a

light chain CDR2 having a sequence of SEQ ID NO: 59; a light chain CDR3 having a sequence of SEQ ID NO: 60; and/or a heavy chain CDR1 having a sequence of SEQ ID NO: 55; a heavy chain CDR2 having a sequence of SEQ ID NO: 56; and a heavy chain CDR3 having a sequence of SEQ ID NO: 57. In some embodiments, the CDRs in the V_H or V_L chain are as set forth in the combinations provided for herein.

~~[0251]~~[0249] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 58; a LCDR2 having a sequence of SEQ ID NO: 59; and a LCDR3 having a sequence of SEQ ID NO: 60; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 55; a HCDR2 having a sequence of SEQ ID NO: 56; and a HCDR3 having a sequence of SEQ ID NO: 57.

~~[0252]~~[0250] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 58, wherein the LCDR1 comprises at most 1 conservative amino acid substitution, a LCDR2 having a sequence of SEQ ID NO: 59, wherein the LCDR2 comprises at most 1 conservative amino acid substitution, and a LCDR3 having a sequence of SEQ ID NO: 60, wherein the LCDR3 comprises at most 1 conservative amino acid substitution; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 55, wherein the HCDR1 comprises at most 1 conservative amino acid substitution, a HCDR2 having a sequence of SEQ ID NO: 56, wherein the HCDR2 comprises at most 1 conservative amino acid substitution, and a HCDR3 having a sequence of SEQ ID NO: 57, wherein the HCDR3 comprises at most 1 conservative amino acid substitution.

~~[0253]~~[0251] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_H peptide and a V_L peptide comprise a light chain CDR1 having a sequence of SEQ ID NO: 58; a light chain CDR2 having a sequence of SEQ ID NO: 59; a light chain CDR3 having a sequence of SEQ ID NO: 60; and/or a heavy chain CDR1 having a sequence of SEQ ID NO: 61; a heavy chain CDR2 having a sequence of SEQ ID NO: 62; and a heavy chain CDR3 having a sequence of SEQ ID NO: 57. In some embodiments, the CDRs in the V_H or V_L chain are as set forth in the combinations provided for herein.

~~[0254]~~[0252] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 58; a LCDR2 having a sequence of SEQ ID NO: 59; and a LCDR3 having a sequence of SEQ ID NO: 60; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 61; a HCDR2 having a sequence of SEQ ID NO: 62; and a HCDR3 having a sequence of SEQ ID NO: 57.

~~[0255]~~[0253] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 58, wherein the LCDR1 comprises at most 1 conservative amino acid substitution, a LCDR2 having a sequence of SEQ ID NO: 59, wherein the LCDR2 comprises at most 1 conservative amino acid substitution, and a LCDR3 having a sequence of SEQ ID NO: 60, wherein the LCDR3 comprises at most 1 conservative amino acid substitution; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 61, wherein the HCDR1 comprises at most 1 conservative amino acid substitution, a HCDR2 having a sequence of SEQ ID NO: 62, wherein the HCDR2 comprises at most 1

conservative amino acid substitution, and a HCDR3 having a sequence of SEQ ID NO: 57, wherein the HCDR3 comprises at most 1 conservative amino acid substitution.

~~[0256]~~[0254] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_H peptide and a V_L peptide comprise a light chain CDR1 having a sequence of SEQ ID NO: 66; a light chain CDR2 having a sequence of LA; a light chain CDR3 having a sequence of SEQ ID NO: 60; and/or a heavy chain CDR1 having a sequence of SEQ ID NO: 63; a heavy chain CDR2 having a sequence of SEQ ID NO: 64; and a heavy chain CDR3 having a sequence of SEQ ID NO: 65. In some embodiments, the CDRs in the V_H or V_L chain are as set forth in the combinations provided for herein.

~~[0257]~~[0255] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 66, a LCDR2 having a sequence of LA; and a LCDR3 having a sequence of SEQ ID NO: 60; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 63; a HCDR2 having a sequence of SEQ ID NO: 64; and a HCDR3 having a sequence of SEQ ID NO: 65.

~~[0258]~~[0256] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 67; and the V_L peptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 68; provided that the V_L peptide comprises a LCDR1 having a sequence of SEQ ID NO: 66, wherein the LCDR1 comprises at most 1 conservative amino acid substitution, a LCDR2 having a sequence of LA, wherein the LCDR2 comprises at most 1 conservative amino acid substitution, and a

LCDR3 having a sequence of SEQ ID NO: 60, wherein the LCDR3 comprises at most 1 conservative amino acid substitution; and the V_H peptide comprises a HCDR1 having a sequence of SEQ ID NO: 63, wherein the HCDR1 comprises at most 1 conservative amino acid substitution, a HCDR2 having a sequence of SEQ ID NO: 64, wherein the HCDR2 comprises at most 1 conservative amino acid substitution, and a HCDR3 having a sequence of SEQ ID NO: 65, wherein the HCDR3 comprises at most 1 conservative amino acid substitution.

~~[0259]~~[0257] In some embodiments, a polypeptide comprises a V_H peptide and a V_L peptide, wherein the V_H peptide comprises a sequence of SEQ ID NO: 67 and the V_L peptide comprises a sequence of SEQ ID NO: 68.

~~[0260]~~[0258] In some embodiments, a polypeptide as provided herein binds to non-human primate CD8. In some embodiments, a polypeptide as provided herein binds to human CD8.

~~[0261]~~[0259] As provided for herein, the different polypeptides (V_H or V_L) described herein can be linked with a peptide linker or not linked with a peptide linker and instead for a continuous sequence. In some embodiments, the peptide linker comprises a sequence of $(GGGGS)_n$ (SEQ ID NO: 49), wherein each n is independently 1-5. In some embodiments, n is 1. In some embodiments, n is 2. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. The linked peptide format can be represented by a formula of V_H-Z-V_L or V_L-Z-V_H, wherein Z is the peptide linker. In some embodiments, Z is $(GGGGS)_n$ (SEQ ID NO: 49), wherein each n is independently 1-5.

~~[0262]~~[0260] In some embodiments, a polypeptide comprising the linked peptide represented by a formula of V_L-Z-V_H comprises a heavy chain variable region as set forth in SEQ ID NO: 67 linked via a linker sequence GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 50) to a light chain variable region as set forth in SEQ ID NO: 68. In some embodiments, a polypeptide comprising a V_L linked via a peptide linker to a V_H has the sequence as set forth below,

NIVLTQSPASLAVSLGQRATISCRASESVDGFGNSFMNWYQQKPGQSPKLLIYLA
SNLESGVPARFSGSGSRTDFTLTIDPVEADDAATYYCQQNNEDPYTFGGGTKLEI
KRGGGGSGGGGSGGGGSGGGGSEVQLQQSGPELVKPGASVKISCKASRYTFTDY

NLHWVKLSHEKSLEWIGFIYPYNGGTGYNQKFKNKAKLTVDYSSSTAYMELRSL
TSVDAAVYYCARDHRYNEGVSFDYWGQGTTLTVSS (SEQ ID NO: 69).

~~[0263]~~[0261] In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 69. In some embodiments, a polypeptide comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 69. In some embodiments, a polypeptide comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 69. In some embodiments, a polypeptide comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 69. In some embodiments, a polypeptide comprises a sequence as set forth in SEQ ID NO: 69. In some embodiments, the polypeptide as set forth in SEQ ID NO: 69 is an antibody, or an antigen binding fragment thereof. In some embodiments, the antibody is an anti-CD8 antibody.

~~[0264]~~[0262] In some embodiments, a polypeptide comprising the linked peptide represented by a formula of V_H-Z-V_L comprises a light chain variable region as set forth in SEQ ID NO: 68 linked via a linker sequence GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 50) to a heavy chain variable region as set forth in SEQ ID NO: 67. In some embodiments, a polypeptide comprising a V_H linked via a peptide linker to a V_L has the sequence as set forth below,

EVQLQQSGPELVKPGASVKISCKASRYTFTDYNLHWVKLSHEKSLEWIGFIYPYN
GGTGYNQKFKNKAKLTVDYSSSTAYMELRSLTSVDAAVYYCARDHRYNEGVSF
DYWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSNIVLTQSPASLAVSLGQRATI
SCRASESVDGFGNSFMNWYQQKPGQSPKLLIYLASNLESGVPARFSGSGSRTDFT
LTIDPVEADDAATYYCQQNNEDPYTFGGGTKLEIKR (SEQ ID NO: 70).

~~[0265]~~[0263] In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 70. In some embodiments, a polypeptide comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 70. In some embodiments, a polypeptide comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 70. In some

embodiments, a polypeptide comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 70. In some embodiments, a polypeptide comprises a sequence as set forth in SEQ ID NO: 70. In some embodiments, the polypeptide as set forth in SEQ ID NO: 70 is an antibody, or an antigen binding fragment thereof. In some embodiments, the antibody is an anti-CD8 antibody. In some embodiments, the anti-CD8 antibody binds to non-human primate CD8. In some embodiments, the anti-CD8 antibody binds to human CD8.

~~[0266]~~[0264] In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 69 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 69 and comprises an Fc region, such as those provided herein, and a transmembrane domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 69 and comprises an Fc region, such as those provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 69 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0267]~~[0265] In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 70 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 70 and comprises an Fc region, such as those provided herein, and a transmembrane domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 70 and comprises an Fc region, such as those provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises

a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 70 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0268]~~[0266] As provided for herein, the polypeptide, antibodies, or antigen binding fragments thereof can be variants of the sequences.

~~[0269]~~[0267] The sequences of the polypeptides or antibodies can be modified to yield human IgG antibodies. The conversion of the sequences provided herein can be modified to yield other types of antibodies. The CDRs can also be linked to other antibodies, proteins, or molecules to create antibody fragments that bind CD8.

~~[0270]~~[0268] In some embodiments, a polypeptide or an antibody as provided herein is a targeting moiety on the surface of an engineered viral particle. In some embodiments, the targeting moiety allows for binding to a target cell. In some embodiments, the targeting moiety is a CD8 binding moiety, such as a polypeptide or an antibody as provided herein. In some embodiments, the targeting moiety comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 69. In some embodiments, the targeting moiety comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 69. In some embodiments, the targeting moiety comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 69. In some embodiments, the targeting moiety comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 69. In some embodiments, the targeting moiety comprises a sequence as set forth in SEQ ID NO: 69. In some embodiments, the targeting moiety as set forth in SEQ ID NO: 69 is an antibody, or an antigen binding fragment thereof. In some embodiments, the targeting moiety is an anti-CD8 antibody.

~~[0271]~~[0269] In some embodiments, a polypeptide or an antibody as provided for herein is a targeting moiety on the surface of an engineered viral particle. In some embodiments, the engineered viral particle is a pseudotyped viral-like particle. In some embodiments, the targeting moiety allows for binding to a target cell. In some embodiments, the targeting moiety is a CD8 binding moiety, such as a polypeptide or an antibody as provided herein. In some embodiments, the targeting moiety comprises a sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence of SEQ ID NO: 70. In some embodiments, the targeting moiety comprises a sequence that is at least 90% identical to a sequence of SEQ ID NO: 70. In some embodiments, the targeting moiety comprises a sequence that is at least 95% identical to a sequence of SEQ ID NO: 70. In some embodiments, the targeting moiety comprises a sequence that is at least 99% identical to a sequence of SEQ ID NO: 70. In some embodiments, the targeting moiety comprises a sequence as set forth in SEQ ID NO: 70. In some embodiments, the targeting moiety as set forth in SEQ ID NO: 70 is an antibody, or an antigen binding fragment thereof. In some embodiments, the targeting moiety is an anti-CD8 antibody. In some embodiments, the anti-CD8 antibody binds to non-human primate CD8. In some embodiments, the anti-CD8 antibody binds to human CD8.

~~[0272]~~[0270] In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 69 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 69 and comprises an Fc region, such as those provided herein, and a transmembrane domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 69 and comprises an Fc region, such as those provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 69 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0273]~~[0271] In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 70 and comprises an Fc region, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 70 and comprises an Fc region, such as those provided herein, and a transmembrane domain, such as those provided herein. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 70 and comprises an Fc region, such as those provided herein, and a CD8/CD28 transmembrane domain. In some embodiments, a polypeptide comprises a sequence having a sequence as set forth in SEQ ID NO: 70 and comprises an Fc region, such as those provided herein, a transmembrane domain, such as those provided herein, and an Env incorporation motif.

~~[0274]~~[0272] Although, the binders that bind to CD7 and CD8 are illustrated, in part, with a mutant VSV-G protein, other fusogenic proteins can also be used in place of the VSV-G protein. For example, in some embodiments, the pseudotyped viral-like particles can pseudotyped using viral glycoproteins other than VSV-G.

~~[0275]~~[0273] In some embodiments, viral particle can be pseudotyped using a viral glycoprotein from, for example, VSV. In some embodiments, viral particle can be pseudotyped using a viral glycoprotein from, for example, the Paramyxoviridae family. In some embodiments, the pseudotyped viral-like particles are pseudotyped using viral glycoproteins of morbillivirus, such as Measles virus. In some embodiments, the pseudotyped viral-like particles are pseudotyped using viral glycoproteins of the Measles virus. In some embodiments, the pseudotyped viral-like particles are pseudotyped using viral glycoproteins of henipavirus, such as Nipah virus, Cedar virus, or Hendra virus. In some embodiments, the pseudotyped viral-like particles are pseudotyped using viral glycoproteins of the Nipah virus. In some embodiments, a polypeptide or an antibody as provided herein is linked via a linker to an envelope glycoprotein G or H of a virus of the Paramyxoviridae family. In some embodiments, the virus of the Paramyxoviridae family is a morbillivirus, such as Measles virus. In some embodiments, the virus of the Paramyxoviridae family is a henipavirus, such as Nipah virus, Cedar virus, or Hendra virus.

~~[0276]~~[0274] In some embodiments, the pseudotyped viral-like particles comprising the targeting moieties provided for herein are pseudotyped with viral glycoproteins of viruses of the Rhabdoviridae family. In some embodiments, the pseudotyped viral-like particles are pseudotyped using viral glycoproteins of a vesicular stomatitis New Jersey virus, a vesicular stomatitis Indiana virus, a vesicular stomatitis Alagoas virus, a vesicular stromatitis Maraba virus, or a vesicular stomatitis Carajas virus. In some embodiments, a polypeptide or an antibody as provided herein is linked via a linker to a glycoprotein of a virus of the Rhabdoviridae family. In some embodiments, the virus of the Rhabdoviridae family is the vesicular stomatitis New Jersey virus, the vesicular stomatitis Indiana virus, the vesicular stomatitis Alagoas virus, the vesicular stromatitis Maraba virus, or the vesicular stomatitis Carajas virus.

~~[0277]~~[0275] In some embodiments, the pseudotyped viral-like particles comprising the targeting moieties provided for herein are pseudotyped with viral glycoproteins of the Parainfluezna virus, Spodoptera frugiperda virus, Drosophila obscura sigmavirus, Wuhan insect

virus 7, Spring viremia of carp virus, or Perch virus. In some embodiments, a polypeptide or an antibody as provided herein is linked via a linker to a glycoprotein of a virus of the Rhabdoviridae family. In some embodiments, the virus of the Rhabdoviridae family is a Parainfluezna virus. In some embodiments, the virus of the Rhabdoviridae family is a Spodoptera frugiperda virus. In some embodiments, the virus of the Rhabdoviridae family is a Drosophila obscura sigmavirus. In some embodiments, the virus of the Rhabdoviridae family is a Wuhan insect virus 7. In some embodiments, the virus of the Rhabdoviridae family is a Spring viremia of carp virus. In some embodiments, the virus of the Rhabdoviridae family is a Perch virus. In some embodiments, a polypeptide or an antibody as provided herein is linked via a linker to a Parainfluezna virus glycoprotein. In some embodiments, a polypeptide or an antibody as provided herein is linked via a linker to a Spodoptera frugiperda rhabdovirus isolate Sf G virus glycoprotein.

~~[0278]~~[0276] In some embodiments, the pseudotyped viral-like particles comprising the targeting moieties provided for herein are pseudotyped with viral glycoproteins of viruses of the Arenaviridae family. In some embodiments, the pseudotyped viral-like particles are pseudotyped using viral glycoproteins of the Machupo virus. In some embodiments, a polypeptide or an antibody as provided herein is linked via a linker to a glycoprotein of a virus of the Arenaviridae family. In some embodiments, the virus of the Arenaviridae family is a Machupo virus.

~~[0279]~~[0277] In some embodiments, the viral particle comprising a mutant VSV-G protein (or other viral glycorptein) and/or or targeting moiety as provided for herein comprises a nucleic acid molecule encoding **for a heterologous molecule of interest or "cargo." For example,** heterologous molecule of interest is meant to refer to any product that may be encoded by a nucleic acid molecule. As non-**limiting examples, "cargo" or "heterologous molecule of interest" may** refer to an siRNA, an shRNA, a peptide, a polypeptide, a protein, a viral payload, a viral genome, or a combination thereof. In some embodiments, the polypeptide is a chimeric antigen receptor **("CAR").** In some embodiments, the heterologous molecule of interest is an siRNA, an shRNA, a non-coding RNA (e.g. a guide RNA for a CRISPR system), a peptide, a polypeptide, a protein, **a viral payload, a viral genome, a chimeric antigen receptor ("CAR"), or a combination thereof.** In some embodiments, the heterologous molecule of interest is a CAR.

~~[0280]~~[0278] **A "chimeric antigen receptor" or "CAR" as used herein refers to an antigen-**binding domain that is fused, directly, or indirectly (e.g. via a hinge or transmembrane domain to

an intracellular signaling domain capable of activating or stimulating an immune cell. Most commonly, the CAR's extracellular binding domain is composed of a single chain variable fragment (scFv) derived from fusing the variable heavy and light regions of a murine or humanized monoclonal antibody. Alternatively, scFvs may be used that are derived from Fab's (instead of from an antibody, e.g., obtained from Fab libraries). In various embodiments, this scFv is fused to a transmembrane domain and then to an intracellular signaling domain. However, the antigen binding domain can be any molecule that can bind to the to target on the cell. For example, the antigen binding domain of a CAR can be an antibody, a scFv antibody, an antigen binding domain, an ankyrin repeat (e.g. DARPIN), a VHH domain antibody, a nanobody, single domain antibody, a FN3 domain, or any combination thereof. In some embodiments, a CAR includes those that solely provide CD3ζ signals upon antigen binding. In some embodiments, the CAR includes those that provide both costimulation (e.g. CD28 or CD137) and activation (CD3 ζ). In some embodiments, the CARs include those that provide multiple costimulation (e.g. CD28 and CD137) and activation (CD3ζ). In various embodiments, the CAR is selected to have high affinity or avidity for the antigen. In some embodiments, the antigen-binding domain binds to CD20. In some embodiments, the antigen-binding domain comprises a CD20 antibody, or fragment thereof. In some embodiments, antibody fragments are as provided for herein, such as but not limited to a scFv antibody, an antigen binding domain, an ankyrin repeat (e.g. DARPIN), a VHH domain antibody, a nanobody, single domain antibody, a FN3 domain, or any combination thereof.

~~[0281]~~[0279] In some embodiments, the antigen-binding domain of the CAR comprises a V_H domain, a V_L domain, or a V_H and a V_L domain. In some embodiments, the V_H domain comprises an amino acid sequence having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 73, or any value or range in-between.

EVQLVESGGGLVQPGRSLRLSCAASGFTFNDYAMHWVRQAPGKGLEWVSTISW
NSGSIGYADSVKGRFTISRDNAKKSLYLQMNSLRAEDTALYYCAKDIQYGNYYY
GMDVWGQGTTVTVSS (SEQ ID NO: 73)

In some embodiments, the V_H domain comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 73. In some embodiments, the V_H domain comprises an amino acid

sequence having at least 95% identity to SEQ ID NO: 73. In some embodiments, the V_H domain comprises an amino acid sequence having at least 98% identity to SEQ ID NO: 73. In some embodiments, the V_H domain comprises an amino acid sequence having at least 99% identity to SEQ ID NO: 73. In some embodiments, the V_H domain comprises an amino acid sequence having the sequence of SEQ ID NO: 73.

~~[0282]~~[0280] In some embodiments, the V_L domain comprises an amino acid sequence having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 74, or any value or range in-between.

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRA TGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPITFGQGTRLEIK (SEQ ID NO: 74)

In some embodiments, the V_L domain comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 74. In some embodiments, the V_L domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 74. In some embodiments, the V_L domain comprises an amino acid sequence having at least 98% identity to SEQ ID NO: 74. In some embodiments, the V_L domain comprises an amino acid sequence having at least 99% identity to SEQ ID NO: 74. In some embodiments, the V_L domain comprises an amino acid sequence having the sequence of SEQ ID NO: 74.

~~[0283]~~[0281] In some embodiments, the antigen-binding domain of the CAR comprises a V_H domain and a V_L domain. In some embodiments, the V_H and V_L domain are not linked by a linker peptide. In some embodiments, the V_H and V_L domain are linked by a linker peptide, such as those as provided for herein, including but not limited to: $(GGGGS)_n$ (SEQ ID NO: 49), wherein each n is independently 1-5. In some embodiment n is 1. In some embodiment n is 2. In some embodiment n is 3. In some embodiment n is 4. In some embodiment n is 5.

~~[0284]~~[0282] In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 73, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 74. In some

embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 73, and comprises a V_L having at least 90% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 73, and comprises a V_L having at least 95% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 73, and comprises a V_L having at least 98% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 73, and comprises a V_L having at least 99% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 73, and comprises a V_L having the sequence of SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 90% identity to SEQ ID NO: 73, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 95% identity to SEQ ID NO: 73, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 98% identity to SEQ ID NO: 73, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a

V_H domain having at least 99% identity to SEQ ID NO: 73, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having an amino acid sequence of SEQ ID NO: 73, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 90% identity to SEQ ID NO: 73, and comprises a V_L having at least 90% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 95% identity to SEQ ID NO: 73, and comprises a V_L having at least 90% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 90% identity to SEQ ID NO: 73, and comprises a V_L having at least 95% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 95% identity to SEQ ID NO: 73, and comprises a V_L having at least 95% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 98% identity to SEQ ID NO: 73, and comprises a V_L having at least 98% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 99% identity to SEQ ID NO: 73, and comprises a V_L having at least 99% identity to SEQ ID NO: 74. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having an amino acid sequence of SEQ ID NO: 73, and comprises a V_L having an amino acid sequence of SEQ ID NO: 74.

~~[0285]~~[0283] In some embodiments, the antigen-binding domain of the CAR comprises a formula of V_H-Z-V_L, wherein V_H is a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 73, Z is a linker comprising the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO: 77), and V_L is a light chain variable region comprising the amino acid sequence of SEQ ID NO: 74. In some embodiments, the antigen-binding domain

of the CAR comprising a formula of V_H-Z-V_L has an amino acid sequence as set forth below:

EVQLVESGGGLVQPGRSLRLSCAASGFTFNDYAMHWVRQAPGKGLEWVSTISW
NSGSIGYADSVKGRFTISRDNAKKSLYLQMNSLRAEDTALYYCAKDIQYGNYYY
GMDVWGQGTTVTVSSGGGGSGGGGSGGGGSEIVLTQSPATLSLSPGERATLSCR
ASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEP
EDFAVYYCQQRSNWPITFGQGTRLEIK (SEQ ID NO: 75)

~~[0286]~~[0284] In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a sequence of SEQ ID NO: 75. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 90% identity to a sequence of SEQ ID NO: 75. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 95% identity to a sequence of SEQ ID NO: 75. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 98% identity to a sequence of SEQ ID NO: 75. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 99% identity to a sequence of SEQ ID NO: 75. In some embodiments, the antigen-binding domain of the CAR comprises the amino acid sequence of SEQ ID NO: 75.

~~[0287]~~[0285] In some embodiments, the antigen-binding domain of the CAR comprises a formula of V_L-Z-V_H, wherein V_L is a light chain variable region comprising the amino acid sequence of SEQ ID NO: 74, Z is a linker comprising the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO: 77), and V_H is a light chain variable region comprising the amino acid sequence of SEQ ID NO: 73. In some embodiments, the antigen-binding domain of the CAR comprising a formula of V_L-Z-V_H has an amino acid sequence as set forth below:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRA
TGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPITFGQGTRLEIKGGGGS
GGGGSGGGGSEVQLVESGGGLVQPGRSLRLSCAASGFTFNDYAMHWVRQAPG
KGLEWVSTISWNSGSIGYADSVKGRFTISRDNAKKSLYLQMNSLRAEDTALYYC
AKDIQYGNYYYGMDVWGQGTTVTVSS (SEQ ID NO: 76)

~~[0288]~~[0286] In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a sequence of SEQ ID NO: 76. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 90% identity to a sequence of SEQ ID NO: 76. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 95% identity to a sequence of SEQ ID NO: 76. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 98% identity to a sequence of SEQ ID NO: 76. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 99% identity to a sequence of SEQ ID NO: 76. In some embodiments, the antigen-binding domain of the CAR comprises the amino acid sequence of SEQ ID NO: 76.

~~[0289]~~[0287] In some embodiments, the antigen-binding domain of the CAR comprises a V_H domain, a V_L domain, or a V_H and a V_L domain. In some embodiments, the V_H domain comprises an amino acid sequence having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 78, or any value or range in-between.

DIVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYATSNLAS
GVPARFSGSGSGTSYSLTISRVEAEDAATYYCQQWSFNPPTFGGGTKLEIKGSTS
(SEQ ID NO: 78)

In some embodiments, the V_H domain comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 78. In some embodiments, the V_H domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 78. In some embodiments, the V_H domain comprises an amino acid sequence having at least 98% identity to SEQ ID NO: 78. In some embodiments, the V_H domain comprises an amino acid sequence having at least 99% identity to SEQ ID NO: 78. In some embodiments, the V_H domain comprises an amino acid sequence having the sequence of SEQ ID NO: 78.

~~[0290]~~[0288] In some embodiments, the V_L domain comprises an amino acid sequence having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 79, or any value or range in-between.

EVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGAIYP GNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSNYYGSSY WFFDVWGAGTTVTVSS (SEQ ID NO: 79)

In some embodiments, the V_L domain comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 79. In some embodiments, the V_L domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 79. In some embodiments, the V_L domain comprises an amino acid sequence having at least 98% identity to SEQ ID NO: 79. In some embodiments, the V_L domain comprises an amino acid sequence having at least 99% identity to SEQ ID NO: 79. In some embodiments, the V_L domain comprises an amino acid sequence having the sequence of SEQ ID NO: 79.

~~[0291]~~[0289] In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 78, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 78, and comprises a V_L having at least 90% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 78, and comprises a V_L having at least 95% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 78, and comprises a V_L having at least 98% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 78, and comprises a V_L having at least

99% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 78, and comprises a V_L having the sequence of SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 90% identity to SEQ ID NO: 78, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 95% identity to SEQ ID NO: 78, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 98% identity to SEQ ID NO: 78, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 99% identity to SEQ ID NO: 78, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having an amino acid sequence of SEQ ID NO: 78, and comprises a V_L having at least 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 90% identity to SEQ ID NO: 78, and comprises a V_L having at least 90% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 95% identity to SEQ ID NO: 78, and comprises a V_L having at least 90% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 90% identity to SEQ ID NO: 78, and comprises a V_L having at least 95% identity to SEQ ID NO: 79. In some embodiments,

the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 95% identity to SEQ ID NO: 78, and comprises a V_L having at least 95% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 98% identity to SEQ ID NO: 78, and comprises a V_L having at least 98% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having at least 99% identity to SEQ ID NO: 78, and comprises a V_L having at least 99% identity to SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a V_H domain and a V_L domain comprises a V_H domain having an amino acid sequence of SEQ ID NO: 78, and comprises a V_L having an amino acid sequence of SEQ ID NO: 79.

~~[0292]~~[0290] In some embodiments, the antigen-binding domain of the CAR comprises a formula of V_H-Z-V_L, wherein V_H is a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 78, Z is a linker comprising the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO: 77), and V_L is a light chain variable region comprising the amino acid sequence of SEQ ID NO: 79. In some embodiments, the antigen-binding domain of the CAR comprising a formula of V_H-Z-V_L has an amino acid sequence as set forth below:

DIVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYATSNLAS
GVPARFSGSGSGTSYSLTISRVEAEDAATYYCQQWSFNPPTFGGGTKLEIKGSTS
GGGGSGGGGSGGGGSSEVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHW
VKQTPGQGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSED
SADYYCARSNYYGSSYWFFDVWGAGTTVTVSS (SEQ ID NO: 80)

~~[0293]~~[0291] In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a sequence of SEQ ID NO: 80. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 90% identity to a sequence of SEQ ID NO: 80. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 95% identity to a sequence of SEQ ID NO: 80. In some embodiments, the antigen-binding domain of the CAR comprises an

amino acid sequence having at least 98% identity to a sequence of SEQ ID NO: 80. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 99% identity to a sequence of SEQ ID NO: 80. In some embodiments, the antigen-binding domain of the CAR comprises the amino acid sequence of SEQ ID NO: 80.

~~[0294]~~[0292] In some embodiments, the antigen-binding domain of the CAR comprises a formula of V_L-Z-V_H, wherein V_L is a light chain variable region comprising the amino acid sequence of SEQ ID NO: 79, Z is a linker comprising the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO: 77), and V_H is a light chain variable region comprising the amino acid sequence of SEQ ID NO: 78. In some embodiments, the antigen-binding domain of the CAR comprising a formula of V_L-Z-V_H has an amino acid sequence as set forth below:

SEVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGAIY
PGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSNYYGSSY
WFFDVWGAGTTVTVSSGGGGSGGGGSGGGGSDIVLTQSPAILSASPGEKVTMTC
RASSSVNYMDWYQKKPGSSPKPWIYATSNLASGVPARFSGSGSGTSYSLTISRVE
AEDAATYYCQQWSFNPPTFGGGTKLEIKGSTS (SEQ ID NO: 81)

~~[0295]~~[0293] In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a sequence of SEQ ID NO: 81. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 90% identity to a sequence of SEQ ID NO: 81. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 95% identity to a sequence of SEQ ID NO: 81. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 98% identity to a sequence of SEQ ID NO: 81. In some embodiments, the antigen-binding domain of the CAR comprises an amino acid sequence having at least 99% identity to a sequence of SEQ ID NO: 81. In some embodiments, the antigen-binding domain of the CAR comprises the amino acid sequence of SEQ ID NO: 81.

~~[0296]~~[0294] In some embodiments, the antigen-binding domain of the CAR comprises rituximab, ocrelizumab, obinutuzumab, ofatumumab, ibritumomab tiuxetan, tositumomab, or ublituximab. In some embodiment, the antigen-binding domain comprises rituximab. In some

embodiment, the antigen-binding domain comprises ofatumumab. In some embodiments, the CAR comprises the 4-1BB domain as well. These are merely illustrative in nature and are not limiting to the present embodiments and any chimeric antigen receptor can be delivered in conjunction with the viral particles and vectors provided for herein. These are non-limiting examples of CARs and any CAR construct could be encoded for by the nucleic acid molecule.

~~[0297]~~[0295] In some embodiments, the pseudotyped viral particle further comprises a heterologous nucleic acid molecule encoding a cargo of interest. The nucleic acid molecule may be useful for modulating the expression of a target gene. In some embodiments, the cargo can be used to modulate the activity of a cell or express a protein that is trafficked to the surface of the target cell. Therefore, in some embodiments, the nucleic acid may comprise an siRNA or an shRNA. The nucleic acid may also encode for a cargo of interest. Therefore, in some embodiments, the cargo of interest may comprise a polypeptide or portion thereof, a protein or portion thereof, a chimeric antigen receptor or portion thereof, or a tumor antigen or a portion thereof. In some embodiments, the cargo of interest is an antibody that is produced by the virus, which can then be secreted by the cell that is infected with the virus. The term "protein" can refer to any polypeptide that carries a native function in a cellular environment. Therefore, in some embodiments, the protein encoded by the nucleic acid cargo of interest may comprise an enzyme, a nuclear receptor, a transporter, a ribosomal protein, a membrane bound protein, a cytoplasmic protein, a G-protein coupled receptor, a voltage gated ion channel, a secretory protein, a mitochondria protein, a cytokine, a chimeric antigen receptor, a tumor antigen, or a portion or chimeric species thereof.

~~[0298]~~[0296] Without being bound to any particular theory, the viral particle comprising the mutant VSV-G protein or other viral glycoprotein as provided for herein that comprises a targeting moiety can be used to express the heterologous molecule of interest in the target cell. Thus, for example, the CAR can be expressed in a T cell that is targeted by a viral particle pseudotyped with a VSV-G protein as provided for herein. Where the T cell is the intended target the viral particle can comprise a targeting moiety that binds to a target on the surface of a T cell, such as, but not limited to CD2, CD3, CD4, CD5, CD7 or CD8. In some embodiments, the target is CD2. In some embodiments, the target is CD3. In some embodiments, the target is CD4. In some embodiments, the target is CD5. In some embodiments, the target is CD6. In some

embodiments, the target is CD7. In some embodiments, the target is CD8.

~~[0299]~~[0297] Also provided herein are viral particles comprising a targeting moiety that binds to CD7 or CD8, or a pharmaceutical composition comprising the same. In some the viral particle comprises a targeting moiety that binds to CD7. . In some the viral particle comprises a targeting moiety that binds to CD7.

~~[0300]~~[0298] In some embodiments, the viral particle that comprises a targeting moiety that binds to CD7 or CD8 is a pseudotyped viral particle. The viral particle can be pseudotyped with a viral glycoprotein, such as those, but not limited to, described herein. In some embodiments, the viral particle is pseudotyped with a VSV-G protein or mutant thereof, including, but not limited to, those provided for herein. The targeting moiety can be any antibody that or binder that binds to CD7 or CD8. Non-limiting examples are provided for herein.

~~[0301]~~[0299] In addition to being pseudotyped, the viral particles comprising a targeting moiety that binds to CD7 or CD8 can comprise a heterologous nucleic acid molecule encoding a molecule of interest. This can be, for example, a siRNA, an shRNA, a non-coding RNA (e.g. a guide RNA for a CRISPR system), a peptide, a polypeptide, a protein, a viral payload, a viral genome, or a combination thereof. In some embodiments, the heterologous molecule of interest is a chimeric antigen receptor ("CAR").

~~[0302]~~[0300] In some embodiments, the pseudotyped viral particle is a recombinant lentivirus. In some embodiments, the recombinant pseudotyped viral particle is replication competent. In some embodiments, the recombinant pseudotyped viral particle is replication incompetent.

~~[0303]~~[0301] In some embodiments, a pharmaceutical composition is provided comprising any composition, particle, or component provided for herein, such as an envelope pseudotyped viral particles or vectors as provided for herein, i.e. a particle that comprises a VSV-G mutant protein provided for herein or other viral glycoprotein, or particles that comprise a targeting moiety and/or a heterologous nucleic acid molecule as provided for herein.

~~[0304]~~[0302] In some embodiments, methods of delivering a cargo of interest to a cell are provided. In some embodiments, the methods comprise contacting the cell with the pseudotyped viral-like particles or viral vectors as provided for herein, or a pharmaceutical composition comprising the same.

~~[0305]~~[0303] In some embodiments, methods of delivering a cargo of interest to a cell in a subject are provided. In some embodiments, the methods comprise administering to the subject the pseudotyped viral-like particles or viral vectors as provided for herein, or a pharmaceutical composition comprising the same. In some embodiments, the cargo is a chimeric antigen receptor or as otherwise provided for herein.

~~[0306]~~[0304] In some embodiments, methods for of delivering a chimeric antigen receptor to a T-cell in a subject are provided. In some embodiments, the methods comprising administering to the subject the pseudotyped viral-like particles or viral vectors as provided for herein, or a pharmaceutical composition comprising the same, wherein the pseudotyped viral-like particle or viral vector comprises a heterologous nucleic acid molecule encoding the chimeric antigen receptor.

~~[0307]~~[0305] Also provided herein are nucleic acid molecules encoding a mutant VSV-G protein as provided for herein.

~~[0308]~~[0306] Also provided herein are nucleic acid molecules encoding a targeting moiety, such as those provided for herein.

~~[0309]~~[0307] Methods of making the viral like particles or vectors are also provided. In some embodiments, the methods comprise making a viral like particles or vectors comprising a mutant VSV-G protein or other viral glycoprotein. In some embodiments, the methods comprise transfecting or transducing a packaging cell line with the nucleic acid molecules encoding a mutant VSV-G protein or another viral glycoprotein as provided for herein under conditions sufficient to produce the pseudotyped viral-like particles or viral vectors. In some embodiments, the methods comprise transfecting or transducing a packaging cell line with the plurality of nucleic acid molecules provided for herein under conditions sufficient to produce the pseudotyped viral-like particles or viral vectors and optionally with a targeting moiety, such as one that binds to a target antigen, such as those provided for herein. In some embodiments, the targeting moiety binds to CD7 or CD8. In some embodiments, the targeting moiety comprises a sequence that binds to CD7 or CD8, such as those provided for herein. In some embodiments, methods further comprise isolating the pseudotyped viral-like particle or viral vector. In some embodiments, the nucleic acid molecules also comprise a nucleic acid molecule encoding a targeting moiety and/or a cargo that is to be delivered by the viral vector that is produced.

~~[0310]~~[0308] Accordingly, in some embodiments, cells are provided comprising heterologous nucleic acid molecules that encode the components to produce the virus. Packaging cell lines are known in the art and can be modified with the molecules of interest to produce the viral particle of interest.

~~[0311]~~[0309] For example, a cell or population of cells is provided that comprises one or more heterologous nucleic acid molecules encoding for a targeting moiety and/or a viral glycoprotein. In some embodiments, the cell comprises heterologous nucleic acid molecules encoding for a targeting moiety and a viral glycoprotein. In some embodiments, the heterologous nucleic acid molecules comprise a nucleic acid sequence encoding for a targeting moiety that binds to CD7 or CD8. In some embodiments, the targeting moiety that is encoded for comprises an amino acid sequence as provided for herein. In some embodiments, the viral glycoprotein encoded for is any viral glycoprotein that can function as a fusogenic protein to facilitate entry of the viral particle into a cell that expresses CD7 or CD8. In some embodiments, the viral glycoprotein is wild-type or a mutant VSV-G protein, such as those provided for herein. Other viral glycoproteins that can be encoded for are also described herein and can be used.

~~[0312]~~[0310] The viral particles provided for herein can be produced or made by, for example, culturing the cell under conditions sufficient to make the viral particle. In some embodiments, the cell is what is called a packaging cell line that provides components of the virus to produce the viral particle. These can be structural or non-structural viral components or proteins.

~~[0313]~~[0311] Also provided for herein are methods of treating cancer in a subject. In some embodiments, the methods comprise administering to the subject the pseudotyped viral-like particles or viral vectors as provided for herein, or a pharmaceutical composition comprising the same, wherein the pseudotyped viral-like particle or viral vector comprises a heterologous nucleic acid molecule encoding the chimeric antigen receptor.

~~[0314]~~[0312] Also provided herein are methods of treating a disease in a subject in need thereof.

~~[0315]~~[0313] In some embodiments, the methods provided include, but are not limited to, methods of treating a disease in a subject in need thereof, comprising administering to the subject the viral particle(s) provided herein to treat the disease.

~~[0316]~~[0314] In certain embodiments, the disease is a cancer. In addition, the compositions provided for herein can be used in methods for the treatment of any condition related to a cancer, such as a cell-mediated immune response against a tumor cell(s), where it is desirable to treat or alleviate the disease. The types of cancers to be treated include, but are not limited to, carcinoma, blastoma, sarcoma, certain leukemia or lymphoid malignancies, benign and malignant tumors, malignancies e.g., sarcomas, carcinomas, and melanomas. Other exemplary cancers include, but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, thyroid cancer, and the like. In some embodiments, the cancer is the cancer is carcinoma, blastoma, sarcoma, leukemia, lymphoid malignancies, benign tumors, malignant tumors, sarcoma, carcinoma, melanoma, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, thyroid cancer, B cell related cancer or T cell related cancer. The cancers may be non-solid tumors (such as hematological tumors) or solid tumors. Adult tumors/cancers and pediatric tumors/cancers are also included. In one embodiment, the cancer is a hematological tumor. In one embodiment, the cancer is a carcinoma. In one embodiment, the cancer is a sarcoma. In one embodiment, the cancer is a leukemia. In one embodiment the cancer is a solid tumor.

~~[0317]~~[0315] Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, CNS tumors (such as a glioma (such

as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma, retinoblastoma and brain metastases).

~~[0318]~~[0316] Carcinomas that can be amenable to therapy by the methods disclosed herein include, but are not limited to, esophageal carcinoma, hepatocellular carcinoma, basal cell carcinoma (a form of skin cancer), squamous cell carcinoma (various tissues), bladder carcinoma, including transitional cell carcinoma (a malignant neoplasm of the bladder), bronchogenic carcinoma, colon carcinoma, colorectal carcinoma, gastric carcinoma, lung carcinoma, including small cell carcinoma and non-small cell carcinoma of the lung, adrenocortical carcinoma, thyroid carcinoma, pancreatic carcinoma, breast carcinoma, ovarian carcinoma, prostate carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, renal cell carcinoma, ductal carcinoma in situ or bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical carcinoma, uterine carcinoma, testicular carcinoma, osteogenic carcinoma, epithelial carcinoma, and nasopharyngeal carcinoma.

~~[0319]~~[0317] In certain exemplary embodiments, the compositions provided herein can be used in methods to treat a myeloma, or a condition related to myeloma. Examples of myeloma or conditions related thereto include, without limitation, light chain myeloma, non-secretory myeloma, monoclonal gamopathy of undertermined significance (MGUS), plasmacytoma (e.g., solitary, multiple solitary, extramedullary plasmacytoma), amyloidosis, and multiple myeloma. In some embodiments, methods of treating multiple myeloma are provided. In some embodiments, the multiple myeloma is refractory myeloma. In some embodiments, the multiple myeloma is relapsed myeloma.

~~[0320]~~[0318] In certain exemplary embodiments, the in vivo modified immune cells produced using the compositions provided herein are used to treat a melanoma, or a condition related to melanoma. Examples of melanoma or conditions related thereto include, without limitation, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, amelanotic melanoma, or melanoma of the skin (e.g., cutaneous, eye, vulva, vagina, rectum melanoma). In some embodiments, the melanoma is cutaneous melanoma.

In some embodiments, the melanoma is refractory melanoma. In some embodiments, the melanoma is relapsed melanoma.

~~[0321]~~[0319] In some embodiments, the compositions provided herein are used to treat a sarcoma, or a condition related to sarcoma. Examples of sarcoma or conditions related thereto include, without limitation, angiosarcoma, chondrosarcoma, chordoma, endotheliosarcoma, Ewing's sarcoma, fibrosarcoma, gastrointestinal stromal tumor, leiomyosarcoma, liposarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, mesothelioma, malignant peripheral nerve sheath tumor, myxosarcoma, osteogenic sarcoma, osteosarcoma, pleomorphic sarcoma, rhabdomyosarcoma, synovioma, synovial sarcoma, and other soft tissue sarcomas. In some embodiments, the sarcoma is synovial sarcoma. In some embodiments, the sarcoma is liposarcoma such as myxoid/round cell liposarcoma, differentiated/dedifferentiated liposarcoma, or pleomorphic liposarcoma. In some embodiments, the sarcoma is myxoid/round cell liposarcoma. In some embodiments, the sarcoma is refractory sarcoma. In some embodiments, the sarcoma is relapsed sarcoma.

~~[0322]~~[0320] In some embodiments, the subject has been treated with a therapeutic agent targeting the disease or condition, e.g. the tumor, prior to administration of the composition. In some aspects, the subject is refractory or non-responsive to the other therapeutic agent. In some embodiments, the subject has persistent or relapsed disease, e.g., following treatment with another therapeutic intervention, including chemotherapy, radiation, and/or hematopoietic stem cell transplantation (HSCT), e.g., allogenic HSCT. In some embodiments, the administration effectively treats the subject despite the subject having become resistant to another therapy.

~~[0323]~~[0321] In some embodiments, the subject is responsive to the other therapeutic agent, and treatment with the therapeutic agent reduces disease burden. In some aspects, the subject is initially responsive to the therapeutic agent, but exhibits a relapse of the disease or condition over time. In some embodiments, the subject has not relapsed. In some such embodiments, the subject is determined to be at risk for relapse, such as at a high risk of relapse, and thus the composition is administered prophylactically, e.g., to reduce the likelihood of or prevent relapse. In some aspects, the subject has not received prior treatment with another therapeutic agent.

~~[0324]~~[0322] The administration of the compositions may be carried out in any convenient manner known to those of skill in the art. For example, the compositions may be

administered to a subject by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient transarterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, intraperitoneally, intranasally, intracranially, or intraosseously. In other instances, the compositions are injected directly into a site of a local disease site in the subject, a lymph node, an organ, a tumor, and the like.

~~[0325]~~[0323] For the prevention or treatment of disease, the appropriate dosage may depend on the type of disease to be treated, the severity and course of the disease, whether the composition is administered for preventive or therapeutic purposes, previous therapy, the subject's clinical history and response to the treatment, and the discretion of the attending physician. The composition is, in some embodiments, suitably administered to the subject at one time or over a series of treatments.

~~[0326]~~[0324] In some embodiments, the composition is administered as part of a combination treatment, such as simultaneously with or sequentially with, in any order, another therapeutic intervention, such as an antibody or produced cell or receptor or agent, such as a cytotoxic or therapeutic agent. The composition(s), in some embodiments, is co-administered with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In some contexts, the composition is co-administered with another therapy sufficiently close in time such that the composition enhances the effect of one or more additional therapeutic agents, or vice versa. In some embodiments, the composition is administered prior to the one or more additional therapeutic agents. In some embodiments, the composition is administered after the one or more additional therapeutic agents. In some embodiments, the one or more additional agents includes a cytokine, such as IL-2, for example, to enhance persistence. In some embodiments, the methods comprise administration of a chemotherapeutic agent. In some embodiments, the methods do not comprise the administration of a chemotherapeutic agent.

~~[0327]~~[0325] In certain embodiments, the compositions may be administered to a subject in combination with an immune checkpoint antibody (e.g., an anti-PD1, anti-CTLA-4, or anti-PDL1 antibody). For example, viral vectors may be administered in combination with an antibody or antibody fragment targeting, for example, PD-1 (programmed death 1 protein). Examples of

anti-PD-1 antibodies include, but are not limited to, pembrolizumab (KEYTRUDA®, formerly lambrolizumab, also known as MK-3475), and nivolumab (BMS-936558, MDX-1106, ONO-4538, OPDIVO®) or an antigen-binding fragment thereof. In certain embodiments, the compositions may be administered in combination with an anti-PD-L1 antibody or antigen-binding fragment thereof. Examples of anti-PD-L1 antibodies include, but are not limited to, BMS-936559, MPDL3280A (TECENTRIQ®, Atezolizumab), and MEDI4736 (Durvalumab, Imfinzi). In certain embodiments, the composition may be administered in combination with an anti-CTLA-4 antibody or antigen-binding fragment thereof. An example of an anti- CTLA-4 antibody includes, but is not limited to, Ipilimumab (trade name Yervoy). Other types of immune checkpoint modulators may also be used including, but not limited to, small molecules, siRNA, miRNA, and CRISPR systems. Immune checkpoint modulators may be administered before, after, or concurrently with the viral vector. In certain embodiments, combination treatment comprising an immune checkpoint modulator may increase the therapeutic efficacy of a therapy comprising a composition as provided herein. The other therapeutic can be administered simultaneously, before, or after the compositions provided herein are administered to the subject.

~~[0328]~~[0326] In certain embodiments, the subject is provided a secondary treatment. Secondary treatments include but are not limited to chemotherapy, radiation, surgery, and medications. In some embodiments, the subject is not provided a secondary treatment.

~~[0329]~~[0327] In some embodiments, the methods are performed without a lymphodepletion step, such as the administration of cyclophosphamide and/or fludarabine.

~~[0330]~~[0328] In some embodiments, the subject can be administered a conditioning therapy after the administration of the compositions to kill certain immune cells that are not transduced with the CAR encoded by the compositions. This can be done by including a selection marker that is encoded by the nucleic acid cargo of interest. In some embodiments, the conditioning therapy comprises administering an effective amount of cyclophosphamide to the subject. In some embodiments, the conditioning therapy comprises administering an effective amount of fludarabine to the subject. In some embodiments, the conditioning therapy comprises administering an effective amount of a combination of cyclophosphamide and fludarabine to the subject.

~~[0331]~~[0329] In some embodiments, a specific dosage regimen of the present disclosure

includes a lymphodepletion step after the administration of the composition. In an exemplary embodiment, the lymphodepletion step includes administration of cyclophosphamide and/or fludarabine.

~~[0332]~~[0330] In some embodiments, the lymphodepletion step includes administration of cyclophosphamide at a dose of between about 200 mg/m2/day and about 2000 mg/m2/day (e.g., 200 mg/m2/day, 300 mg/m2/day, or 500 mg/m2/day). In an exemplary embodiment, the dose of cyclophosphamide is about 300 mg/m2/day. In some embodiments, the lymphodepletion step includes administration of fludarabine at a dose of between about 20 mg/m2/day and about 900 mg/m2/day (e.g., 20 mg/m2/day, 25 mg/m2/day, 30 mg/m2/day, or 60 mg/m2/day). In an exemplary embodiment, the dose of fludarabine is about 30 mg/m2/day.

~~[0333]~~[0331] In some embodiment, the lymphodepletion step includes administration of cyclophosphamide at a dose of between about 200 mg/m2/day and about 2000 mg/m2/day (e.g., 200 mg/m2/day, 300 mg/m2/day, or 500 mg/m2/day), and fludarabine at a dose of between about 20 mg/m2/day and about 900 mg/m2/day (e.g., 20 mg/m2/day, 25 mg/m2/day, 30 mg/m2/day, or 60 mg/m2/day). In an exemplary embodiment, the lymphodepletion step includes administration of cyclophosphamide at a dose of about 300 mg/m2/day, and fludarabine at a dose of about 30 mg/m2/day.

~~[0334]~~[0332] In an exemplary embodiment, the dosing of cyclophosphamide is 300 mg/m2/day over three days, and the dosing of fludarabine is 30 mg/m2/day over three days.

~~[0335]~~[0333] It is known in the art that one of the adverse effects of the use of CAR T cells can be the onset of immune activation, known as cytokine release syndrome (CRS). CRS is immune activation resulting in elevated inflammatory cytokines. CRS is a known on-target toxicity, development of which likely correlates with efficacy. Clinical and laboratory measures range from mild CRS (constitutional symptoms and/or grade-2 organ toxicity) to severe CRS (sCRS; grade ≥3 organ toxicity, aggressive clinical intervention, and/or potentially life threatening). Clinical features include: high fever, malaise, fatigue, myalgia, nausea, anorexia, tachycardia/hypotension, capillary leak, cardiac dysfunction, renal impairment, hepatic failure, and disseminated intravascular coagulation. Dramatic elevations of cytokines including interferon-gamma, granulocyte macrophage colony-stimulating factor, IL-10, and IL-6 have been shown following CAR T-cell infusion. One CRS signature is elevation of cytokines including IL-

6 (severe elevation), IFN-gamma, TNF-alpha (moderate), and IL-2 (mild). Elevations in clinically available markers of inflammation including ferritin and C-reactive protein (CRP) have also been observed to correlate with the CRS syndrome. The presence of CRS generally correlates with expansion and progressive immune activation of adoptively transferred cells. It has been demonstrated that the degree of CRS severity is dictated by disease burden at the time of infusion as patients with high tumor burden experience a more sCRS.

~~[0336]~~[0334] Accordingly, in some embodiments, the methods comprise, following the diagnosis of CRS, appropriate CRS management strategies to mitigate the physiological symptoms of uncontrolled inflammation without dampening the antitumor efficacy of the in vivo generated cells (e.g., CAR T cells). CRS management strategies are known in the art. For example, systemic corticosteroids may be administered to rapidly reverse symptoms of sCRS (e.g., grade 3 CRS) without compromising initial antitumor response.

~~[0337]~~[0335] In some embodiments, an anti-IL-6R antibody may be administered. An example of an anti-IL-6R antibody is the Food and Drug Administration-approved monoclonal antibody tocilizumab, also known as atlizumab (marketed as Actemra, or RoActemra). Tocilizumab is a humanized monoclonal antibody against the interleukin-6 receptor (IL-6R). Administration of tocilizumab has demonstrated near-immediate reversal of CRS.

~~[0338]~~[0336] CRS is generally managed based on the severity of the observed syndrome and interventions are tailored as such. CRS management decisions may be based upon clinical signs and symptoms and response to interventions, not solely on laboratory values alone.

~~[0339]~~[0337] Mild to moderate cases generally are treated with symptom management with fluid therapy, non-steroidal anti-inflammatory drug (NSAID) and antihistamines as needed for adequate symptom relief. More severe cases include patients with any degree of hemodynamic instability; with any hemodynamic instability, the administration of tocilizumab is recommended. The first-line management of CRS may be tocilizumab, in some embodiments, at the labeled dose of 8 mg/kg IV over 60 minutes (not to exceed 800 mg/dose); tocilizumab can be repeated Q8 hours. If suboptimal response to the first dose of tocilizumab, additional doses of tocilizumab may be considered. Tocilizumab can be administered alone or in combination with corticosteroid therapy. Patients with continued or progressive CRS symptoms, inadequate clinical improvement in 12-18 hours or poor response to tocilizumab, may be treated with high-dose corticosteroid therapy,

generally hydrocortisone 100 mg IV or methylprednisolone 1-2 mg/kg. In patients with more severe hemodynamic instability or more severe respiratory symptoms, patients may be administered high-dose corticosteroid therapy early in the course of the CRS. CRS management guidance may be based on published standards (Lee et al. (2019) Biol Blood Marrow Transplant, doi.org/10.1016/j.bbmt.2018.12.758; Neelapu et al. (2018) Nat Rev Clin Oncology, 15:47; Teachey et al. (2016) Cancer Discov, 6(6):664-679).

~~[0340]~~[0338] Features consistent with Macrophage Activation Syndrome (MAS) or Hemophagocytic lymphohistiocytosis (HLH) have been observed in patients treated with CAR-T therapy (Henter, 2007), coincident with clinical manifestations of the CRS. MAS appears to be a reaction to immune activation that occurs from the CRS, and should therefore be considered a manifestation of CRS. MAS is similar to HLH (also a reaction to immune stimulation). The clinical syndrome of MAS is characterized by high grade non-remitting fever, cytopenias affecting at least two of three lineages, and hepatosplenomegaly. It is associated with high serum ferritin, soluble interleukin-2 receptor, and triglycerides, and a decrease of circulating natural killer (NK) activity.

~~[0341]~~[0339] In some embodiments, methods of treating cancer in a subject in need thereof are provided, the methods comprising administering to the subject any of the compositions, such as the viral particle(s), provided herein.

~~[0342]~~[0340] The compositions disclosed herein can comprise a pharmaceutical composition, and for example include a pharmaceutically acceptable carrier, and/or a pharmaceutical formulation.

~~[0343]~~[0341] The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative. In some aspects, the choice of carrier is determined in part by the particular cell and/or by the method of administration. Accordingly, there are a variety of suitable formulations. For example, the pharmaceutical composition can contain preservatives. Suitable preservatives may include, for example, methylparaben, propylparaben, sodium benzoate,

and benzalkonium chloride. In some aspects, a mixture of two or more preservatives is used. The preservative or mixtures thereof are typically present in an amount of about 0.0001% to about 2% by weight of the total composition. Carriers are described, e.g., by Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980). Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG).

~~[0344]~~[0342] Buffering agents in some aspects are included in the compositions. Suitable buffering agents include, for example, citric acid, sodium citrate, phosphoric acid, potassium phosphate, and various other acids and salts. In some aspects, a mixture of two or more buffering agents is used. The buffering agent or mixtures thereof are typically present in an amount of about 0.001% to about 4% by weight of the total composition. Methods for preparing administrable pharmaceutical compositions are known. Exemplary methods are described in more detail in, for example, Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins; 21st ed. (May 1, 2005).

~~[0345]~~[0343] The formulations can include aqueous solutions. The formulation or composition may also contain more than one active ingredient useful for the particular indication, disease, or condition being treated with the composition, preferably those with activities complementary to the composition, where the respective activities do not adversely affect one another. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended. Thus, in some embodiments, the pharmaceutical composition further