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Molecular Immunology 39 (2003) 941–952

Molecular
Immunologywww.elsevier.com/locate/molimm

Humanization of the anti-CD18 antibody 6.7: an unexpected effect of a framework residue in binding to antigen

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Received 7 November 2002; accepted 6 February 2003

Abstract

Humanization of monoclonal antibodies by complementary determinant region (CDR)-grafting has become a standard procedure to improve the clinical usage of animal antibodies. However, antibody humanization may result in loss of activity that has been attributed to structural constraints in the framework structure. In this paper, we report the complete humanization of the 6.7 anti-human CD18 monoclonal antibody in a scFv form. We used a germline-based approach to design a humanized VL gene fragment and expressed it together with a previously described humanized VH. The designed humanized VL has only 14 mutations compared to the closest human germline sequence. The resulting humanized scFv maintained the binding capacity and specificity to human CD18 expressed on the cell surface of peripheral blood mononuclear cells (PBMC), and showed the same pattern of staining T-lymphocytes sub-populations, in comparison to the original monoclonal antibody. We observed an unexpected effect of a conserved mouse–human framework position (L37) that hinders the binding of the humanized scFv to antigen. This paper reveals a new framework residue that interferes with paratope and antigen binding and also reinforces the germline approach as a successful strategy to humanize antibodies.

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Keywords: Humanization; Framework; CDR-grafting; CD18

1. Introduction

Antibody engineering has become a popular technology for development of a new generation of drugs (Presta, 2002; Maynard and Georgiou, 2000). The amount knowledge available with regards to antibody structure at atomic resolution, allied to its conserved conformation, permits the elaboration of general rules for antigen binding site structural and functional features. To date, the commonest antibody manipulation is the process of humanization. In this process, a murine antibody sequence is replaced by a homologous human sequence yielding a human-like antibody, reducing its immunogenicity, and preserving the binding to the original antigen (Jones et al., 1986; Riechmann et al., 1988; Verhoeyen et al., 1988). This methodology has revolutionized the clinical use of monoclonal antibodies (mAb),

since the patients' response to heterologous rodent sequences prevents the full biological effect of the monoclonal antibody (Winter and Harris, 1993). Moreover, heterologous mAbs present a reduced half-life and a diminished capacity to induce effector functions (Isaacs et al., 1992). The limit of this process is in the preservation of the antibody's affinity and specificity. After a decade of research in this process, many rules have been established that help maintain the overall antibody structure while the mouse primary sequence is being saturated with human residues. The use of human germline sequences (Rosok et al., 1996; Caldas et al., 2000) and the maintenance of key framework residues are examples of these rules (Tempest et al., 1991). Many successfully humanized antibodies have been reported, including in this group those that are already being routinely used in therapeutics (Glennie and Johnson, 2000).

Antibody humanization is becoming a trivial methodology, where many different experimental procedures result in an active antibody. In spite of this, many of the designed human antibodies do not fully mimic the behavior of the original murine protein. Loss of affinity, specificity, or stability are the main parameters affected by this experimental

Abbreviations: bp, base-pair; CDR, complementary determinant region; Fab, antigen binding fragment; FR, framework; PCR, polymerase chain reaction; scFv, single chain variable fragment; mAb, monoclonal antibody; VL, variable light chain; VH, variable heavy chain

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procedure. Mild losses are normally associated with this procedure (Carter et al., 1992; Hsiao et al., 1994). A lower affinity is normally tolerated for humanized antibodies.

These effects have been partially remedied by reengineering the framework (Kettleborough et al., 1991; Singer et al., 1993; Nakatani et al., 1994; Zhu and Carter, 1995; Saldanha et al., 1999). Therefore, it is crucial to describe more subtle structural features that allow the preservation of antibody paratope integrity during this process. The rules are far from being completely understood. Long-range effects promoted by certain residues are among those forces responsible for subtle changes in the global structure. Unfortunately these are the most difficult issues to probe, since even small deviations could be responsible for removing residue–residue contact, or reducing surface complementarity. Therefore, even distant residues could interfere with paratope conformation and antigen binding (Chien et al., 1989).

We used an anti-human CD18 as a model antibody for humanization. CD18 is an integrin family membrane protein involved in cell adhesion. It is found in many different cell types and it is always associated with one of the different isoforms of CD11. Antibodies to CD18 may inhibit cell–cell attachment and especially leukocyte-tissue adhesion. Therefore, anti-CD18 antibodies have been proposed as adjuvant in many therapies that involves leukocytes infiltration and inflammation. They have been tested successfully as protective agents in ischemic myocardial injury in animal models (Gao et al., 2002), but much of this enthusiasm was lost after failure of human trials (Dove, 2000). Its potential application is much wider, and it has also been cited as a potential treatment for preventing meningitis sequels or graft rejection (Tuomanen et al., 1989; Isobe et al., 1997). The antibody used in this work, mAb 6.7, binds to CD18 (David et al., 1991) in a unique inhibitory epitope recently mapped to residues 350–432 (Lu et al., 2001).

In this work we describe the complete humanization of murine mAb 6.7 anti-human CD18. In a previous paper we described the successful humanization of the VH domain by means of a germline human VH gene fragment sequence using an expanded CDR1 (CDR1 + H1) graft (Caldas et al., 2000). We apply this same concept to the design of two versions of the humanized VL domain. While testing them, we have shown the effect of a mutation far from the antibody's binding site that resulted in a loss of affinity for the intact CD18 molecule on the cell surface. We propose it could be the result of a new, unexpected effect of a residue that interferes with binding of the completely humanized scFv even though it is located at a distance from the binding site.

2. Materials and methods

2.1. Computational analysis

Similarity analysis was initially performed using FASTA (Pearson, 2000) and the Swiss-prot database (Bairoch and

Apweiler, 2000). Blastp (Altschul et al., 1997) was also used either through the IgBlast page (<http://www.ncbi.nlm.nih.gov/igblast/>) or to analyze the PDB database (Berman et al., 2000). Amino acid residues usage in mouse and human VL was calculated from all-mouse and all-human VL files from Kabat Database (Johnson and Wu, 2000), using perl scripts. Germline VL sequences were obtained from the IgBlast page. Clustal W (Higgins et al., 1996) was used for multi-sequence alignment that was visualized using BioEdit version 5.0.9 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Tri-dimensional structure was visualized using RASMOL version 2.6 (Bernstein, 2000). This version of RASMOL permits direct distance calculations, but we also used perl scripts to perform such calculations. Accessibility of each atom in PDB file was calculated using the program Surface version 1.1 (Tsodikov et al., 2002). Variable region numbering follows Kabat's convention (Kabat et al., 1991).

2.2. Synthetic oligonucleotides

The overlapping oligonucleotides used for the synthesis of the humanized versions were supplied by DNA-gency (Malvern, PA). The oligonucleotides used were: L1 (5' AGAAGATCTGACGTGGTTATGACCCAAAGCCCC-TTGTCCCTGCCAGTCACTCTGGGC3'); L2 (5'GTGCA-CCAAGCGTTGGCTA GACCTGCAGCTTATAGAGGCA-GGCTGGCCCCAGAGTGACTGG3'); L3L (5'CAACGCTT-GGTGCACACCAACGGTAACACCTACTTCCACTGGT-TTCTTCAAAGACCAGGACAG3'); L3Q (5'CAACGCTT-GGTGCACACCAACGGTAACACCTACTTCCACTGGT-TTCAAACAAAGACCAGGACAG3'); L4 (5'AAAGAATCT-ATTGGAAACCTTGTAATCAACAGACGGGGGCTCT-GTCCTGGTCTTTG3'); L5 (5' TCCAATAGATTCTTTGG-AGTCCCAGACAGGTTTTCTGGCTCTGGTAGCGGGA-CTGATTTCT3'); L6 (5'ATACACCCCGACATCCTCAGCT-TCTACCCTGGAAATTTTGAGTGTGAAATCAGTCCC-GCT3'); L7 (5'GATGTCGGGGTGATTATTGTTACAG-TCAACACATGTTCCCCGGACTTTCGGTGGTGGC3'); L8 (5'ACCATGGGCTCTCTTGATCTCGAGCTTTGTGC-CACCACCGAAAGT3'); EXTL1 (5'GC TAGTAGAAGAT-CT3') and EXTL2 (5'CACACCATGGGCTCT3').

The 5' L1 oligonucleotide contains a *Bgl*II restriction site and the 3' L8 carries a *Nco*I and a *Xho*I site (these sites are underlined). The L3L and L3Q contain the codon change between the two VL humanized versions in bold (see results).

2.3. Assembly of the VL humanized versions

The 6.7 VL sequence was previously determined (Caldas et al., 2000) and its amino acid sequence was used for a search for the closest human germline sequence. The closest human germline sequence chosen was used as a framework to graft the murine CDRs. The DNA fragments for two humanized VL versions were generated using eight overlapping oligonucleotides ranging from 45 to 63 bp, with 15 bp of complementarity, in a PCR-based-protocol. Aliquots of

10 pmol of each pair of complementary oligonucleotides were annealed separately in a 50 μ l reaction containing 9 mM Tris–HCl (pH 7.6), 13 mM MgCl₂, 21 mM DTT and 200 μ M dNTPs. The samples were incubated in 400 ml of boiling water for 5 min and left standing until the water reached room temperature. Each pair of primers were elongated by the addition of 24 U DNA polymerase I (Klenow fragment, Biolabs) for 30 min at room temperature. The two pairs of primers coding for the N-terminus were mixed and amplified by PCR, and the same procedure was followed for the two pairs of primers coding for the C-terminus. The DNA fragments for the two N-terminus and C-terminus regions were amplified by 20 thermal cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 2 min and analyzed in agarose gel. Finally, the full-length DNA fragment was amplified using as DNA templates the amplified fragments from the first PCR, extracted directly from the agarose gel with a pipet tip. The 5' and 3' external primers (EXTL1 and EXTL2) were used in this second PCR; the DNA fragments were PCR-amplified after 25 thermal cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 2 min, purified with Quiaquick gel purification system (QIAGEN), using the manufacturer's recommendation, cloned in the pGEM-T vector (Promega) and sequenced using the T7 Sequencing Kit (Pharmacia).

2.4. Construction of the expression vectors

The constructs that code for the humanized scFvs were assembled based on the pIg17hVH/mVL (Caldas et al., 2000) derived from pIg17Z22 (Brigido et al., 1993). In this system, the proteins can be expressed as a fusion product with a staphylococcal protein A domain, in order to allow the detection of the recombinant protein and also to facilitate the purification in an IgG sepharose chromatography column. The DNA fragments of the humanized VL were cloned in the vector pIg17hVH/mVL, which had its murine VL replaced by the humanized VLs, through digestion with *Bgl*II and *Nco*I. After the construction and verification of the expression cassettes, these were transferred to the *Pichia pastoris* expression vector pPIg16 (Andrade et al., 2000) by replacing the existing scFv, through digestion with the restriction enzymes *Xma*I and *Eco*RI.

2.5. Expression of the humanized scFvs in *Pichia pastoris*

P. pastoris GS115 cells (Invitrogen, San Diego, CA) were grown in liquid medium and made competent by resuspension in 1 M sorbitol. The cells were electroporated by pulse discharge (1500 V, 25 μ F, 400 Ω ; Bio-Rad Gene Pulser) for 5 ms in the presence of 5–10 μ g of plasmid DNA linearized with *Sa*II. This enzyme cuts within the plasmid-encoded *HIS4* gene and favors homologous recombination with the endogenous, non-functional *his4* gene of GS115 cells. Therefore, transformants (His⁺) were screened by their capacity to grow in the absence of histidine as described by the manufacturer (Invitrogen). Protein expression kinetics

were determined by growing clones expressing the two humanized scFvs in 25 ml of BMGY medium (1% yeast extract, 2% peptone, 10 mM potassium phosphate, pH 6.0, 1,34% yeast nitrogen base, 4×10^{-5} % biotin, 1% glycerol) at 30 °C in a shaking incubator (250 rpm) until the culture reached $A_{600} = 2.0$ – 6.0 . Cells were then centrifuged and resuspended in 100–200 ml of BMMY medium (which has 0.5% methanol instead of 1% glycerol of the BMGY medium, while the other components are the same) to induce protein expression. Cells were incubated for 4 days at 30 °C in a shaking incubator (250 rpm). Aliquots of culture supernatants were taken daily, and examined by SDS–PAGE and Western blotting. For large scale expression, the clones were grown exactly the same way as above, for 80 h at 30 °C under agitation. The supernatants were harvested following centrifugation and filtration through a 0.45 μ m cellulose acetate filter. After the addition of 80 μ g of Pepstatin A and 14 μ g of PMSF to the supernatants, these were concentrated to about 5 ml using an ultrafiltrating stirred cell (Corning) with a membrane filter with a cut-off of 10,000 Da according to manufacturer's instructions.

2.6. Purification of recombinant scFvs

The concentrated supernatants were run through an IgG Sepharose 6B Fast Flow column (Pharmacia) previously activated by three alternating washes with 0.5 M acetic acid, pH 3.4, and PBST (PBS and Tween 20, 0.1%) and finally equilibrated with PBS. ScFv fragments were eluted with 0.5 M acetic acid, immediately neutralized with 1.5 M Tris–HCl, pH 8.8. The purified proteins were dialyzed against PBS and quantified using the BCA Protein Assay Kit (Pierce).

2.7. Flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) obtained from a normal individual by gradient centrifugation were used for immunofluorescence assays. Antibodies utilized were: recombinant Z22 scFv (Andrade et al., 2000) as a negative control; rabbit anti-human IgG–FITC (Dakopatts, Denmark; used in the second step of the indirect immunofluorescence reaction to bind to the protein A domain present in the recombinant anti-CD18 scFvs, through the Fc fragment); rabbit anti-mouse IgG (Sigma); 6.7 anti-CD18 FITC (Instituto Butantan-InCor, Brazil); anti-CD19PE (Dakopatts, Denmark); anti-CD3 FITC (Dakopatts, Denmark); anti-CD4 FITC (Dakopatts, Denmark); anti-CD8 FITC (Dakopatts, Denmark) and anti-CD45RO PE (Pharmingen). The sample incubated with both anti-CD19PE and rabbit anti-human IgG–FITC was used to evaluate binding of the rabbit antibody to IgG expressed on B cells and exclude any other unspecific binding from the tests with the scFvs. Anti-CD3 was used as a positive control of the assays. 2×10^5 cells were incubated with the different antibodies for 30 min at 4 °C and washed three times. For the samples with the recombinant humanized scFvs, a second incubation was

performed with rabbit anti-human IgG-FITC. All samples were resuspended in 400 µl of FACS buffer (PBS, 2% FCS and 0.01% sodium azide) and analyzed using a FAC-Scan flow cytometer (Becton Dickinson, CA, USA). Ten thousand events were analyzed for each sample, inside the gate of lymphocytes. Recombinant proteins were added in equimolar quantities. Results are expressed as the percentage of stained cells. The antibodies anti-CD4, anti-CD8 and anti-CD45RO were used in order to characterize the T-lymphocyte sub-populations that the humanized scFvs were able to bind.

2.8. Blocking capacity of the humanized scFvs

In order to analyze the binding specificity of the two humanized scFvs, a blocking experiment was performed. The capacity of the scFvs to block the binding of the original 6.7 anti-CD18 FITC to surface CD18 molecules was tested. Cells were initially incubated with the two humanized scFvs, washed, incubated with rabbit anti-mouse IgG (to block the protein A domain present in the recombinant scFvs) and then incubated with 6.7 FITC. The percentage of positive cells and the intensity of immunofluorescence (IF) were compared in samples with 6.7 FITC alone and samples with the different humanized scFvs plus 6.7 FITC. The percentage of inhibition was calculated considering these differences.

3. Results

3.1. Selection of the framework Vκ and Jκ for CDR-grafting

The human framework used to accept the murine CDRs was selected based on the closest germline sequence (Caldas

et al., 2000). We used the FASTA program to search for human Vκ sequences deposited in the Swiss-prot database. The closest human sequence found was the germline Vκ fragment KV2F (Klobeck et al., 1985) with 76% identity and 89% similarity to the mouse 6.7 Vκ gene fragment (AF135165). A similar result was obtained using the NCBI IgBlast tool. In this case, two human germline Vκ sequences exhibit good hits with the mouse Vκ. The closest was the A17 (X63403) with 76% identity followed by the A18 (X63396) with 74% identity (Lautner-Rieske et al., 1992). The alignment of the original anti-CD18 Vκ gene segment to the human related sequences is shown in Fig. 1. The A17 coding sequence is identical to the KV2F Swiss-prot record, while A18 is 81% identical (91% similar) to either A17 or KV2F. Either Vκ could be used for grafting the 6.7 Vκ complementary determinant region (CDR), but the KV2F/A17 was chosen due to its closer proximity. The 6.7 Jκ (AF135165) closest human Jκ gene segment is the Jκ4 with only one difference. Thus the human germline Jκ4 sequence was chosen for completing the FR4 of the humanized VL. The conservative V → L codon change observed in the recombinant FR4 (Fig. 1) is due to an XhoI site at the end of VL used for the expression vector manipulation.

3.2. Identification of putative constraints

The visual inspection of two of the closest murine (1MRC) and human (1AD9) Fab crystals suggests an overall conserved structure in the VL domain. The systematic survey of the tri-dimensional structure at the replacing residues reveals two positions that could be important for maintaining the framework structure. The murine residue Leu⁴⁶ was found to be buried in the VH–VL interface. By using a cut-off distance of 4.0 Å, VL residue 46 makes many contacts

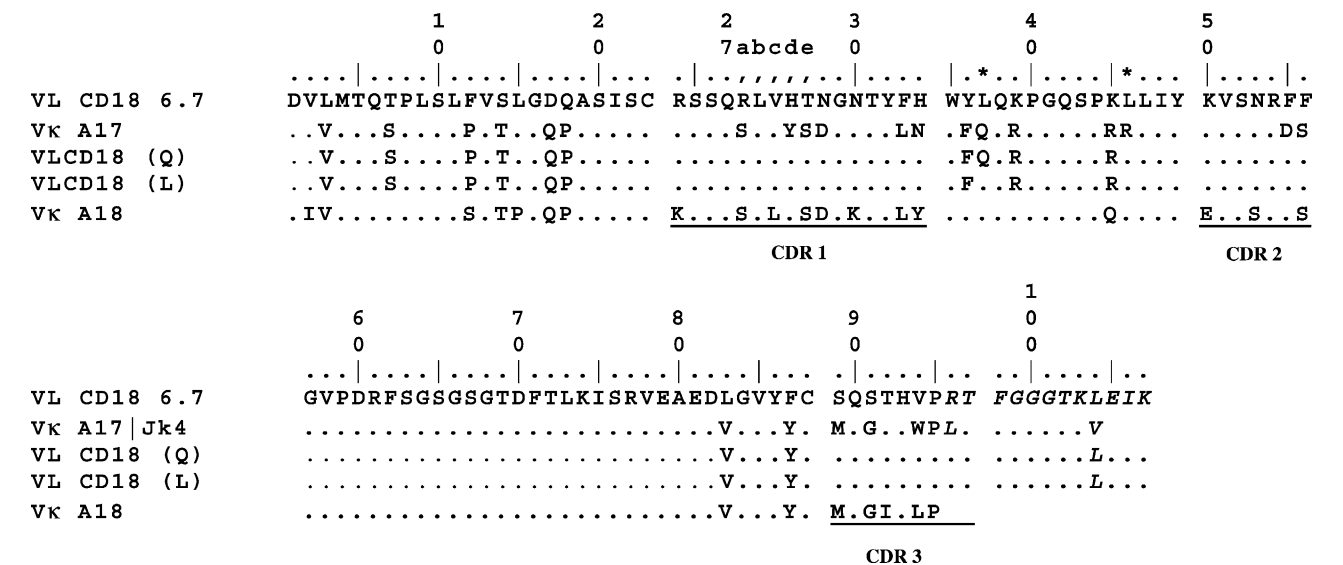


Fig. 1. Design of anti-CD18 VL humanized versions. Amino acid sequence alignments of original murine VL (VL 6.7), closest human germline VL (Vκ A17 and Vκ A18) and humanized VL versions (VLCD18 (Q) and VLCD18 (L)). CDR residues, according to Kabat et al. (1991) are indicated. Asterisks indicate the residue 37 and 46, maintained in the humanized VL as discussed in the text. Jκ residues are shown in italic.

including one to the LCDR 2 residue Phe⁵⁵, two “Vernier” zone residues (Tyr³⁶ and Trp³⁵), framework residues Ile⁴⁸, Val⁵⁸, and the HCDR3 residues Asp¹⁰¹ e Gly⁹⁷. Therefore, we decided to preserve the original murine Leu⁴⁶ due to its array of contacts that includes contacts to VH and VL CDRs.

Murine residue Leu³⁷ was found to be superficially buried, making many contacts within the VL domain core. Its contact residues in 1MRC structure at 4.0 Å cut-off are: Pro⁴⁴, Lys⁴⁵, Leu⁴⁷ and Tyr⁸⁶. The Lys⁴⁵ contact is one of the conserved hydrogen bonds involved in the maintenance of the variable domain structure (Chothia et al., 1985). In the 1AD9 structure, Gln³⁷ contacts the same subset of residues as above. The only discrepancy is a hypothetical hydrogen bond between the hydroxyl O of the Tyr⁸⁶ and the amide N^ε of Gln³⁷ ($d = 282 \text{ Å}$). In the 1MRC VL, the residue 37 is partially exposed to the solvent making a contact to a water molecule in the crystal. No water was found close to residue Gln³⁷ in the VL of 1AD9. A survey of the Kabat database showed that position 37 is filled with either glutamine or leucine. For the mouse light chain, leucine appears in 20% of the antibodies while glutamine responds for 78% of all-mouse antibodies; other amino acid residues make up less than 3%. Similar numbers occur for human light chain. Due to its location in the VL-solvent interface and to its close proximity to the CDR1, we chose this position to construct two humanized versions for the VL domain of the 6.7. The first construction carries the original L³⁷ residue (named version L) while the other has the human germline Gln³⁷ residue (version Q). Both constructions retained the murine Leu⁴⁶ residue.

3.3. Design of the recombinant humanized VL

The complete humanized VL sequence was obtained by grafting the CDR1, 2 and 3 from the 6.7 VL in the proposed human germline V κ (KV2F/A17) fused to J κ 4 gene fragment. CDR1 was defined as residues 24 to 34 (24–39 in sequential numbering), CDR2, as residues 50–56 (55–61), and CDR3, residues 89–97 (94–102), following the Kabat definition (Kabat et al., 1991). The grafting process introduced six changes in CDR1, 2 in CDR2 and 5 in CDR3 of the human VL, resulting in 13 differences out of the 32 residues in the CDRs. The complete humanized sequences are shown in Fig. 1. The final proposed humanized sequence had 12 differences compared to the original 6.7 VL (89% identity) and 14 differences to the original V κ KV2F/A17/J κ 4 (87.6% identity) in the Leu³⁷ version (Fig. 1). The J κ fragment was used as is, except for the Leu¹⁰⁴ introduced by an XhoI site. The proposed recombinant VL was chemically synthesized for cloning in an scFv expression vector.

3.4. Construction and expression of the humanized scFv

As shown in Fig. 2, a set of ten oligonucleotides was used to generate the humanized VL using recombinant PCR

as previously described for the VH domain (Caldas et al., 2000). Briefly, the primers were initially annealed, filled in by Klenow and then amplified as pairs until they reached the full sized VL (Fig. 2). The synthetic DNA fragment was cloned in pGEM-T easy vector. The recombinant clones were initially checked for size of insert prior to being repeatedly sequenced. Several clones with the correct sized insert were tested and one clone (out of six) of the L version and one (out of 8) for Q version had a correct sequence. Correctly synthesized DNA inserts were digested with BglII and XhoI, and isolated from gel for cloning in the pIg17hVH/mVL plasmid cleaved with the same restriction endonucleases. This plasmid already harbored a hemi-humanized version of the 6.7 anti-CD18 scFv composed of a humanized VH fused to the original murine VL (Caldas et al., 2000). The cloning procedure eliminates the original murine VL, replacing it with the synthetically humanized gene fragment. Two new constructions were obtained: pIg17hVH/hVL(L) and pIg17hVH/hVL(Q). For simplicity, these constructions were named pIg17LL and pIg17LQ, respectively. The whole scFv cassette, digested with XmaI/EcoRI, was used to replace the scFv cassette of pPIg16, a *P. pastoris* expression vector (Andrade et al., 2000). According to the notation used above, the resulting plasmids were named pPIg LL and pPIg LQ.

A protease defective strain of *P. pastoris* was used to receive the expression vectors by electroporation. Both plasmids were used to transform electrocompetent yeast cells. Many clones were obtained and screened for scFv production in the colony filter assay, where scFv producing cells were detected by immunostaining (Andrade et al., 2000). Two clones of each construction, found to be positive in the filter assay, were selected for growth on an analytical scale. In both cases, the detection of recombinant scFv was low. Filter assay positive clones were barely visible and the yield of purified scFv was also limiting. From 200 ml of yeast cultures, we normally obtained about 0.5–1 mg/l. Even so, we were able to purify around 1 mg of recombinant scFv of both L and Q version of the humanized anti-CD18 for further characterization.

3.5. Immunological characterization of recombinant humanized antibodies

The CD18 antigen is expressed at the cell surface of all leucocytes. Therefore, we tested the two variants of recombinant humanized antibody (LL and LQ) directly for binding to peripheral blood mononuclear cells (PBMC) by Flow Cytometry analysis. Our results show that the humanized LL version presents essentially the same binding capacity to lymphocytes as the original anti-CD18 monoclonal antibody, staining 85% of gated cells. In contrast, the LQ version only stained 53% of the cells in the gate of lymphocytes (Fig. 3). The original 6.7 anti-CD18 mAb and an anti-DNA scFv were used as positive and negative controls, respectively. The small population stained with high intensity in

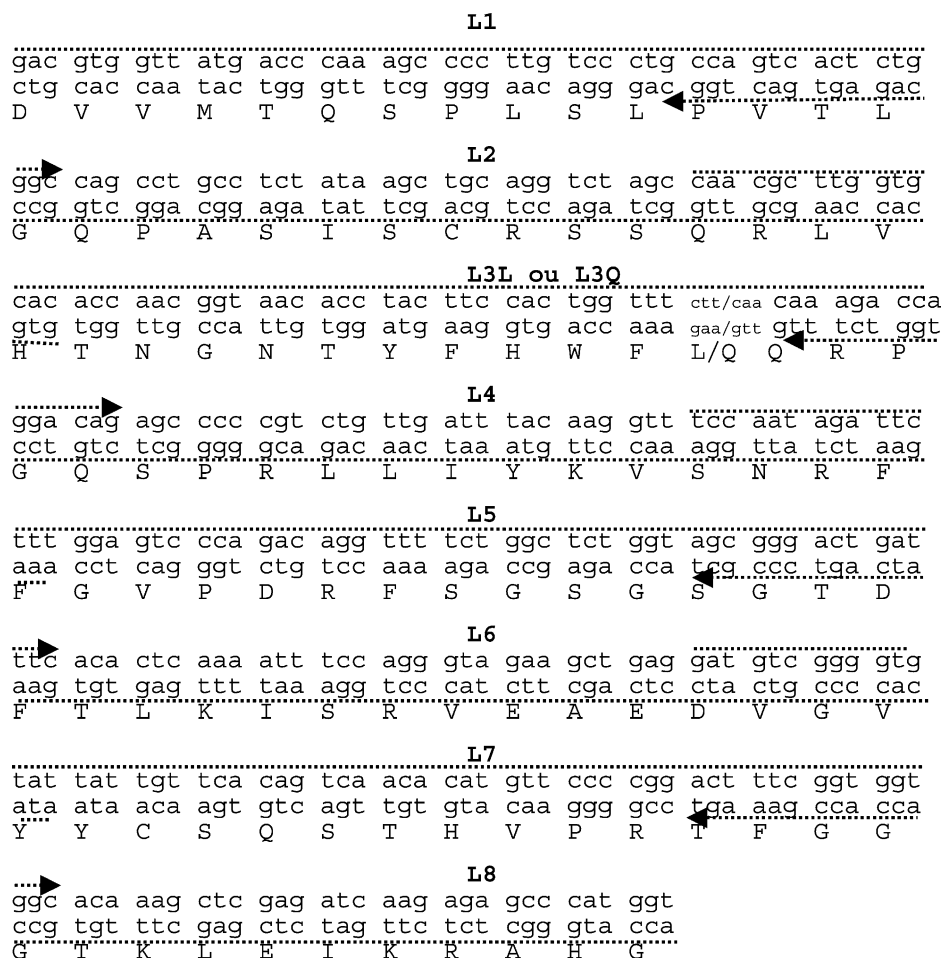


Fig. 2. The VL humanized versions were assembled using a PCR-based protocol. Arrows indicated the sense (→) and anti-sense (←) oligonucleotides used for VL synthesis. Two variations of the L3 oligonucleotides were synthesized (L3L and L3Q), that create the difference among the versions L and Q.

the humanized versions of anti-CD18 was interpreted as un-specific staining, since it was also detected with anti-human IgG-FITC alone. The negative control anti-DNA scFv did not show any significant staining.

The humanized scFvs were also able to compete efficiently with the 6.7 mAb for binding CD18 on lymphocytes, as shown in Fig. 4. In this experiment, decreasing dilutions of scFv were incubated with PBMC prior to incubation with the original anti-CD18 mAb. Both humanized versions were able to efficiently block the binding of the mAb to CD18 molecules at the cell surface. Again, the LL version was also more efficient than LQ, blocking up to 80% of mAb's binding activity in a 1:5 dilution.

In order to compare the cell subsets recognized by the humanized LL version and the original murine antibody, we performed FACS analysis, using different markers. We found that both the original anti-CD18 and the humanized LL version displayed the same pattern of staining to CD4⁺, CD8⁺ and to memory CD45RO⁺ lymphocytes (Fig. 5).

4. Discussion

The rules for transferring ligand specificity between donor and acceptor antibody are partially known. Today it is conceivable to transfer the CDR regions to a closely related framework acceptor, retaining the antibody's specificity. The fact that the three-dimensional structure of the variable domain is highly conserved makes this strategy feasible. In addition, the traditional model of antibody ontogeny relies on a flexible variable domain structure for evolving B cell response for an infinite antigen repertoire. Somatic hypermutation molds the final high affinity antibody. Such flexibility is so impressive that even Ig transgenic mice are able to make antibodies successfully even with a very limited Ig repertoire (Cascalho et al., 1996). It is likely that antibodies are made for binding, and transposing specificity is an operation controlled by simple rules. When Wu and Kabat (1970) systematically analyzed the primary structure of the variable domain of antibodies, they observed that interspaced in the overall variable structure of the amino terminus domain of

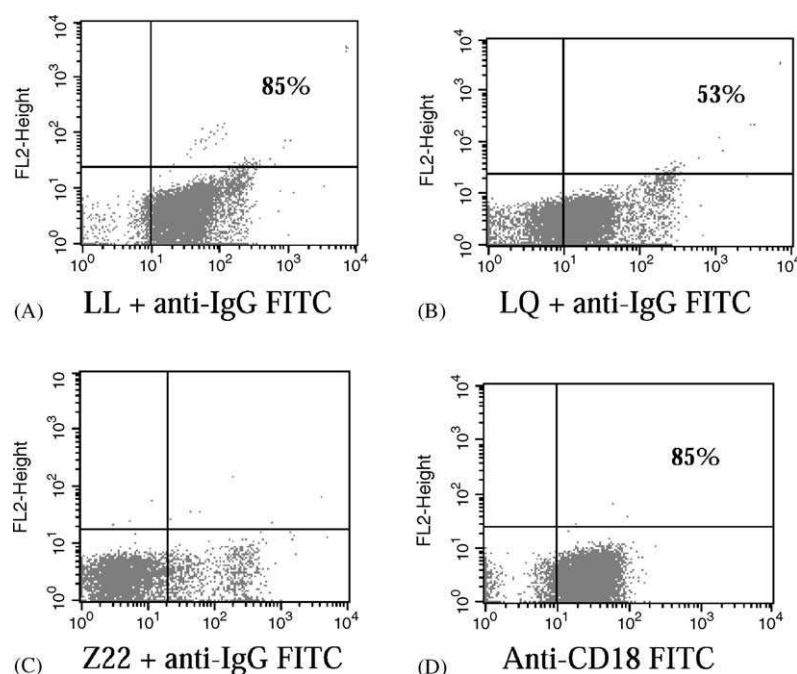


Fig. 3. The scFv anti-CD18 humanized versions bind to human lymphocyte cell surface; 84, 53 and 85% of the cells were positive when incubated with the humanized scFv versions LL (A), LQ (B), or the original monoclonal anti-CD18 antibody (D), respectively. The Z22 anti-Z-DNA scFv was used as negative control (C).

both heavy and light chain of antibody, there were hypervariable regions. These regions were termed complementary determining regions after a new paradigm that implicates this region as the determinant for an antibody's binding specificity. This paradigm is still accepted today, and it is the basis of CDR-grafting (CDR replacement) as it was initially proposed by Jones et al. (1986). From that time on, transferring CDRs from mouse antibody to a human antibody framework became a general rule for antibody humanization.

The first humanization procedures were based on transferring mouse CDRs to human frameworks derived from known human antibodies. Even though this simple opera-

tion works, in most cases affinity was lost. To achieve an antibody with a better affinity, different authors have applied different strategies such as using other human framework V genes (Singer et al., 1993), exchanging sterically hindering residues (Zhu and Carter, 1995), or replacing residues that was known to interfere with CDR conformation (Tempest et al., 1991). Therefore the design of such humanized antibodies requires correction of the framework.

In a broader view, most humanized antibodies lose part of their affinity compared to the original antibody (Studnicka et al., 1994; Tempest et al., 1995; Co et al., 1996). This loss is enhanced as the similarity of the chosen framework and the donor variable sequence decreases. Many authors elegantly demonstrated this framework effect (Tramontano et al., 1990; Foote and Winter, 1992; Studnicka et al., 1994). Tramontano et al. (1990) demonstrated the effect of the residue 71 heavy chain as a prototype of framework bias on the CDR conformation. Many other trending framework positions were revealed by systematically analyzing antibody X-ray crystals (Foote and Winter, 1992; Studnicka et al., 1994). Thus, an influence of the framework in paratope assembling became widely accepted. It may appear contradictory because CDRs are the most variable portion of the variable domain, but recent studies bring evidence that CDRs indeed determine the overall shape of the paratope (Holmes and Foote, 1997; Holmes et al., 1998). The best scene is of a framework that sustains the paratope but may constrain certain conformational spaces. CDR would shape the paratope and bias the whole variable region structure inside the framework intrinsic limitations.

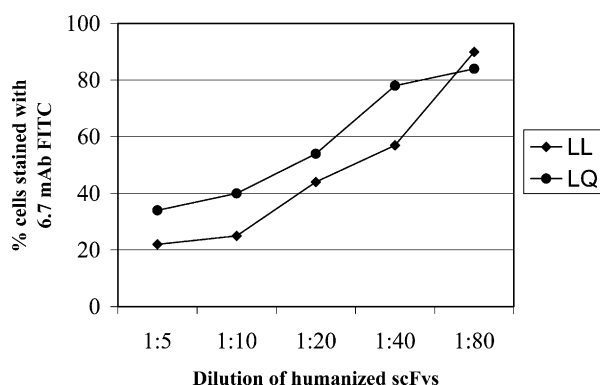


Fig. 4. Humanized scFvs block the binding of anti-CD18mAb FITC to CD18+ cells. Cells were incubated with different concentrations of recombinant humanized scFvs, washed, and then incubated with the mAb anti-CD18 FITC. The percentage of cells stained with the anti-CD18mAb FITC after the addition of the humanized scFvs is shown.

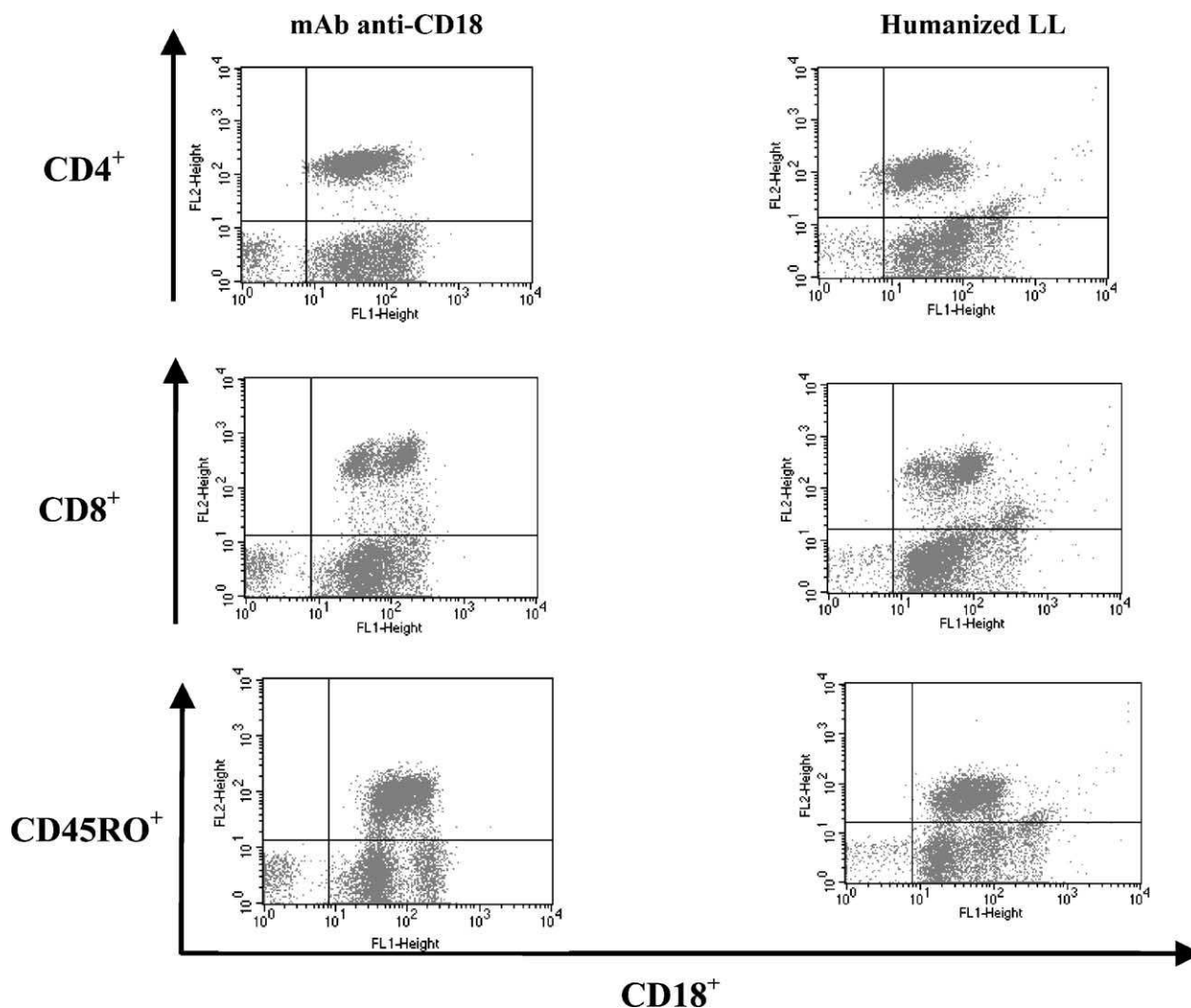


Fig. 5. T cell subpopulations stained by the humanized LL version compared to the original mAb.

In this report we synthesized the light chain variable region as part of a humanized anti-human CD18 antibody. The strategy to design the humanized light chain variable region was based on the closest germline sequence (Caldas et al., 2000). Such a strategy allows us to reduce the framework derived constraints due to the use of the closest germline framework sequence. Many human germline sequences are available to date, facilitating the search for homologous V gene candidates (Tomlinson et al., 1995). After design we proceeded to the visual inspection of the three-dimensional structure of similar antibodies. In this step, we chose to use X-ray derived information instead of the molecular model for the humanized structure, because there is a large amount of data on crystal structures of antibodies in the PDB. (This is probably the most studied protein family at the three-dimensional level.) We were able to find, in the PDB, two sequences which were very similar to our VL sequence; one is a mouse Fab and the other is a humanized Fab. There are 12 differences between the murine and the humanized VL. Most of them correspond to exposed residues

or residues far from the paratope. There was one important residue, Leu⁴⁶, that was shown to make contacts to LCDR2 and 3, besides playing an important role in the interface of VL/VH, making close contact to HCDR3 (Padlan, 1994). The exchange of a leucine, an aliphatic amino acid, for the arginine that appears in the human closest germline, would probably disrupt many atomic contacts and destabilize the overall structure. Therefore this residue was kept as in the original murine antibody.

Another observed difference was at position 37. This residue sits in the bottom of the molecule between the structurally conserved Gln³⁸, involved in light chain-heavy chain interface (Chothia et al., 1985), and the “Vernier” zone residues after CDR1 (Foote and Winter, 1992). In the mouse antibody structure 1MRC, the Leu³⁷ is partially accessible to solvent where it is close to a water molecule (3.37 Å). It is probably not involved in a direct H-bond since the distance found is over the expected distance of a regular water-carbonyl hydrogen bond (Creighton, 1984). In the human V κ germline sequence used in this work, a glutamine

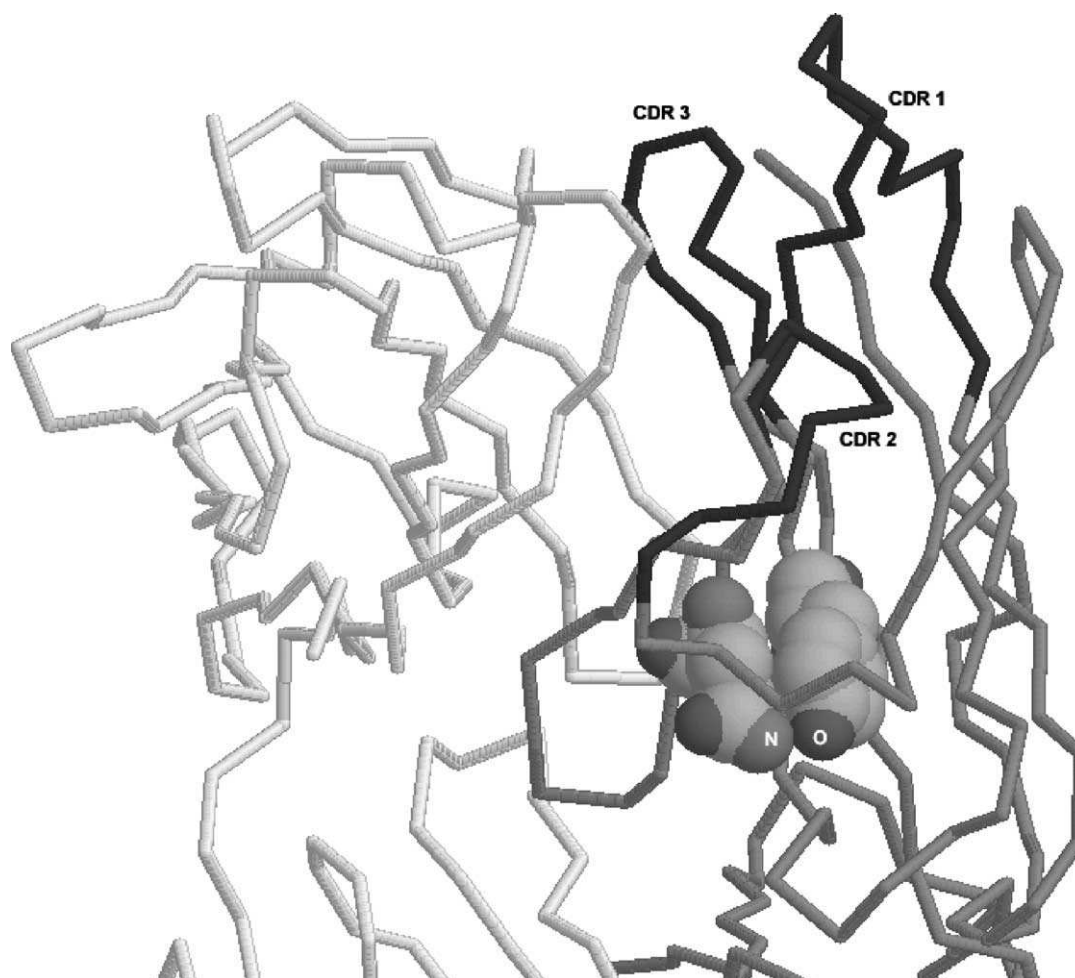


Fig. 6. Structural visualization of the Gln³⁷–Tyr⁸⁶ interaction. The structure of variable domain of 1AD9 was visualized using RASMOL. VL backbone is shown in gray and its three CDRs (black) are labeled. Gln³⁷ and Tyr⁸⁶ residues are shown in a space filling representation. The atoms labeled with N and O denotes the glutamine amide group and the tyrosine hydroxyl group, respectively. Heavy chain backbone (light gray) is shown in the background.

residue fills the position 37. This residue is found in many humanized antibodies, including our closest PDB crystal structure, 1AD9. In this crystal, Gln³⁷ is mostly buried and it contacts the same set of residues as Leu³⁷ in the murine structure. The only difference is a hypothetical H-bond between the amide group of Gln³⁷ and the hydroxyl group of Tyr⁸⁶ (Fig. 6). The short distance between them (2.82 Å) supports this assumption. Besides that the environment around position 37 is conserved in both structures, as is the conserved H-bond to residue 45.

The leucine residue is less common than glutamine in either human and mouse VL at position 37. This position seems to be exclusively filled by one of these large residues and therefore there should be a restriction for using either one of them, but polar residues seem more appropriate for occupying this position. Analysis of the germline genes reveals a similar proportion of Gln/Leu residues at position 37 in mouse and human antibodies (Lautner-Rieske et al., 1992). Interestingly, Chothia et al. (1998) classified this position as a buried polar region, barely variable, that is involved in the stabilization of FR2. It is possible that the

formation of the H-bond between the Gln³⁷ and Tyr⁸⁶ stabilizes the antibody hydrophobic core.

We tested both versions of humanized antibodies using as the VH domain a previously characterized humanized heavy chain (Caldas et al., 2000). The synthesis of both humanized VL was performed using recombinant PCR to achieve a full-length gene segment. We found that only a small proportion of clones carry the correct construction and we attributed to the purity of the synthetic oligonucleotides. We had to screen a large number of clones to find a correct one. A single extensively checked clone for each version (Q and L) was chosen. The VL gene segment was isolated and transferred to the *P. pastoris* expression vector along with the heavy chain gene fragment version L (Caldas et al., 2000). The recombinant scFv produced by this expression system had a staphylococcal protein A tag in its carboxi-terminus, which allowed for purification and detection of the recombinant protein. The yield of secreted scFv for this expression system was lower than expected when compared to previous results (Andrade et al., 2000), but it was sufficient to test for binding in fluorescence activated cell cytometry.

The β_2 -integrin antigen CD18 is a cell membrane associated high molecular weight glycoprotein. Although it would be appropriate to test the specificity of the humanized antibodies directly on CD18 antigen, the binding of the 6.7 antibody to soluble CD18 in simple immunoassays such as enzyme linked immunosorbent assay (ELISA) has not been reported. Therefore, we tested the humanized antibodies directly on cells from a healthy donor expressing the CD18 antigen, by FACS analysis. Similarly to the 6.7 mAb, both humanized antibodies were able to bind to PBMC. No binding was observed with the negative control, suggesting that the observed binding of the humanized scFv was due to its affinity for PBMC antigens, excluding the possibility of a fortuitous activity due to misfolded proteins or due its protein A affinity tag. In this experiment, the LL version showed higher cell labeling when compared to the LQ version. The specificity of constructions was analyzed in a blocking experiment in which we showed that binding of 6.7 mAb was specifically blocked by the humanized scFv, but not by a similar but non-specific scFv (anti-DNA, data not shown). This experiment indicates that the humanized scFv binds to PBMC specifically through the CD18 antigen and there is no unspecific binding to some other cellular structure. Moreover, both versions of the humanized antibody fragments bind to the same epitope as the original 6.7 mAb. The blocking experiment also reveals that the LL version is more effective than the LQ version in competing for the 6.7 binding site. The experimental design in this work does not permit a direct comparison of the affinity of mAb with that of the humanized versions. However, it does allow us to infer that the LL version affinity is similar to that of 6.7 mAb due to the blocking efficiency of this scFv compared to the whole 6.7 mAb. The original murine mAb is divalent and this attribute contributes to an increased avidity compared to a monomeric scFv, hence quantitating true affinity was not possible for this system.

To be useful in therapeutics, a humanized antibody has to maximally mimetize the affinity and specificity of the original heterologous antibody. The CD18 antigen is found in leukocytes, including lymphocytes and monocytes. Here, we used the binding profile in different lymphocyte populations of cells as evidence that the humanized antibody retained its specificity. The comparison of the 6.7 antibody with its L humanized version reveals a very similar profile for binding to CD18 in three lymphocyte sub-populations: CD4⁺, CD8⁺ and CD45RO⁺. FACS analysis revealed that both murine and the L humanized version stain approximately the same number of cells. The similarity of binding profile for the LL humanized antibody, compared to the 6.7 mAb, suggests that it can be used for the replacement of the murine antibody. However, further in vivo studies will be necessary for confirming its efficacy and clinical applicability.

All the experiments used to verify and to compare the humanized scFv to de 6.7 mAb result in a consistently better performance of the LL over the LQ version. For some reason the presence of a leucine residue at position 37 enhances

binding activity, although the specificity was maintained in both humanized versions compared to 6.7 mAb. The net effect of this Leu $\rightarrow$ Gln change in the binding activity of the humanized scFv was expected. Its volume and buried character associated with a discrepancy in the hydrophilicity suggests an impact on the overall structure. The difference observed in this work may be explained by the formation of an H-bond between residue 37 and 86 only in the case of an electron acceptor side-chain, such as a glutamine, which is indeed present. This could reduce the flexibility required for the FR2 and FR3 reconfiguration of the framework, which is required for the successful graft of the CDR. The result may be a more rigid paratope that hampers the association with the antigen. Moreover, this framework rigidity may result in reduced paratope flexibility that seems fundamental for binding of some antigens, as suggested by others (Herron et al., 1991; Stanfield et al., 1993).

We have shown the complete humanization of the 6.7 mAb. The procedure used here was very simple but is reiteratively successful. It suggests that the principle of CDR-grafting using the closest germline is indeed a step forward. Recent publications on humanization rely on more complex grafting procedures that do not necessarily lead to more efficient antibodies. The SDR (specificity determining residues) approach was based in the humanization of specific contact residues (Iwahashi et al., 1999; Tamura et al., 2000; De Pascalis et al., 2002). Even though the procedure leads to active antibodies, it does demand either the “a priori” knowledge of the antibody’s tri-dimensional structure, or a tantalizing screening for binding specific residues. Moreover, since many human CDR residues were maintained in the final structure, the determinant role of the CDRs for the overall antibody structure was partially lost. Tan et al. (2002) have recently proposed a humanization procedure based on a canonical region based germline sequence search. Their results were indeed disappointing. The authors argue that their process allows a very “human” antibody based on their comparison of the number of introduced mutations in their germline sequence and FDA approved antibodies. It should be pointed out, however, that the number of mutations introduced in their chosen VL germline sequence was 21. In contrast, in the humanized 6.7 VL we introduced 14 mutations including one due to an *XhoI* restriction site introduced in FR4 far from the binding site. Hence, our approach is robust, as it leads to a preservation of binding activity while minimizing the number of murine residues. Moreover the systematic analysis of related structures leads to the detection of framework residues that potentially interfere with paratope formation, as we have shown for the VL position 37.

Acknowledgements

We thank Sandra Maria Monteiro for help in the FACS analyses, Dr. Linda Caldas and Adilton Guedes Oliveira for

English corrections to the manuscript, and Dr. Armand Bensussan and Dr. Laurence Boumsell for providing the original hybridoma.

References

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Andrade, E.V., Albuquerque, F.C., Moraes, L.M., Brigido, M.M., Santos-Silva, M.A., 2000. Single-chain Fv with Fc fragment of the human IgG1 tag: construction, *Pichia pastoris* expression and antigen binding characterization. *J. Biochem. (Tokyo)* 128, 891–895.
- Bairoch, A., Apweiler, R., 2000. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucleic Acids Res.* 28, 45–48.
- Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourn, P.E., 2000. The Protein Data Bank. *Nucleic Acids Res.* 28, 235–242.
- Bernstein, H.J., 2000. Recent changes to RasMol, recombining the variants. *Trends Biochem. Sci.* 25, 435–453.
- Brigido, M.M., Polymenis, M., Stollar, B.D., 1993. Role of mouse VH10 and VL gene segments in the specific binding of antibody to Z-DNA, analyzed with recombinant single chain Fv molecules. *J. Immunol.* 150, 469–479.
- Caldas, C., Coelho, V.P., Rigden, D.J., Neschich, G., Moro, A.M., Brigido, M.M., 2000. Design and synthesis of germline-based hemi-humanized single-chain Fv against the CD18 surface antigen. *Protein Eng.* 13, 353–360.
- Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E., Shepard, H.M., 1992. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc. Natl. Acad. Sci. U.S.A.* 89, 4285–4289.
- Cascalho, M., Ma, A., Lee, S., Masat, L., Wabl, M., 1996. A quasi-monoclonal mouse. *Science* 272, 1585–1588.
- Chien, N.C., Roberts, V.A., Giusti, A.M., Scharff, M.D., Getzoff, E.D., 1989. Significant structural and functional change of an antigen-binding site by a distant amino acid substitution: proposal of a structural mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 86, 5532–5536.
- Chothia, C., Novotny, J., Brucoleri, R., Karplus, M., 1985. Domain association in immunoglobulin molecules. The packing of variable domains. *J. Mol. Biol.* 186, 651–663.
- Chothia, C., Gelfand, I., Kister, A., 1998. Structural determinants in the sequences of immunoglobulin variable domain. *J. Mol. Biol.* 278, 457–479.
- Co, M.S., Baker, J., Bednarik, K., Janzek, E., Neruda, W., Mayer, P., Plot, R., Stumper, B., Vasquez, M., Queen, C., Loibner, H., 1996. Humanized anti-Lewis Y antibodies: in vitro properties and pharmacokinetics in rhesus monkeys. *Cancer Res.* 56, 1118–1125.
- Creighton, T.E., 1984. Physical forces that determine the properties of proteins. *Proteins*. Freeman Press, New York, Chapter 4, pp. 133–157.
- David, V., Leca, G., Corvaia, N., Deist, F., Boumsell, L., Bensussan, A., 1991. Proliferation of resting lymphocytes is induced by triggering T cells through an epitope common to the three CD18/CD11 leukocyte adhesion molecules. *Cell Immunol.* 136, 519–524.
- De Pascalis, R., Iwahashi, M., Tamura, M., Padlan, E.A., Gonzales, N.R., Santos, A.D., Giuliano, M., Schuck, P., Schlom, J., Kashmiri, S.V., 2002. Grafting of abbreviated complementarity-determining regions containing specificity-determining residues essential for ligand contact to engineer a less immunogenic humanized monoclonal antibody. *J. Immunol.* 169, 3076–3084.
- Dove, A., 2000. CD18 trials disappoint again. *Nat. Biotechnol.* 18, 817–818.
- Foote, J., Winter, G., 1992. Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* 224, 487–499.
- Gao, F., Yue, T.L., Shi, D.W., Christopher, T.A., Lopez, B.L., Ohlstein, E.H., Barone, F.C., Ma, X.L., 2002. p38 MAPK inhibition reduces myocardial reperfusion injury via inhibition of endothelial adhesion molecule expression and blockade of PMN accumulation. *Cardiovasc. Res.* 53, 414–422.
- Glennie, M.J., Johnson, P.W.M., 2000. Clinical trials of antibody therapy. *Immunol. Today* 21, 403–410.
- Herron, J.N., He, X.M., Ballard, D.W., Blier, P.R., Pace, P.E., Bothwell, A.L., Voss Jr., E.W., Edmundson, A.B., 1991. An autoantibody to single-stranded DNA: comparison of the three-dimensional structures of the unliganded Fab and a deoxynucleotide–Fab complex. *Proteins* 11, 159–175.
- Higgins, D.G., Thompson, J.D., Gibson, T.J., 1996. Using CLUSTAL for multiple sequence alignments. *Methods Enzymol.* 266, 383–402.
- Holmes, M.A., Foote, J., 1997. Structural consequences of humanizing an antibody. *J. Immunol.* 158, 2192–2201.
- Holmes, M.A., Buss, T.N., Foote, J., 1998. Conformational correction mechanisms aiding antigen recognition by a humanized antibody. *J. Exp. Med.* 187, 479–485.
- Hsiao, K.C., Bajorath, J., Harris, L.J., 1994. Humanization of 60.3, an anti-CD18 antibody: importance of the L2 loop. *Protein Eng.* 7, 815–822.
- Isaacs, J.D., Clark, M.R., Greenwood, J., Waldmann, H., 1992. Therapy with monoclonal antibodies. An in vivo model for the assessment of therapeutic potential. *J. Immunol.* 148, 3062–3071.
- Isobe, M., Suzuki, J., Yamazaki, S., Yazaki, Y., Horie, S., Okubo, Y., Maemura, K., Yazaki, Y., Sekiguchi, M., 1997. Regulation by differential development of Th1 and Th2 cells in peripheral tolerance to cardiac allograft induced by blocking ICAM-1/LFA-1 adhesion. *Circulation* 96, 2247–2253.
- Iwahashi, M., Milenic, D.E., Padlan, E.A., Bei, R., Schlom, J., Kashmiri, S.V., 1999. CDR substitutions of a humanized monoclonal antibody (CC49): contributions of individual CDRs to antigen binding and immunogenicity. *Mol. Immunol.* 36, 1079–1091.
- Johnson, G., Wu, T.T., 2000. Kabat database and its applications: 30 years after the first variability plot. *Nucleic Acids Res.* 28, 214–218.
- Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S., Winter, G., 1986. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321, 522–525.
- Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., Foeller, C., 1991. *Sequences of Proteins of Immunological Interest*, fifth ed. Public Health Service, National Institutes of Health, Bethesda, MD.
- Kettleborough, C.A., Saldanha, J., Heath, V.J., Morrison, C.J., Bendig, M.M., 1991. Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation. *Protein Eng.* 4, 773–783.
- Klobeck, H.G., Meindl, A., Combriato, G., Solomon, A., Zachau, H.G., 1985. Human immunoglobulin kappa light chain genes of subgroups II and III. *Nucleic Acids Res.* 13, 6499–6513.
- Lautner-Rieske, A., Huber, C., Meindl, A., Pargent, W., Schable, K.F., Thiebe, R., Zocher, I., Zachau, H.G., 1992. The human immunoglobulin kappa locus. Characterization of the duplicated A regions. *Eur. J. Immunol.* 22, 1023–1029.
- Lu, C., Ferzly, M., Takagi, J., Springer, T.A., 2001. Epitope mapping of antibodies to the C-terminal region of the integrin beta 2 subunit reveals regions that become exposed upon receptor activation. *J. Immunol.* 166, 5629–5637.
- Maynard, J., Georgiou, G., 2000. Antibody engineering. *Annu. Rev. Biomed. Eng.* 2, 339–376.
- Nakatani, T., Lone, Y.C., Yamakawa, J., Kanaoka, M., Gomi, H., Wijdenes, J., Noguchi, H., 1994. Humanization of mouse anti-human IL-2 receptor antibody B-B10. *Protein Eng.* 7, 435–443.
- Padlan, E.A., 1994. Anatomy of the antibody molecule. *Mol. Immunol.* 31, 169–217.
- Pearson, W.R., 2000. Flexible sequence similarity searching with the FASTA3 program package. *Methods Mol. Biol.* 132, 185–219.
- Presta, L.G., 2002. Engineering antibodies for therapy. *Curr. Pharm. Biotechnol.* 3, 237–256.

- Riechmann, L., Foote, J., Winter, G., 1988. Expression of an antibody Fv fragment in myeloma cells. *J. Mol. Biol.* 203, 825–828.
- Rosok, M.J., Yelton, D.E., Harris, L.J., Bajorath, J., Hellstrom, K.E., Hellstrom, I., Cruz, G.A., Kristensson, K., Lin, H., Huse, W.D., Glaser, S.M., 1996. A combinatorial library strategy for the rapid humanization of anticarcinoma BR96 Fab. *J. Biol. Chem.* 271, 22611–22618.
- Saldanha, J.W., Martin, A.C., Leger, O.J., 1999. A single backmutation in the human kIV framework of a previously unsuccessfully humanized antibody restores the binding activity and increases the secretion in cos cells. *Mol. Immunol.* 36, 709–719.
- Singer, I.I., Kawka, D.W., DeMartino, J.A., Daugherty, B.L., Elliston, K.O., Alves, K., Bush, B.L., Cameron, P.M., Cuca, G.C., Davies, P., 1993. Optimal humanization of 1B4, an anti-CD18 murine monoclonal antibody, is achieved by correct choice of human V-region framework sequences. *J. Immunol.* 150, 2844–2857.
- Stanfield, R.L., Takimoto-Kamimura, M., Rini, J.M., Profy, A.T., Wilson, I.A., 1993. Major antigen-induced domain rearrangements in an antibody. *Structure* 1, 83–93.
- Studnicka, G.M., Soares, S., Better, M., Williams, R.E., Nadell, R., Horwitz, A.H., 1994. Human-engineered monoclonal antibodies retain full specific binding activity by preserving non-CDR complementary-modulating residues. *Protein Eng.* 7, 805–814.
- Tamura, M., Milenic, D.E., Iwahashi, M., Padlan, E., Schlom, J., Kashmiri, S.V., 2000. Structural correlates of an anticarcinoma antibody: identification of specificity-determining residues (SDRs) and development of a minimally immunogenic antibody variant by retention of SDRs only. *J. Immunol.* 164, 1432–1441.
- Tan, P., Mitchell, D.A., Buss, T.N., Holmes, M.A., Anasetti, C., Foote, J., 2002. Superhumanized antibodies: reduction of immunogenic potential by complementarity-determining region grafting with human germline sequences: application to an anti-CD28. *J. Immunol.* 169, 1119–1125.
- Tempest, P.R., Bremner, P., Lambert, M., Taylor, G., Furze, J.M., Carr, F.J., Harris, W.J., 1991. Reshaping a human monoclonal antibody to inhibit human respiratory syncytial virus infection in vivo. *Biotechnology* 9, 266–271.
- Tempest, P.R., White, P., Buttle, M., Carr, F.J., Harris, W.J., 1995. Identification of framework residues required to restore antigen binding during reshaping of a monoclonal antibody against the glycoprotein gB of human cytomegalovirus. *Int. J. Biol. Macromol.* 17, 37–42.
- Tomlinson, I.M., Cox, J.P., Gherardi, E., Lesk, A.M., Chothia, C., 1995. The structural repertoire of the human V kappa domain. *EMBO J.* 14, 4628–4638.
- Tramontano, A., Chothia, C., Lesk, A.M., 1990. Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins. *J. Mol. Biol.* 215, 175–182.
- Tsodikov, O.V., Record Jr., M.T., Sergeev, Y.V., 2002. Novel computer program for fast exact calculation of accessible and molecular surface areas and average surface curvature. *J. Comput. Chem.* 23, 600–609.
- Tuomanen, E.I., Saukkonen, K., Sande, S., Cioffe, C., Wright, S.D., 1989. Reduction of inflammation, tissue damage, and mortality in bacterial meningitis in rabbits treated with monoclonal antibodies against adhesion-promoting receptors of leukocytes. *J. Exp. Med.* 170, 959–969.
- Winter, G., Harris, W.J., 1993. Humanized antibodies. *Immunol. Today* 14, 243–252.
- Wu, T.T., Kabat, E.A., 1970. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J. Exp. Med.* 132, 211–250.
- Zhu, Z., Carter, P., 1995. Identification of heavy chain residues in a humanized anti-CD3 antibody important for efficient antigen binding and T cell activation. *J. Immunol.* 155, 1903–1910.