diisopropylfluorophosphate (DEEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Sterna *et al.* (1984) *J. Neuroimmunol.* 7:27).

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound (*e.g.*, a B7-4 or PD-1 polypeptide) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the

preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (*e.g.*, peptide) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the protein can be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. 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 the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

In one embodiment of the present invention a therapeutically effective amount of an antibody to a B7-4 or PD-1 protein is administered to a subject. As defined herein, a therapeutically effective amount of antibody (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or

disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result from the results of diagnostic assays as described herein.

Monitoring the influence of agents (*e.g.*, drugs or compounds) on the expression or activity of a B7-4 or PD-1 protein can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase B7-4 or PD-1 gene expression, protein levels, or upregulate B7-4 or PD-1 activity, can be monitored in clinical trials of subjects exhibiting decreased B7-4 or PD-1 gene expression, protein levels, or downregulated B7-4 or PD-1 activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease B7-4 or PD-1 gene expression, protein levels, or downregulate B7-4 or PD-1 activity, can be monitored in clinical trials of subjects exhibiting increased B7-4 or PD-1 gene expression, protein levels, or upregulated B7-4 or PD-1 activity. In such clinical trials, the expression or activity of a B7-4 or PD-1 gene, and preferably, other genes that have been implicated in a disorder can be used as a "read out" or markers of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including B7-4 or PD-1, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) which modulates B7-4 or PD-1 activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on a B7-4 or PD-1 associated disorder, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of B7-4 or PD-1 and other genes implicated in the B7-4 or PD-1 associated disorder, respectively. The levels of gene expression (*i.e.*, a gene expression pattern) can be quantified by Northern blot analysis

or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of B7-4 or PD-1 or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state can be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a B7-4 or PD-1 protein, mRNA, or genomic DNA in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the B7-4 or PD-1 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the B7-4 or PD-1 protein, mRNA, or genomic DNA in the pre-administration sample with the B7-4 or PD-1 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of B7-4 or PD-1 to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of B7-4 or PD-1 to lower levels than detected, *i.e.* to decrease the effectiveness of the agent. According to such an embodiment, B7-4 or PD-1 expression or activity can be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference.

EXAMPLES

Example 1. Isolation of B7-4 cDNA Molecules.

The protein sequence of the extracellular domain of human B7-1 was used to search the public databases for nucleic acid molecules encoding homologous polypeptides. Two overlapping sequences in the EST database, AA292201 and AA399416, were identified. These sequences were used to isolate full-length B7-4 cDNAs from human activated keratinocyte and placental cDNA libraries as follows.

Oligonucleotides with the sequence 5'-CAGCTATGGTGGTGCCGACTACAA-3' (SEQ ID NO:5) and 5'-AGGTGCTAGGGGACAGTGTTAGACA-3' (SEQ ID NO:6) from these ESTs were synthesized. These oligonucleotides were used to prime a PCR reaction using as template cDNA prepared by reverse transcription of mRNAs from the spleen of a case of follicular lymphoma, activated B cells, INF-γ activated keratinocytes, normal spleen, and placenta. Conditions were 94°C, 1 min; 94°C, 30 sec, 56°C, 30 sec, 68°C, 1 min for 35 cycles; 68°C, 3 min, hold 4°C. All templates gave a band of the expected size of 389 bp. The 389 bp product from the PCR of INF-γ activated keratinocytes was purified by agarose gel electrophoresis and 0.12 ng was used as a template in a PCR reaction containing 0.05 mM biotin-21-dUTP and the above primers. Conditions were 94°C, 1 min; 94°C, 30 sec, 56°C, 30 sec, 68°C, 2 min for 20 cycles; 68°C, 5 min, hold 4°C. The biotinylated PCR product was purified on a Nucleospin column (Clontech) and used as a probe in the ClonCapture cDNA selection procedure (Clontech). 60 ng of denatured, biotinylated PCR product was incubated with 2 mM $CoCl_2$, 1 X RecA buffer, 1 μg of RecA protein, 1X ATP in a final volume of 30 μl. The reaction was incubated at 37°C for 15 min. To that mixture, 0.7 μg of plasmid DNA of an activated keratinocyte cDNA library and 0.4 μg of a human placental cDNA library was added and incubation continued for 20 min. 50 ng of EcoRV digested lambda DNA was added to the reaction and incubated 5 min. 0.6 μl of 10% SDS and 5.6 μg of proteinase K were added and incubated at 37°C for 10 min. Proteinase K was inactivated by adding 1 μl of 0.1 M PMSF. Streptavidin magnetic beads were preincubated with 5 μg of sheared salmon sperm DNA for 10 min and the beads

captured with a magnet, the supernatant removed, and the beads resuspended in 30 µl of binding buffer (1 mM EDTA, 1 M NaCl, 10 mM Tris-HCl, pH 7.5). The beads were added to the reaction and the reaction incubated for 30 min at room temperature with gentle mixing. The beads were captured with a magnet and the supernatant removed. The beads were washed with 1 ml of washing buffer (1 mM EDTA, 2 M NaCl, 10 mM Tris-HCl, pH 7.5), beads were captured with a magnet and the supernatant removed. The wash procedure was repeated 3 times. One ml of sterile H_2O was added to the washed beads, incubated 5 min at 37°C, beads were captured on a magnet and the supernatant removed. Captured DNA was eluted by adding 0.1 ml of elution buffer (1 mM EDTA, 0.1 N NaOH)., incubating 5 min at room temperature, beads were captured with a magnet and the supernatant removed and saved in a new tube. 22.5 µl of precipitation mix containing carrier and pH neutralizers was added along with 2.5 volumes of ethanol. The plasmid DNA was concentrated by centrifugation and re-dissolved in H_2O. Plasmid DNA was re-introduced into E. coli DH10B/P3 by electroporation and selected on LB-agar plates containing 7.5 µg/ml tetracycline and 25 µg/ml ampicillin. Colonies were lifted onto Nytran filters and hybridized with ^{32}P-labeled oligonucleotides with the sequence 5'-CAGCTATGGTGGTGCCGACTACAA-3' (SEQ ID NO:7), 5'-AGGTGCTAGGGGACAGTGTTAGACA-3' (SEQ ID NO:8) , and 5'-TCGCTTGTAGTCGGCACCACCATA-3' (SEQ ID NO:9). All oligos are from AA292201 sequence. Final wash conditions were 2 X SSC, 0.1 % SDS at 55° for 20 min. The two hybridizing colonies were picked and the sequence of the cDNA inserts was determined.

Sequencing revealed two forms of B7-4 molecules. The first form, B7-4 secreted (B7-4S) encodes a protein having a short hydrophilic domain without a membrane anchor. The nucleotide and amino acid sequences of this form are shown in SEQ ID NO:1 and 2, respectively. The second form, B7-4 membrane (B7-4M) encodes a protein having a transmembrane and short cytoplasmic domain. The nucleotide and amino acid sequences of this form are shown in SEQ ID NO:3 and 4, respectively. Both members of the B7-4 family identified have signal, IgV, and IgC domains, as illustrated in Figures 3 and 4. The B7-4M form has approximately 21% amino acid identity to

human B7-1 and about 20% amino acid identity to human B7-2 as calculated using the default Blosum62 matrix with gap penalties set at existence 11 and extension 1 (See http://www.ncbi.nlm.nih.gov), under conditions where B7-1 and B7-2 have about 26% identity.

Example 2. Expression of B7-4 mRNA: Northern Blot Analysis

An mRNA of the soluble form of B7-4 is predicted to be about 1.2kb though other sizes are possible. The mRNA of the second form is about 3.8 kb, with minor mRNAs of 1.6 and 6.5 kb.

Expression of B7-4 polypeptides was analyzed. RNA was prepared by guanidine thiocyanate homogenization and cesium chloride centrifugation. Equal amounts of RNA (approximately 2 μg poly(A)+ RNA) were electrophoresed on an agarose gel, blotted, and hybridized to a portion of ^{32}P-labeled B7-4 cDNA common to both the B7-4S and B7-4 M forms. These B7-4 mRNAs are highly expressed in placenta, lung, and heart and are moderately expressed in the thymus. In addition, these B7-4 mRNAs are weakly expressed in skeletal muscle, kidney, pancreas, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. They were also found to be very weakly expressed in liver or brain. B7-4 mRNAs were not expressed in unstimulated monocytes, but were strongly induced by IFN-γ. Similarly, the expression of these polypeptides was found to be induced in keratinocytes by TPA/IFN- γ and in dendritic cells by IFN- γ. These B7-4 mRNAs were not expressed in unstimulated B cells, but were induced by Ig crosslinking.

Expression of these B7-4 mRNAs was also examined in a variety of cell lines. They were not found to be expressed in B cell lines such as Raji, Ramos, LBL, Nalm 6, and DHL-4. They were also not expressed in T cell lines, such as Jurkat, Rex, CEM, HPB-ALL, Peer4, and H9 or in HTLV-1 transformed T cell lines such as SPP and MT2 or in the myeloid line U937.

Example 3. Further Characterization of B7-4 mRNA Expression: Northern Blot Analysis

Mouse and human multiple tissue Northern blots (Clontech, Palo Alto, CA) were probed with ^{32}P-dCTP radiolabeled cDNA probes in QuikHyb (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The human B7-4 probe consisted of a 1 kb BamHI/NotI fragment of the cDNA spanning the coding region and 3' untranslated region of SEQ ID NO:1. The mouse B7-4 probe consisted of a 300 bp cDNA fragment from the coding region. Control actin probes were supplied by Clontech. Blots were washed twice at room temperature in 2X SSC, 0.1% SDS, followed by 0.2X SSC, 0.1% SDS at 65°C, and examined by autoradiography.

B7-4 mRNA was expressed at high levels in heart, human placenta, and human fetal liver, and at lower levels in spleen, lymph nodes, thymus, and mouse liver.

B7-4 mRNA was expressed in a variety of transformed mouse cell lines, including PU5-1.8, RAW 264.7, K-Balb, M-MSV-Balb/3T3, Hepa 1-6, R1.1, L1210, P38D1, P815, and NB41A3 cells.

Example 4. Further Characterization of B7-4 mRNA Expression: Quantitative PCR, Genechip Hybridization, and RNA Blot Analysis

B7-4 mRNA expression on antigen presenting cells was examined and compared to the expression of B7-1 and B7-2 on those cells. For quantitative PCR analysis, cellular RNA was deoxyribonuclease-treated, re-extracted and converted to first strand cDNA. FAM (6-caroxyfluorescein)-labeled human B7-4, B7-1, B7-2, and GAPDH probes were purchased from PE Biosystems (B7-4: primers 5'-GCCGAAGTCATCTGGACAAG-3' (SEQ ID NO:13) and 5'-TCTCAGTGTGCTGGTCACAT-3' (SEQ ID NO:14), probe 5'-FAM-CACCACCACCAATTCCAAGA-3' (SEQ ID NO:15); B7-1: primers 5'-ACGTGACCAAGGAAGTGAAAGAA-3' (SEQ ID NO:16) and 5'-TGCCAGCTCTTCAACAGAAACAT-3' (SEQ ID NO:17), probe 5'-FAM-TGGCAACGCTGTCCTGTGGTCAC-3' (SEQ ID NO:18); B7-2: primers 5'-GGGCCGCACAAGTTTTGAT-3' (SEQ ID NO:19) and 5'-GCCCTTGTCCTTGATCTGAAGA-3' (SEQ ID NO:20), probe 5'-FAM-CGGACAGTTGGACCCTGAGACTTCACA-3' (SEQ ID NO:21).

PCR reactions were set up in 96-well plates using reagents from the Perkin Elmer TaqMan™ EZ kit, according to the manufacturer's instructions. Standard curves were set up for each of the four genes analyzed. Forty cycles of PCR were run in an ABI Prism 7700 Sequence Detector and GAPDH was used to normalize the B7-4, B7-1, and B7-2 results.

The Affymetrix Mu19KsubA chip was used for Genechip hybridization analysis. The sequence of a portion of murine B7-4 is represented by expressed sequence tag TC17781 of The Institute for Genomic Research on this chip. RNA isolation, chip hybridization and scanning was performed as described in Byrne, M. C. *et al.* (2000) Curr. Prot. Mol. Biol. Suppl. 49:22.2.1-22.2.13.

For RNA blot hybridization, the 1.6 kb human and 3.6 kb murine B7-4 cDNAs were excised by digestion with Xba I and labeled by random priming with γ-^{32}P-ATP and the Klenow fragment of DNA polymerase I. RNA blots were hybridized as described in Freeman, G. J. *et al.* (1992) J. Immunol. 149:3795-3801.

Human dendritic cells were derived from peripheral blood. Mononuclear cells were isolated after fractionation on a Ficoll gradient. Non-adherent cells were removed and the remaining cells cultured in 150 ng/ml human GM-CSF (R&D Systems) and 100 ng/ml human IL-4 (R&D Systems) for 2 days. The non-adherent dendritic cells were isolated ($CD80^+$ $CD86^+$ HLA-DR^+ $CD54^+$ $CD58^+$ $CD1a^+$) and cultured in GM-CSF alone or activated with GM-CSF, 2.5 μg/ml LPS (Sigma Chemicals), and 10 ng/ml human Interferon-γ. At 4 hours and 20 hours after activation, cells were harvested and RNA isolated using the RNeasy kit (Qiagen).

Murine bone marrow mononuclear cells were immuno-depleted of granulocytes, lymphocytes and Ia^+ cells by magnetic activated cell sorting and cultured in petri dishes with GM-CSF and IL-4. Dendritic cells were harvested as the non-adherent population after 7 days of culture, and demonstrated to be 75-80% $CD11c^+$, high IA^+ cells. Cells were activated with LPS and human interferon-γ.

Analysis of expression in human blood monocytes by RNA blot hybridization demonstrated that B7-2 is not expressed by unstimulated monocytes, but is rapidly upregulated upon interferon-γ treatment. Treatment of monocytes with another pro-

inflammatory cytokine, tumor necrosis factor (TNF)-α led to a low level induction similar to that found with medium alone, presumably as a result of activation by adherence to plastic. In addition to the major 4.2 kb B7-4 mRNA, a minor 1.8 kb B7-4 mRNA species was also observed in interferon-γ treated monocytes. Expression of B7-4 by human B-cells activated by cell surface immunoglobulin cross-linking, but not by the Raji cell line, was also observed. Similarly, B7-1 is not expressed by unstimulated monocytes, but is upregulated in response to interferon-γ with kinetics similar to B7-4 expression. In contrast, B7-2 mRNA is constitutively expressed in monocytes and levels are unaffected by interferon-γ or TNF-α treatment.

B7-4, B7-1, and B7-2 mRNA expression by human dendritic cells was also examined by quantitative PCR. Human peripheral blood-derived dendritic cells were treated with granulocyte-macrophage colony stimulated factor (GM-CSF) alone or activated with GM-CSF, lipopolysaccharide (LPS), and interferon-γ. As a result of activation by LPS and interferon-γ, B7-4 mRNA was rapidly induced with a 16-fold increase at 4 hours and a 34-fold increase at 20 hours, relative to non-induced cells. B7-1 and B7-2 mRNAs were also induced upon activation: B7-1 was induced 21-fold at 4 hours and 22-fold at 20 hours. B7-2 showed little induction at 4 hours; however, expression was induced 5-fold at 20 hours. Expression of B7-4 by murine bone marrow-derived dendritic cells treated with LPS and interferon-γ was examined using Genechip™ hybridization. B7-4 expression in these cells follows a pattern similar to that observed on human dendritic cells: a 5-fold induction of the B7-4 mRNA relative to the uninduced cells at 6 and 20 hours after induction. These data demonstrate that B7-4 is expressed by antigen presenting cells and lymphocytes, and it is induced on dendritic cells in a manner similar to B7-1 and B7-2. Treatment of human keratinocytes with phorbol ester and interferon-γ also induced B7-4.

In murine tissues, an approximately 3.7kb B7-4 mRNA transcript was detected by northern blot hybridization. The distribution of the murine B7-4 mRNA closely resembled that of the human B7-4, with high levels in heart, thymus and lung, and low levels in kidney, spleen and liver.

Example 5. Chromosomal Localization of B7-4

The chromosomal localization of the B7-4 genes was determined using a monochromosomal blot kit commercially available from Quantum (Toronto, Canada). The blots were probed with a sequence that recognizes both B7-4S and B7-4M. Using this method, the B7-4 polypeptides have been localized to human chromosome 9, whereas B7-1 and B7-2 have been localized to human chromosome 3. The butyrophilins, which also share limited amino acid sequence identity with the B7-4 family have been localized to the major histocompatability complex on chromosome 6. The chromosomal location of B7-4 was confirmed using B7-4 specific primers in PCR amplification of monochromosomal somatic cell hybrid DNA templates available from Quantum Technologies (Canada).

Example 6. Binding of B7-4 Molecules to T Cell Ligands or Antibodies.

COS cells were transfected with either vector DNA (pcDNAI), or an expression plasmid containing the B7-4M cDNA. After 72 hours, the transfected COS cells were detached by incubation in PBS containing 0.5 mM EDTA for 30 min. at 37°C.

The ability of COS cells expressing B7-4M to bind to various T cell receptors and antibodies was tested. FACS analysis of binding of CD28Ig, CTLA4-Ig, and control Ig by B7-4-transfected COS cells showed that neither CD28Ig nor CTLA4-Ig was bound by B7-4 (Figure 8). The ability of COS cells expressing B7-4M to bind to IgG and murine ICOS-his fusion protein was also tested. No binding of human B7-4 to murine ICOS was detected (Figure 9). As shown in Figure 10, FACS analysis revealed binding of BB1 (anti B7-1 and anti B7-3), but not IgM or 133 (anti-B7) antibodies to B7-4-transfected COS cells.

Example 7. Costimulation of T Cell Proliferation By B7-4 molecules

The ability of B7-4 polypeptides to costimulate human T cell proliferation was tested.

Human $CD28^+$ T cells were isolated by immunomagnetic bead depletion using monoclonal antibodies directed against B cells, natural killer cells and macrophages as previously described (Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci. USA* 90, 6586-6590). B7-4 and vector transfected COS cells were harvested 72 hours after transfection, incubated with 25μg/ml of mitomycin-C for 1 hour, and then extensively washed. 10^5 naïve T Cells were stimulated with plate bound anti-CD3 mAb plus 20,000 mitomycin-c treated COS cells transfected with the indicated DNA construct.

T cell proliferation was measured by 3H-thymidine (1 μCi) incorporated for the last 12 hours of a 72 hour incubation. As shown in Figures 11 and 12, COS cells expressing B7-4 can costimulate T cell proliferation.

Example 8. Generation of Murine Antibodies to B7-4

Mammalian expression vectors (pEF6 or pcDNA3.1 (Invitrogen)) were prepared comprising the entire murine or human B7-4 cDNA. The cDNA/vector construct was dissolved in 0.9% saline at 1 mg/ml (not TE or PBS).

Before immunization, 78 μl of 1 mg/ml cardiotoxin (Sigma #C-1777) in 0.9% saline was injected into the tibialis anterior muscle of each hind limb of the mouse being immunized. Each mouse was then left alone for 5 days.

After anesthetizing the mice, 50 μl of 1 mg/ml purified B7-4 cDNA/vector construct (in 0.9% saline) was injected into each regenerating tibialis anterior muscle.

Antibody titers were measured approximately six days after immunization using standard methods, for example, in an ELISA assay. The cDNA immunization was repeated every 2-4 weeks for three cycles (until the antibody titre was >1:10,000). Mice were then boosted with CHO cells transfected with PDL-1.

Spleen cells isolated from mice having appropriate antibody titers were harvested. The spleen cells were fused to fusion partners SP2-0) to make hybridomas. Hybridomas and antibodies were manipulated using standard methods (see, e.g., "Antibodies: A Laboratory Manual", Harlow, E. and Lane, D., Cold Spring Harbor Laboratory (1988), which is incorporated herein by reference).

Antibodies 2A3, 10D9, 5A9, and 11D12 were among those selected in screening assays. These antibodies were found to bind to COS or CHO cells transfected with human B7-4 and not to mock transfected cells or to cells transfected with mouse B7-4. The antibodies were used to detect the presence of B7-4 on various cell populations. B7-4 expression was observed, inter alia, on heart tissue, tumor cells (including some lung tumor cells, some ovarian tumor cells, some breast tumor cells, some epithelial tumor cells, and some squamous cell carcinomas), placenta, and thymic epithelium.

Example 9. Generation of Fully Human Antibodies to B7-4

In this example, fully human antibodies against B7-4 of PD-1 are made in mice that are transgenic for human immunoglobulin genes. Transgenic mice are made using standard methods, *e.g.*, according to Hogan, *et al.*, "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory, which is incorporated herein by reference, or are purchased commercially. Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and embryonic stem cells: a practical approach, Robertson, E. J. ed., IRL Press, Washington, D.C., 1987; Zjilstra *et al.* (1989) *Nature* 342:435-438; and Schwartzberg *et al.* (1989) *Science* 246:799-803, each of which is incorporated herein by reference). DNA cloning procedures are carried out according to Sambrook, J. *et al.* in Molecular Cloning: A Laboratory Manual, 2d ed., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference. Oligonucleotides are synthesized, *e.g.*, on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer or are purchased commercially.

Transgenic mice are immunized using a purified or recombinant B7-4 or PD-1 or a fusion protein comprising at least an immunogenic portion of the extracellular domain of B7-4 or PD-1. Approximately four hundred μg of B7-4 or PD-1 in 100 μL of phosphate buffered saline (PBS) is injected intraperitoneally into each mouse. Serum samples are collected approximately six days later by retro-orbital sinus bleeding.

Antibody reactivity and specificity for B7-4 or PD-1 are assessed using an indirect enzyme-linked immunosorbent assay (ELISA). Several immunoglobulin superfamily molecules are tested as controls (*e.g.*, CTLA4 and CD28) to analyze the

antibody specificity of the antibody for B7-4 or PD-1. Antibodies having human variable regions which bind to B7-4 or PD-1 are detected by enzyme conjugates specific for human IgM and human IgG sub-classes with no cross reactivity to mouse immunoglobulin. Briefly, PVC microtiter plates are coated with B7-4 or PD-1 by coating wells overnight at 37°C with 5 μg/mL B7-4 in PBS. Serum samples are diluted in PBS, 5% serum, 0.5% Tween-20 and are incubated in the wells for 1 hour at room temperature, followed by anti-human IgG Fc and IgG F(ab')-horseradish peroxidase or anti-human IgM Fc-horseradish peroxidase in the same diluent. After 1hour at room temperature enzyme activity is assessed by addition of ABTS substrate (Sigma, St. Louis, Mo.) and read after 30 minutes at 415-490 nm. In pre-immunization serum samples from the same mice, titers of human antibodies to the same target antigens are also tested.

Spleen cells isolated from mice having appropriate antibody titers are harvested. The spleen cells are fused to appropriate fusion partners (*e.g.*, myeloma cells) to make hybridomas. Hybridomas and antibodies are manipulated according to "Antibodies: A Laboratory Manual", Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), which is incorporated herein by reference.

The complementarity determining sequences of the murine VH and VL domains of a murine antibody could be used to graft into the framework of human immunoglobulins in order to generate a humanized antibody against B7-4 or PD-1 (Riechmann et al. 1988. Nature 332:323; Verhoeyen et al. 1988. Science 239:1534).

Example 10. Generation of Human Single Chain Fvs Reactive with B7-4 or PD-1

As an alternative to preparing monoclonal antibody-secreting hybridomas, anti B7-4 or anti-PD-1 antibodies (single chain Fv-like portions of antibodies) were identified and isolated by screening a combinatorial library of human immunoglobulin sequences displayed on M13 bacteriophage from Cambridge Antibody Technology. Ltd., Melbourn, UK (Winter et al. 1994 Annu. Rev. Immunol. 1994 12:433; Hoogenboom et al., 1998, Immunotechnology 4: 1) PD-1.Fc or B7-4.Fc was used to thereby isolate immunoglobulin library members that bind a B7-4 or PD-1 polypeptide. Kits for generating and screening phage display libraries are commercially available and

standard methods were employed to generate the scFv (Helfrich et al. J. Immunol Methods 2000. 237: 131-45; Cardoso et al. Scand J. Immunol 2000. 51: 337-44.)PD-1.Fc or B7-4.Fc were immobilized on plastic and phage expressing specific scFv were selected by panning and multiple rounds of enrichment (Griffiths et al. 1993 EMBO J. 12:725).

Example 11. Identification of a receptor for B7-4

Fusion proteins consisting of the extracellular region of human PD-1 fused to the hinge-CH2-CH3 domains of either human immunoglobulin gamma 1 or murine Ig gamma2a (with mutations blocking FcR and complement interaction) were used to search for a ligand that binds to PD-1. As part of this search, staining of the cell surface of monocytes stimulated with gamma-interferon was found. B7-4 is induced in monocytes after stimulation with gamma-interferon, as observed by northern blot hybridization.

The binding of PD-1-Fc (human Ig gamma1) to the surface of COS cells transiently transfected with a B7-4-expression vector was tested. COS cells were transfected with either B7-4M or B7-1 using LipofectAMINE transfection reagent. After 48 hours, the cells were stained with human PD-1-Fc, murine PD-1-Fc, CTLA4-Fc, Flt4-Fc, or IgG followed by anti-IgG conjugated to phycoerythrin (PE). The cells were then analyzed by flow cytometry. As shown in Figure 13, COS cells expressing B7-4 bound both human PD-1-Fc and murine PD-1-Fc, but did not bind CTLA4-Fc, Flt4-Fc, or human IgG. As a positive control, it was demonstrated that B7-1 expressing COS cells bound CTLA4-Fc, but not PD-1-Fc or IgG.

In addition, an in situ assay of transfected COS cell monolayers was performed. Monolayers were probed with PD-1Fc, CTLA4Fc or human IgG1 and binding was detected with a secondary antibody directed against the Fc portion and conjugated to alkaline phosphatase. Binding was visualized with chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium and light microscopy. In parallel, cells transfected with B7-4 were found to bind to PD-1-Fc, and not CTLA4-Fc (human Ig gamma 1) or Flt4-Fc, the extracellular region of murine Flt4 linked to human Ig

gamma 1. In parallel, PD-1Fc did not bind the surface of mock-transfected, B7-1 or B7-2 transfected COS cells.

In another experiment, no binding of PD-1-Fc to soluble forms of B7-1 or B7-2 and binding to B7-4 was detected using a BIACORE-based assay. In parallel, hCTLA4 was shown to bind to B7-1 and not to B7-4. PD-1-Fc or CTLA4-Fc was immobilized and conditions were essentially as described by Fitz *et al.* (1997) *Oncogene* 15:613). Concentrated COS cell medium from cells that had been transfected with full length B7-4M or B7-4-Fc was injected and interactions were measured using real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705). Human B7-4 was found to bind human and mouse PD-1 and this binding was inhibited by competition with a coinjected PD-1-Fc, but not CTLA4-Fc. These experiments demonstrate not only the binding of soluble B7-4-Fc fusion protein to immobilized PD-1-Fc, but also demonstrate the presence of a soluble form of B7-4 in the conditioned medium of B7-4M cDNA transfected cells, presumably as a result of shedding.

Figure 14 illustrates the ability of PD-1 and not Flt4 (the receptor for vascular endothelial growth factor C) to competitively inhibit the binding of PD-1 to B7-4. The binding of human PD-1 gamma 2a fusion protein to COS cells expressing B7-4M is shown in Panel A. The binding was detected with anti-gamma 2a specific reagents linked to PE. Human PD-1 linked to IgG1 was added at: 50 μg/ml, 6.25 μg/ml, 100 μg/ml, or 25 μg/ml and was found to compete for binding. As a control, Flt4IgG1 at 100 μg/ml was not found to compete for binding of PD-1 to B7-4.

In yet another experiment, the ability of B7-4 to bind to PD-1 was determined by flow cytometry and BIACORE-binding assays. Human and murine PD-1.Ig fusion proteins bound to both human and murine B7-4 expressed on CHO cells, as detected by flow cytometry (Figure 15). However, neither human CTLA-4.Ig, human CD28.Ig, nor human ICOS.Ig bound to either B7-4 expressing cell line. The PD-1 fusion proteins did not bind CHO cells transfected with vector alone. Further confirmation of the PD-1:B7-4 interaction was obtained using surface plasmon resonance with a BIACORE instrument. The human and murine PD-1.Ig proteins and human CTLA-4.Ig were

immobilized on the flow cell surfaces of a dextran chip and tested for binding to soluble human B7-4.Ig. B7-4.Ig bound to both human and murine PD-1.Ig, but not to human CTLA-4.Ig (Figure 16). This binding was blocked by competition with co-injected soluble PD-1.Ig, but not CTLA-4.Ig. Soluble forms of human B7-1 and B7-2 did not bind immobilized human PD-1.

These data demonstrate that PD-1 binds B7-4, and that this interaction may regulate the action of PD-1.

Example 12. B7-4 Can Transmit a Negative Signal to Immune Cells

In this example, $5x10^5$ Jurkat T cells per well were stimulated with anti-CD3 coated beads (at a 1:1 ratio) and soluble anti-CD28. COS cells expressing B7-4 or a negative control, called EZZ, were titrated into the wells. Supernatants were harvested at 48 hours and assayed by ELISA for human IL-2. Figure 17 shows that increasing numbers of COS B7-4 cells (bars on the right in the figure) lead to a decrease in IL-2 production.

Using similar assay formats, for example in which human PHA-blasts from PBMCs were stimulated with immobilized anti-CD3 and soluble anti-CD28, a decrease in T-cell proliferation was observed by titrating in COS cells expressing B7-4.

Example 13. The PD-1:B7-4 Interaction Inhibits CD3-mediated T-Cell Proliferation

To examine the functional significance of the PD-1:B7-4 interaction, the functional consequences of B7-4 interaction with its receptor were also examined using human T-cells. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation. $CD4^+$ T cell populations (85-90% purity) were purified by negative selection using a cocktail of monoclonal antibodies and immunomagnetic beads (PerseptiveBiosystems). Anti-CD3, control IgG and fusion protein were covalently attached to polyurethane-coated tosyl activated Dynabeads (Dynal) according to manufacturer's instructions and as described previously (Blair, P. J. *et al.* (1998) *J. Immunol.* 160:12-15). Anti-CD3 antibody (UCHT1, Pharmingen) at the indicated concentration was added to 1 x 10^7 beads/ml 0.1 M phosphate buffer pH 7.4. Control IgG was added to the bead suspension in order to maintain a constant total Ig

concentration of 5μg/ml during binding. Similarly, anti-CD3/B7-4.Ig(γ2a) beads were prepared with the indicated anti-CD3 antibody concentration, a constant concentration of either B7-4.Ig representing 40% of the total bound protein ($2\mu g/10^7$ beads), and control IgG to make up the remaining total bound protein. 10^5 T cells were cultured in 96 well flat-bottom plates, and beads were added at a 1 bead to 1 cell ratio in the presence or absence of the indicated concentrations of anti-CD28 antibody (CD28.2, Pharmingen). Proliferation was determined by labeling cultures for the last 6 hr of a 4-day assay with 1 μCi ^{3}H-thymidine/well. For analysis by cytokine ELISAs, cultures were set up as described above and supernatants harvested at the indicated times. Interferon-γ, IL-10 and IL-2 concentrations were determined using commercially available ELISA kits (Genzyme).

Purified $CD4^+$ T-cells obtained from peripheral blood mononuclear cells (PBMC) were activated with beads coated with anti-CD3 mAb and either human B7-4.Ig or a control Ig. Proliferation and cytokine production was assessed 96 hours after stimulation. As shown in Figure 18, cells activated with anti-CD3 mAb/B7-4.Ig coated beads showed a 69% decrease in proliferation relative to anti-CD3 mAb/control Ig activated cells. Furthermore, activation of cells in the presence of B7-4 also impaired cytokine secretion. In the presence of B7-4, interferon-γ and IL-10 secretions were decreased by approximately 80% and 60% , respectively (Figure 18). IL-2 production was below detection under these activation conditions at both 24 and 96 hr. However, under conditions in which costimulation in the form of soluble anti-CD28 was provided, activation of cells in the presence of B7-4 also led to a decrease in IL-2 production. Thus, activation of murine and human T-cells in the presence of B7-4 leads to inhibition of both proliferation and cytokine secretion.

Example 14. The Outcome of PD-1:B7-4 Interaction Depends on the Strength of T-cell Receptor and CD28 Signals

To examine the relationship between T-cell receptor, CD28 and PD-1 mediated signals, human $CD4^+$ T-cells were stimulated with suboptimal or optimal concentrations of anti-CD3 mAb, a fixed concentration of B7-4.Ig and increasing concentrations of

soluble anti-CD28 mAb. Using anti-CD3 mAb-coated beads, the concentrations required for suboptimal and optimal T-cell stimulation were established. Under conditions of suboptimal T-cell receptor engagement (anti-CD3 mAb at 1μg/ml), minimal proliferation was observed in the absence of costimulation (Figure 19A). Addition of increasing concentrations of soluble anti-CD28 mAb led to an up to 30-fold increase in proliferation. Under these conditions, activation of T cells in the presence of B7-4 resulted in an 80% reduction in proliferation (Figure 19A). A maximal level of costimulation (anti-Cd28 at 250 ng/ml) was required to rescue the inhibition of proliferation mediated by B7-4 stimulation. In contrast, under saturating conditions of T-cell receptor activation (anti-CD3 mAb at 2μg/ml), B7-4 mediated inhibition of T-cell proliferation was only observed in the absence of CD28 costimulation (Figure 19B).

Example 15. Ability of B7-4 To Inhibit CD28 Signals and Cytokine Production

The inhibitory effects of the PD-1:B7-4 pathway appear to be determined by the strength of signal through the TCR and CD28 (see previous example), whereby weak CD3/CD28-mediated responses are easily downregulated. To study the interaction of the CD28 signal and the PD-1:B7-4 pathway, pre-activated DO11.10 $CD4^+$ T cells were activated with OVA peptide presented by CHO-IA^d/B7.2 or CHO-IA^d/B7.2/B7-4.

For detection of B7-4, 5 x 10^4 CHO transfectants cells were incubated with 5μg/ml of human PD-1Ig (hPD-1-Ig) (Genetics Institute, Cambridge, MA) and developed with goat anti-murine IgG2a-phycoerythrin (PE) (Southern Biotechnology Associates Inc., Birmingham, AL). In addition, cells were stained separately with 5μg/ml anti-IA^d-PE or B7.2-PE (Pharmingen, San Diego, CA). Following each step, cells were washed three times with PBS/1% BSA/0.02% sodium azide. After the final incubation, cells were fixed with 1% paraformaldehyde. Ten thousand events were analyzed on a FACSCalibar (Becton Dickinson, Mountain View, CA). All isotype controls were all obtained from Pharmingen.

Splenocytes were prepared from DO11.10 mice and treated with Tris-NH_4Cl to deplete erythrocytes. Cells were cultured with 1μg/ml of OVA peptide for 72 hours (Analytical Biotechnology Services, Boston, MA) in RPMI 1640 (Life Technologies, Grand Island, New York) supplemented with 10% FCS (Sigma, St

Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 10 mM HEPES, 50 μM 2-ME (all from Life Technologies) and 15mg/ml of gentamicin (BioWhittaker, Walkersville, MD). $CD4^+$ T cells were purified by positive selection using magnetic-activated cell sorting separation columns (Miltenyi Biotec, Auburn, CA) with resulting purity of >98%. Cells were rested overnight before re-stimulation.

Proliferation of CHO cells was inhibited by incubation with 50 μg/ml of mitomycin C (Bristol Laboratories, Princeton, NJ) for 16 hours at 37°C. At the end of the incubation period, the cells were harvested with 10 mM EDTA in PBS, washed twice and left on ice for 1 hour. The cells were subsequently washed three times and resuspended in culture medium. 10^5 pre-activated $CD4^+$ T cells were cultured with varying concentrations of OVA peptide and 10^4 mitomycin C-treated CHO transfectants in 96 well plates. To assay proliferation, cultures were incubated for 48 hrs and pulsed with 1μCi/well of [^{3}H] thymidine (New England Nuclear, Boston, MA) for the last 6 hours of the incubation period.

The expression of B7 and IA^d was similar on all CHO transfectants (Figure 20). As expected, introduction of B7.2 led to an increase in proliferative responses by T cells at all antigen concentrations (Figure 21). However, B7-4 inhibited responses at lower peptide concentrations (0.01μg/ml and 0.001μg/ml) (Figure 21).

To address the capacity of PD-1:B7-4 pathway to inhibit cytokine production, supernatants from DO11.10 $CD4^+$T cells activated with OVA peptide presented by CHO cell transfectants were analyzed. Aliquots of supernatants were harvested at various times after initiation of cultures. IL-2, IL-4, IFN-γ and IL-10 levels were analyzed using mAbs and recombinant cytokine standards from Pharmingen. Detection limits were as follows: IL-2, 20pg/ml, IL-4, 40pg/ml. IFN-γ, 100pg/ml and IL-10, 200pg/ml. Production of IL-2, IL-4, IFN-γ and IL-10 was inhibited significantly when DO11.10 $CD4^+$T cells were cultured with 0.1 μg/ml peptide and B7-4 (Figure 22). At this concentration there was only a weak inhibition of proliferation. However B7-4 significantly inhibited cytokine production at 0.01μg/ml peptide, consistent with the inhibition of proliferation (Figure 23). IL-10 was not detected under these activation

conditions. Therefore, PD-1 engagement by B7-4 can downregulate cytokine production even when T cell proliferation is not affected.

To determine whether the diminished cytokine production was due to reduced mRNA levels, and RNase protection assay was utilized. $CD4^+$ T cells were restimulated with various CHO cell transfectants and 0.01μg/ml OVA peptide. After 48 hours, cells were harvested and mRNA was isolated using TRIzol® reagent (Life Technologies). 5μg mRNA was analyzed for cytokine levels by RNase protection assay using RiboQuant multiprobe kit mCK1 according to the manufacturer's instructions (Pharmingen). Transcript levels of IL-4, IL-10, IL-13, IL-2, IL6 and IFN-γ mRNA were detected in pre-activated DO11-10 $CD4^+$T cells after stimulation with 0.01μg/ml OVA peptide presented by CHO-IA^d/B7.2. However, the introduction of B7-4 significantly reduced cytokine mRNA levels. There was minimal upregulation of mRNA for cytokines in unstimulated T cell cultures or T cells activated with peptide presented by CHO-IA^d. These results further demonstrate the capacity of the PD-1:B7-4 pathway to antagonize a strong B7/CD28 signal at least when antigenic stimulation is weak or limiting, and the inhibition of at least cytokine production in conditions of strong antigenic stimulation.

Example 16. Mechanism of action of the PD-1:B7-4 pathway

Cross-linking of CTLA-4 has been shown to inhibit cell cycle progression in naïve T cells (Krummel, M. F. and Allison, J. P. (1996) *J. Exp. Med.* 183:2533-2540; Walunas, T. L. *et al.* (1996) *J. Exp. Med.* 183:2541-2550). As PD-1 was isolated from murine cell lines undergoing apoptosis, a possible mechanism of action of the PD-1:B7-4 pathway might be to increase programmed cell death. To address this issue, DO11.10 $CD4^+$ T cells were restimulated with 0.01μg/ml peptide and various CHO transfectants and cell cycle progression was analyzed. $CD4^+$ T cells were restimulated with 0.01μg/ml peptide as described previously. After 36 hours of culture, cells were recovered and stained with anti CD4-FITC. Cells were washed in PBS, fixed in 70% ethanol for 1 hour on ice and then resuspended in PBS containing 10 μg/ml RNase (Sigma) and 50 μg/ml propidium iodide (Sigma). Analysis was performed within an hour of staining.

After 48 hours, cells were recovered and stained with CD4-FITC. After permeabilization, cells were incubated with propidium iodide to analyze the G_0/G_1, S/G_2 and sub-diploid populations. $CD4^+$ T cells restimulated with peptide presented by CHO-IA^d have a large proportion of cells in the sub-diploid population, indicative of apoptosis (Figure 24). In cultures where $CD4^+$ T cells were stimulated by peptide presented by CHO-IA^d/B7-2, there were increased number of cells in the S/G_2 phase, and a decreased number in the sub-diploid population, indicating that cells were in cycle and rescued from apoptosis by B7/CD28 costimulation. The introduction of B7-4 led to an increased number of cells in the G0/G1 phase (Figure 24). There were comparable levels of apoptosis in the B7-4 cultures to the CHO-IA^d/B7 cultures. This was confirmed by annexin staining. The inhibition of cell progression by the PD-1:B7-4 pathway confirms its role in downregulating T cell activation.

Example 17. Inhibition of binding of biotinylated human B7-4 Fc to human PD-1Fc

Fc fusion proteins were generated by linking the extracellular region of PD-1 or B7-4 to the hinge-CH2-CH3 domains of murine Igγ2a. recombinant proteins were produced in COS cells transiently transfected with LipofectAMINE (Gibco-BRL) or stably transfected CHO cell lines and purified from conditioned media using protein A-Sepharose.

The ability of antibodies to B7-4 or PD-1 to inhibit the interaction of human B7-4Fc and human PD-1 Fc was tested using standard ELISA methods. Briefly, human PD-1Fc molecules were immobilized in 96-well plates, blocked, and washed. Biotinylated B7-4Fc molecules (100ng/ml) were added to wells at concentrations of approximately 2000, 700, 200, 70, 25, 8, and 1.18 ng/ml (Figure 25). The wells were incubated with StrepAvidin conjugated horse radish peroxidase, washed, and color was developed using standard methods. The ED50 of B7-4Fc was found to be 108ng/ml.

The ability of murine antibodies to human B7-4 (10D9 and 11D12) or scFv portions of human immunoglobulins (B7-4-1, B7-4-6, and B7-4-12) to inhibit the binding of biotinylated human B7-4Fc to human PD-1Fc was tested at 7 concentrations of inhibitors. The IC50 was found to range from 0.5 nM to 24 nM and the data are presented in Figure 25.

The PD-1 specific scFv were also tested for their ability to inhibit the binding of B7-4 Fc to PD-1Fc using the same ELISA methods described above. Human scFv reactive with PD-1 (PD1-17 scFv) were found to inhibit specific binding (EC50 between 10^{-7} and 10^{-8}) as shown in Figure 26. V_L and V_H domains of the PD1-17scFv were used to generate a complete IgG. In brief, the VH and VL coding regions were linked to genomic CH and CL gene sequences in expression vectors. The resulting expression vectors were transiently transfected into human 293 cells and the IgG harvested from the conditioned medium. The potency of the grafted whole IgG molecule was higher than for the scFv antibody (EC 50 between 10^{-8}M and 10^{-9}M).

Example 18. Administration of Soluble B7-4Fc Exacerbates Disease in a Murine Model.

To determine if modulation of the B7-4/PD-1 pathway has immunoregulatory activity in vivo, the protein was evaluated in a murine model of experimental autoimmune encephalomyelitis (EAE) that shares many clinical and pathological features with the human disease multiple sclerosis. Female SJL/J mice were immunized with 100μg of proteolipid protein (PLP) in complete Freund's adjuvant. Ten days later, spleens were harvested, processed to single cell suspensions and then restimulated in vitro with 5μg of PLP for 96 hours. Cells were washed three times in PBS and then $15x10^6$ cells transferred to naive SJL/J mice by intraperitoneal injection. The adoptive transfer of autoreactive T cells results in acute paralysis of recipient mice which manifests as loss of tail tone with subsequent progression to full hind limb paralysis. This paralytic episode coincides with marked infiltration of activated T cells and macrophages in the CNS. Under most conditions, this is an acute model of disease with spontaneous recovery occurring after a short period of paralysis. For evaluation of B7-4Fc, mice were injected subcutaneously with 200μg of the protein in 100μl of sterile saline on days 0, 2, 4, 7 and 11 after cell transfer (n=10). Control mice (n=10) received an equal volume of saline only. All animals were monitored regularly for clinical signs of disease which were scored as follows: 1. Loss of tail tone; 2. Hind limb weakness/partial hind limb paralysis; 3. Complete hind limb paralysis; 4. Hind and forelimb paralysis; 5. Moribund.

In the experiment shown in Figure 27, the incidence and onset of clinical disease were similar in both groups. Mice treated with the B7-4Fc however, developed severe disease with the majority of animals rapidly progressing to complete hind and forelimb paralysis (9/10 and 1/10 for B7-4Fc and control mice respectively). Mortality associated with clinical signs of disease was 10% in the control group and 70% in the B7-4Fc treated mice. In addition, recovery from clinical disease was substantially delayed in the B7-4Fc treated mice that did survive despite the fact that treatment was discontinued on day 11.

In conclusion, using an adoptive transfer model of T cell mediated autoimmunity, administration of soluble B7-4Fc exacerbates clinical signs of disease resulting in increased mortality and delayed recovery from paralysis. These findings are consistent with enhanced activation/infiltration of inflammatory cells into the CNS and clearly demonstrate the immunoregulatory potential for the B7-4Fc protein in vivo.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A method for modulating an immune response comprising contacting an immune cell with an agent that modulates signaling via PD-1 to thereby modulate the immune response.

2. The method of claim 1, wherein immune response is downregulated.

3. The method of claim1, wherein signaling via PD-1 is stimulated using an agent selected from the group consisting of: an activating antibody that recognizes PD-1, a form of B7-4 that binds to an inhibitory receptor, and a small molecule that binds to PD-1.

4. The method of claim 1, wherein the immune cell is selected from the group consisting of: a T cell, a B cell, and a myeloid cell.

5. The method of claim 2, wherein anergy is induced in the immune cell.

6. The method of claim 2, further comprising contacting the immune cell with an additional agent that downregulates an immune response.

7. The method of claim 1, wherein the immune response is upregulated.

8. The method of claim 1, wherein the signaling via PD-1 is inhibited using an agent selected from the group consisting of: a blocking antibody that recognizes PD-1, a non-activating form of B7-4, an antibody that recognizes B7-4, and a soluble form of PD-1.

9. The method of claim 1, wherein the step of contacting occurs *in vivo*.

10. The method of claim 1, wherein the step of contacting occurs *in vitro*.

11. A method for modulating the interaction of B7-4 with an inhibitory receptor on an immune cell comprising contacting an antigen presenting cell which expresses B7-4 with an agent selected from the group consisting of: a form of B7-4, a form of PD-1, or

an agent that modulates the interaction of B7-4 and PD-1 such that the interaction of B7-4 with an inhibitory receptor on an immune cell is modulated.

12. The method of claim 11, further comprising contacting the immune cell or the antigen presenting cell with an additional agent that modulates an immune response.

13. The method of claim 11, wherein the step of contacting is performed *in vitro*.

14. The method of claim 11, wherein the step of contacting is performed *in vivo*.

15. The method of claim 11, wherein the immune cell is selected from the group consisting of: a T cell, a B cell, and a myeloid cell.

16. A method for inhibiting activation in an immune cell via a non-apoptotic mechanism comprising increasing the activity or expression of PD-1 in a immune cell such that immune cell activation is inhibited.

17. A vaccine comprising an antigen and an agent that inhibits signaling via PD-1 in an immune cell.

18. A composition comprising an antigen and an agent that promotes signaling via PD-1 in an immune cell.

19. A method for treating a subject having a condition that would benefit from upregulation of an immune response comprising administering an agent that inhibits signaling via PD-1 in a immune cell of the subject such that a condition that would benefit from upregulation of an immune response is treated.

20. The method of claim 19, wherein said agent comprises a soluble form of PD-1 or B7-4.

21. The method of claim 19, further comprising administering a second agent that upregulates an immune response to the subject.

22. The method of claim 19, wherein the condition is selected from the group consisting of: a tumor, a neurological disease or an immunosuppressive disease.

23. A method for treating a subject having a condition that would benefit from downregulation of an immune response comprising administering an agent that stimulates signaling via PD-1 in a immune cell of the subject such that a condition that would benefit from downregulation of an immune response is treated.

24. The method of claim 23, wherein said agent is selected from the group consisting of: an antibody that stimulates signaling via PD-1, a bispecific antibody, and soluble B7-4.

25. The method of claim 23, further comprising administering a second agent that downregulates an immune response to the subject.

26. The method of claim 23, wherein the condition is selected from the group consisting of: a transplant, an allergy, and an autoimmune disorder.

27. A cell-based assay for screening for compounds which modulate the activity of B7-4 or PD-1 comprising contacting a cell expressing a B7-4 target molecule or PD-1 target molecule with a test compound and determining the ability of the test compound to modulate the activity of the B7-4 or PD-1 target molecule.

28. A cell-free assay for screening for compounds which modulate the binding of B7-4 or PD-1 to a target molecule comprising contacting a B7-4 or PD-1 protein or biologically active portion thereof with a test compound and determining the ability of the test compound to bind to the B7-4 or PD-1 protein or biologically active portion thereof.

GCTTCCCGAGGCTCCGCACCAGCCGCGCTTCTGTCCGCCTGCAGGGCATTCCA
GAAAGATGAGGATATTTGCTGTCTTTATATTCATGACCTACTGGCATTTGCTG
AACGCATTTACTGTCACGGTTCCCAAGGACCTATATGTGGTAGAGTATGGTA
GCAATATGACAATTGAATGCAAATTCCCAGTAGAAAAACAATTAGACCTGGC
TGCACTAATTGTCTATTGGGAAATGGAGGATAAGAACATTATTCAATTTGTGC
ATGGAGAGGAAGACCTGAAGGTTCAGCATAGTAGCTACAGACAGAGGGCCC
GGCTGTTGAAGGACCAGCTCTCCCTGGGAAATGCTGCACTTCAGATCACAGA
TGTGAAATTGCAGGATGCAGGGGTGTACCGCTGCATGATCAGCTATGGTGGT
GCCGACTACAAGCGAATTACTGTGAAAGTCAATGCCCCATACAACAAAATCA
ACCAAAGAATTTTGGTTGTGGATCCAGTCACCTCTGAACATGAACTGACATGT
CAGGCTGAGGGCTACCCCAAGGCCGAAGTCATCTGGACAAGCAGTGACCATC
AAGTCCTGAGTGGTAAGACCACCACCACCAATTCCAAGAGAGAGGAGAAGC
TTTTCAATGTGACCAGCACACTGAGAATCAACACAACAACTAATGAGATTTT
CTACTGCACTTTTAGGAGATTAGATCCTGAGGAAAACCATACAGCTGAATTG
GTCATCCCAGGTAATATTCTGAATGTGTCCATTAAAATATGTCTAACACTGTC
CCCTAGCACCTAGCATGATGTCTGCCTATCATAGTCATTCAGTGATTGTTGAA
TAAATGAATGAATGAATAACACTATGTTTACAAAATATATCCTAATTCCTCAC
CTCCATTCATCCAAACCATATTGTTACTTAATAAACATTCAGCAGATATTTAT
GGAATAAAAAAAAAAAAAAAAAAAA

FIGURE 1

CGAGGCTCCGCACCAGCCGCGCTTCTGTCCGCCTGCAGGGCATTCCAGAAAGA
TGAGGATATTTGCTGTCTTTATATTCATGACCTACTGGCATTTGCTGAACGCATT
TACTGTCACGGTTCCCAAGGACCTATATGTGGTAGAGTATGGTAGCAATATGAC
AATTGAATGCAAATTCCCAGTAGAAAAACAATTAGACCTGGCTGCACTAATTGT
CTATTGGGAAATGGAGGATAAGAACATTATTCAATTTGTGCATGGAGAGGAAG
ACCTGAAGGTTCAGCATAGTAGCTACAGACAGAGGGCCCGGCTGTTGAAGGAC
CAGCTCTCCCTGGGAAATGCTGCACTTCAGATCACAGATGTGAAATTGCAGGAT
GCAGGGGTGTACCGCTGCATGATCAGCTATGGTGGTGCCGACTACAAGCGAAT
TACTGTGAAAGTCAATGCCCCATACAACAAAATCAACCAAAGAATTTTGGTTGT
GGATCCAGTCACCTCTGAACATGAACTGACATGTCAGGCTGAGGGCTACCCCA
AGGCCGAAGTCATCTGGACAAGCAGTGACCATCAAGTCCTGAGTGGTAAGACC
ACCACCACCAATTCCAAGAGAGAGGAGAAGCTTTTCAATGTGACCAGCACACT
GAGAATCAACACAACAACTAATGAGATTTTCTACTGCACTTTTAGGAGATTAGA
TCCTGAGGAAAACCATACAGCTGAATTGGTCATCCCAGAACTACCTCTGGCACA
TCCTCCAAATGAAAGGACTCACTTGGTAATTCTGGGAGCCATCTTATTATGCCTT
GGTGTAGCACTGACATTCATCTTCCGTTTAAGAAAAGGGAGAATGATGGATGT
GAAAAAATGTGGCATCCAAGATACAAACTCAAAGAAGCAAAGTGATACACATTT
GGAGGAGACGTAATCCAGCATTGGAACTTCTGATCTTCAAGCAGGGATTCTCA
ACCTGTGGTTTAGGGGTTCATCGGGGCTGAGCGTGACAAGAGGAAGGAATGG
GCCCGTGGGATGCAGGCAATGTGGGACTTAAAAGGCCCAAGCACTGAAAATG
GAACCTGGCGAAAGCAGAGGAGGAGAATGAAGAAAGATGGAGTCAAACAGGG
AGCCTGGAGGGAGACCTTGATACTTTCAAATGCCTGAGGGGCTCATCGACGCC
TGTGACAGGGAGAAAGGATACTTCTGAACAAGGAGCCTCCAAGCAAATCATCC
ATTGCTCATCCTAGGAAGACGGGTTGAGAATCCCTAATTTGAGGGTCAGTTCCT
GCAGAAGTGCCCTTTGCCTCCACTCAATGCCTCAATTTGTTTTCTGCATGACTGA
GAGTCTCAGTGTTGGAACGGGACAGTATTTATGTATGAGTTTTTCCTATTTATTT
TGAGTCTGTGAGGTCTTCTTGTCATGTGAGTGTGGTTGTGAATGATTTCTTTTGA
AGATATATTGTAGTAGATGTTACAATTTTGTCGCCAAACTAAACTTGCTGCTTAA
TGATTTGCTCACATCTAGTAAAACATGGAGTATTTGTAAAAAAAAAAAAAAA

FIGURE 2

292 secreted (245 amino acids)

Signal/IgV/IgC/hydrophilic tail
(a) (b) (c) (d)

Ig cysteines in large bold

MRIFAVFIFMTYWHLLNA (signal)

FTVTVPKDLYVVEYGSNMTIE**C**KFPVEKQLDLAALIVYWEMEDKN
IIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQD
AGVYR**C**MISYGGADYKRITVKVNAPY (IgV)

NKINQRILVVDPVTSEHELT**C**QAEGYPKAEVIWTSSDHQVLSGKT
TTTNSKREEKLFNVTSTLRINTTTNEIFY**C**TFRRLDPEENHTAEL
VIP (IgC)

GNILNVSIKICLTLSPST (hydrophilic tail)

FIGURE 3

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292 membrane (290 amino acids)

Signal/IgV/IgC/transmembrane (underlined)
plus cytoplasmic

Ig cysteines in large bold

MRIFAVFIFMTYWHLLNA (signal)

FTVTVPKDLYVVEYGSNMTIE**C**KFPVEKQLDLAALIVYWEMEDKN
IIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQD
AGVYR**C**MISYGGADYKRITVKVNAPY (IgV)

NKINQRILVVDPVTSEHELT**C**QAEGYPKAEVIWTSSDHQVLSGKT
TTTNSKREEKLFNVTSTLRINTTTNEIFY**C**TFRRLDPEENHTAEL
VIP (IgC)

ELPLAHPPNERTHLVILGAILLCLGVALTFIFRLRKGRMMDVKKC
GIQDTNSKKQSDTHLEET (transmembrane plus cytoplasmic)

FIGURE 4

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AGATAGTTCCCAAAACATGAGGATATTTGCTGGCATTATATTCACAGCCTGC
TGTCACTTGCTACGGGCGTTTACTATCACGGCTCCAAAGGACTTGTACGTG
GTGGAGTATGGCAGCAACGTCACGATGGAGTGCAGATTCCCTGTAGAACG
GGAGCTGGACCTGCTTGCGTTAGTGGTGTACTGGGAAAAGGAAGATGAGC
AAGTGATTCAGTTTGTGGCAGGAGAGGAGGACCTTAAGCCTCAGCACAGCA
ACTTCAGGGGGAGAGCCTCGCTGCCAAAGGACCAGCTTTTGAAGGGAAAT
GCTGCCCTTCAGATCACAGACGTCAAGCTGCAGGACGCAGGCGTTTACTGC
TGCATAATCAGCTACGGTGGTGCGGACTACAAGCGAATCACGCTGAAAGTC
AATGCCCCATACCGCAAAATCAACCAGAGAATTTCCGTGGATCCAGCCACTT
CTGAGCATGAACTAATATGTCAGGCCGAGGGTTATCCAGAAGCTGAGGTAA
TCTGGACAAACAGTGACCACCAACCCGTGAGTGGGAAGAGAAGTGTCACCA
CTTCCCGGACAGAGGGGATGCTTCTCAATGTGACCAGCAGTCTGAGGGTCA
ACGCCACAGCGAATGATGTTTTCTACTGTACGTTTTGGAGATCACAGCCAG
GGCAAAACCACACAGCGGAGCTGATCATCCCAGAACTGCCTGCAACACATC
CTCCACAGAACAGGACTCACTGGGTGCTTCTGGGATCCATCCTGTTGTTCC
TCATTGTAGTGTCCACGGTCCTCCTCTTCTTGAGAAAACAAGTGAGAATGCT
AGATGTGGAGAAATGTGGCGTTGAAGATACAAGCTCAAAAAACCGAAATGA
TACACAATTCGAGGAGACGTAAGCAGTGTTGAACCCTCTGATCGTCGATTG
GCAGCTTGTGGTCTGTGAAAGAAAGGGCCCATGGGACATGAGTCCAAAGAC
TCAAGATGGAACCTGAGGGAGAGAACCAAGAAAGTGTTGGGAGAGGAGCC
TGGAACAACGGACATTTTTTCCAGGGAGACACTGCTAAGCAAGTTGCCCAT
CAGTCGTCTTGGGAAATGGATTGAGGGTTCCTGGCTTAGCAGCTGGTCCTT
GCACAGTGACCTTTTCCTCTGCTCAGTGCCGGGATGAGAGATGGAGTCATG
AGTGTTGAAGAATAAGTGCCTTCTATTTATTTTGAGTCTGTGTGTTCTCACTT
TGGGCATGTAATTATGACTGGTGAATTCTGACGACATGATAGATCTTAAGAT
GTAGTCACCAAACTCAACTGCTGCTTAGCATCCTCCGTAACTACTGATACAA
GCAGGGAACACAGAGGTCACCTGCTTGGTTTGACAGGCTCTTGCTGTCTGA
CTCAAATAATCTTTATTTTTCAGTCCTCAAGGCTCTTCGATAGCAGTTGTTCT
GTATCAGCCTTATAGGTGTCAGGTATAGCACTCAACATCTCATCTCATTACA
ATAGCAACCCTCATCACCATAGCAACAGCTAACCTCTGTTATCCTCACTTCA
TAGCCAGGAAGCTGAGCGACTAAGTCACTTGCCCACAGAGTATCAGCTCTC
AGATTTCTGTTCTTCAGCCACTGTCCTTTCAGGATAGAATTTGTCGTTAAGAA
ATTAATTTAAAAACTGATTATTGAGTAGCATTGTATATCAATCACAACATGCC
TTGTGCACTGTGCTGGCCTCTGAGCATAAAGATGTACGCCGGAGTACCGGT
CGGACATGTTTATGTGTGTTAAATACTCAGAGAAATGTTCATTAACAAGGAG
CTTGCATTTTAGAGACACTGGAAAGTAACTCCAGTTCATTGTCTAGCATTAC
ATTTACCTCATTTGCTATCCTTGCCATACAGTCTCTTGTTCTCCATGAAGTGT
CATGAATCTTGTTGAATAGTTCTTTTATTTTTTAAATGTTTCTATTTAAATGATA
TTGACATCTGAGGCGATAGCTCAGTTGGTAAAACCCTTTCCTCACAAGTGTG
AAACCCTGAGTCTTATCCCTAGAACCCACATAAAAAACAGTTGCGTATGTTT
GTGCATGCTTTTGATCCCAGCACTAGGGAGGCAGAGGCAGGCAGATCCTG
AGCTCTCATTGACCACCCAGCCTAGCCTACATGGTTAGCTCCAGGCCTACA
GGAGCTGGCAGAGCCTGAAAAACGATGCCTAGACACACACACACACACACA
CACACACACACACACACACACACACACCATGTACTCATAGACCTAAGTGCACC
CTCCTACACATGCACACACATACAATTCAAACACAAATCAACAGGGAATTGT

Figure 5

CTCAGAATGGTCCCCAAGACAAAGAAGAAGAAAAACACCAAACCAGCTCTA
TTCCCTCAGCCTATCCTCTCTACTCCTTCCTAGAAGCAACTACTATTGTTTTT
GTATATAAATTTACCCAACGACAGTTAATATGTAGAATATATATTAAAGTGTC
TGTCAATATATATTATCTCTTTCTTTCTTTCTTCCTTTCTTTCTTTCTTTCTTTC
TTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTCCTTCCTTCCTTCCTTCCTTC
CTTCCTTCCTTCCTTTCTTTCTTTCTTTCTTTTTTTCTGTCTATCTGTACCTAAA
TGGTTGCTCACTATGCATTTTCTGTGCTCTTCGCCCTTTTTATTTAATGTATG
GATATTTATGCTGCTTCCAGAATGGATCTAAAGCTCTTTGTTTCTAGGTTTTC
TCCCCCATCCTTCTAGGCATCTCTCACACTGTCTAGGCCAGACACCATGTCT
GCTGCCTGAATCTGTAGACACCATTTATAAAGCACGTACTCACCGAGTTTGT
ATTTGGCTTGTTCTGTGTCTGATTAAAGGGAGACCATGAGTCCCCAGGGTA
CACTGAGTTACCCCAGTACCAAGGGGGAGCCTTGTTTGTGTCTCCATGGCA
GAAGCAGGCCTGGAGCCATTTTGGTTTCTTCCTTGACTTCTCTCAAACACAG
ACGCCTCACTTGCTCATTACAGGTTCTCCTTTGGGAATGTCAGCATTGCTCC
TTGACTGCTGGCTGCCCTGGAAGGAGCCCATTAGCTCTGTGTGAGCCCTTG
ACAGCTACTGCCTCTCCTTACCACAGGGGCCTCTAAGATACTGTTACCTAGA
GGTCTTGAGGATCTGTGTTCTCTGGGGGGAGGAAAGGAGGAGGAACCCAG
AACTTTCTTACAGTTTTCCTTGTTCTGTCACATGTCAAGACTGAAGGAACAG
GCTGGGCTACGTAGTGAGATCCTGTCTCAAAGGAAAGACGAGCATAGCCGA
ACCCCCGGTGGAACCCCCTCTGTTACCTGTTCACACAAGCTTATTGATGAGT
CTCATGTTAATGTCTTGTTTGTATGAAGTTTAAGAAAATATCGGGTTGGGCAA
CACATTCTATTTATTCATTTTATTTGAAATCTTAATGCCATCTCATGGTGTTGG
ATTGGTGTGGCACTTTATTCTTTTGTGTTGTGTATAACCATAAATTTTATTTTG
CATCAGATTGTCAATGTATTGCATTAATTTAATAAATATTTTTATTTATTAAAAA
AAAAAAAAAAAAAAA

Figure 5
(continued)

MRIFAGIIFTACCHLLRAFTITAPKDLYVVEYGSNVTMECRFPVERELDLLALVVYWEKEDEQVIQFVAGEE
DLKPQHSNFRGRASLPKDQLLKGNAALQITDVKLQDAGVYCCIISYGGADYKRITLKVNAPYRKINQRISV
DPATSEHELICQAEGYPEAEVIWTNSDHQPVSGKRSVTTSRTEGMLLNVTSSLRVNATANDVFYCTFWR
SQPGQNHTAELIIPELPATHPPQNRTHWVLLGSILLFLIVVSTVLLFLRKQVRMLDVEKCGVEDTSSKNRN
DTQFEET.

Figure 6

mB7-4 vs. hB7-4

69% identity

```
mB7-4   1   MRIFAGIIFTACCHLLRAFTITAPKDLYVVEYGSNVTMECRFPVERELDLLALVVYWEKE 60
            MRIFA  IF    HLL AFT+T PKDLYVVEYGSN+T+EC+FPVE++LDL AL+VYWE E
hB7-4   1   MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEME 60

mB7-4  61   DEQVIQFVAGEEDLKPQHSNFRGRASLPKDQLLKGNAALQITDVKLQDAGVYCCIISYGG 120
            D+ +IQFV GEEDLK QHS++R RA L KDQL  GNAALQITDVKLQDAGVY C+ISYGG
hB7-4  61   DKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGG 120

mB7-4 121   ADYKRITLKVNAPYRKINQRI-SVDPATSEHELICQAEGYPEAEVIWTNSDHQPVSGKRS 179
            ADYKRIT+KVNAPY KINQRI  VDP TSEHEL CQAEGYP+AEVIWT+SDHQ +SGK +
hB7-4  121   ADYKRITVKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTT 180

mB7-4  180   VTTSRTEGMLLNVTSSLRVNATANDVFYCTFWRSQPGQNHTAELIIPELPATHPPQNRTH 239
             T S+ E  L NVTS+LR+N T N++FYCTF R  P +NHTAEL+IPELP  HPP  RTH
hB7-4  181   TTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNERTH 240

mB7-4  240   WVLLGSILLFLIVVSTVLLFLRKQVRMLDVEKCGVEDTSSKNRNDTQFEET 290
             V+LG+ILL L V  T +  LRK  RM+DV+KCG++DT+SK ++DT  EET
hB7-4  241   LVILGAILLCLGVALTFIFRLRKG-RMMDVKKCGIQDTNSKKQSDTHLEET 290
```

Figure 7

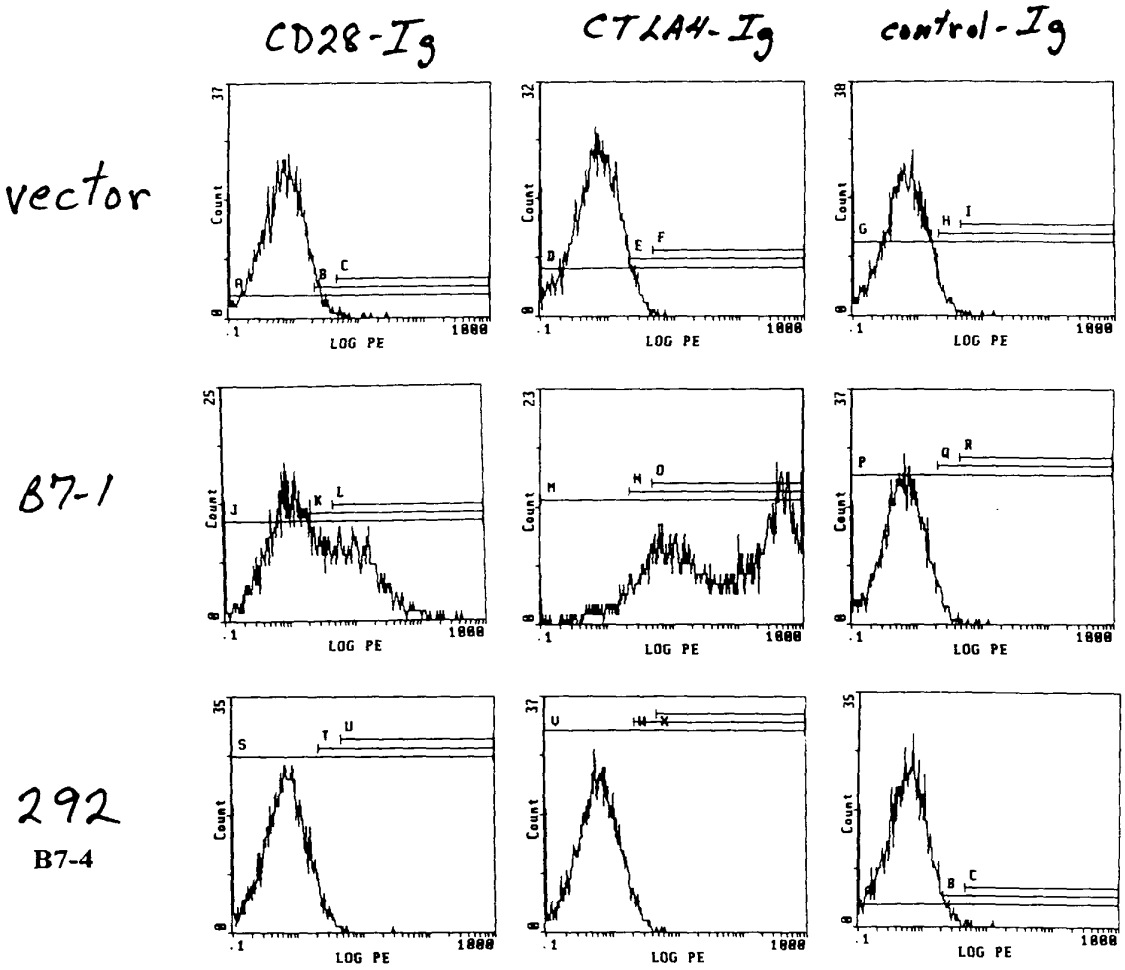

CD28-Ig
CTLA4-Ig
control-Ig
vector
B7-1
292
B7-4
Count
LOG PE
.1
1000

Figure 8

Ig G mICOS - His

Vector

B7-1

292

B7-4

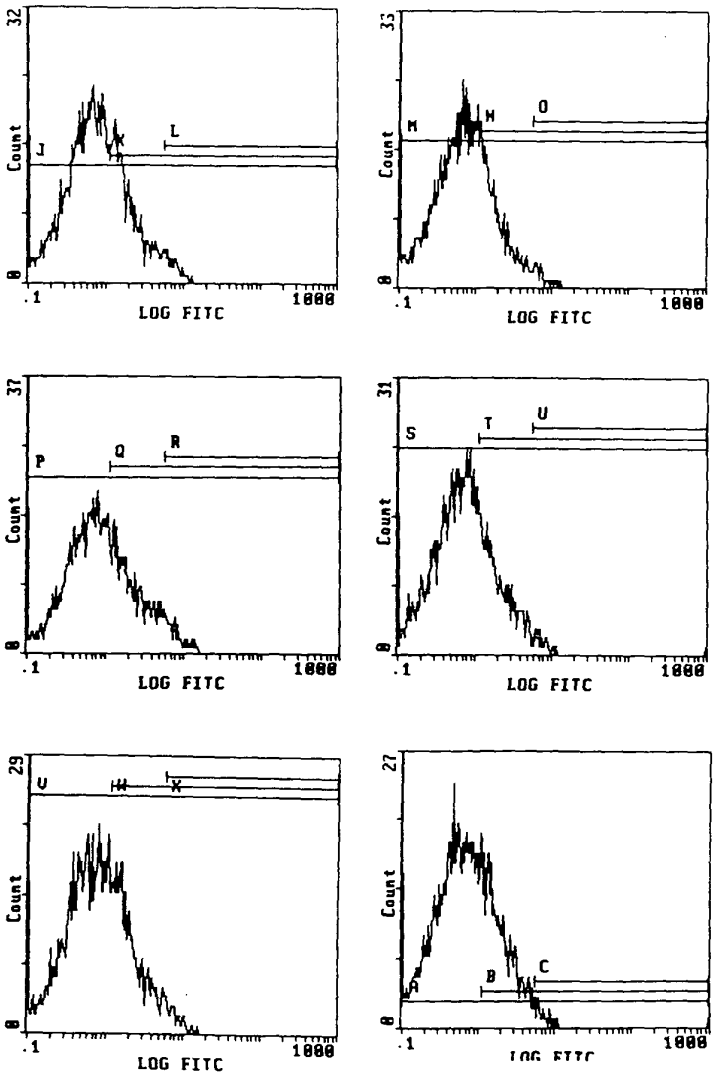


Figure 9

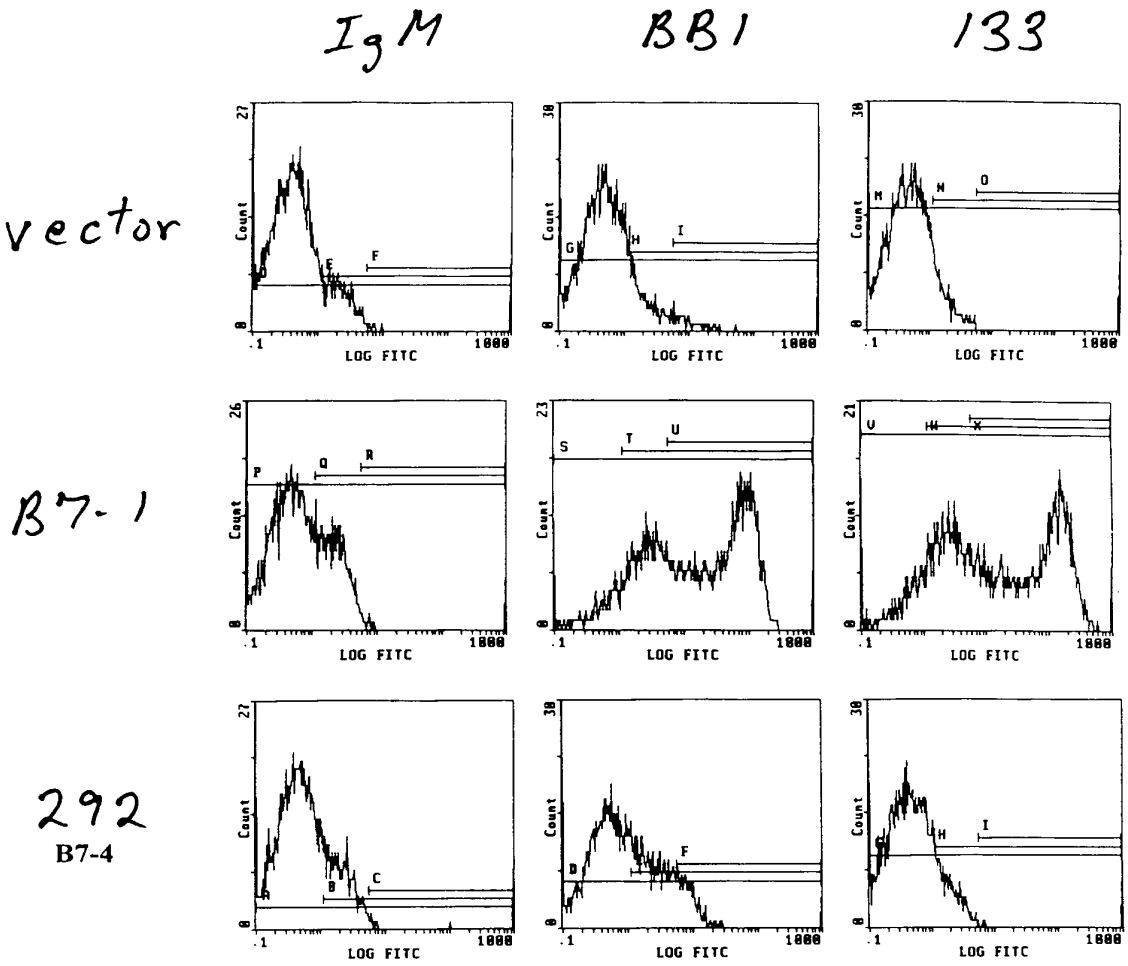


Figure 10

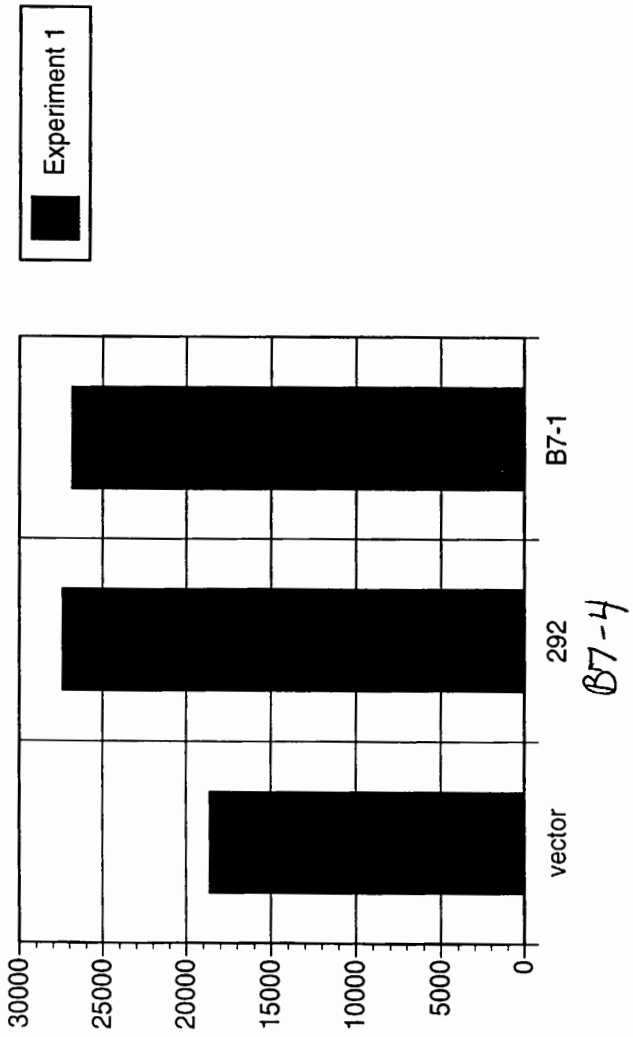


Figure 11

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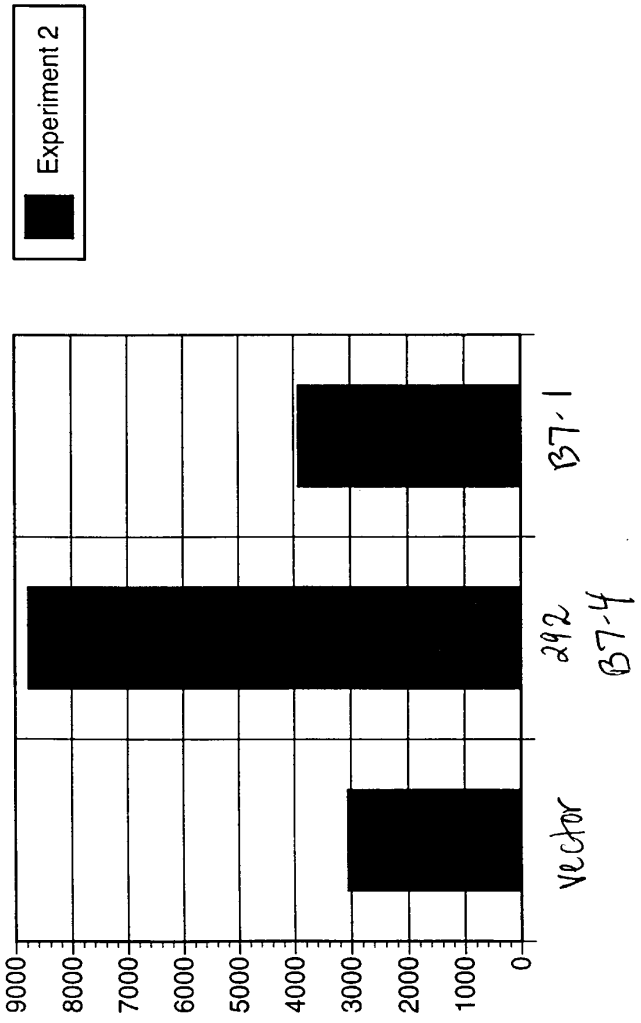


Figure 12

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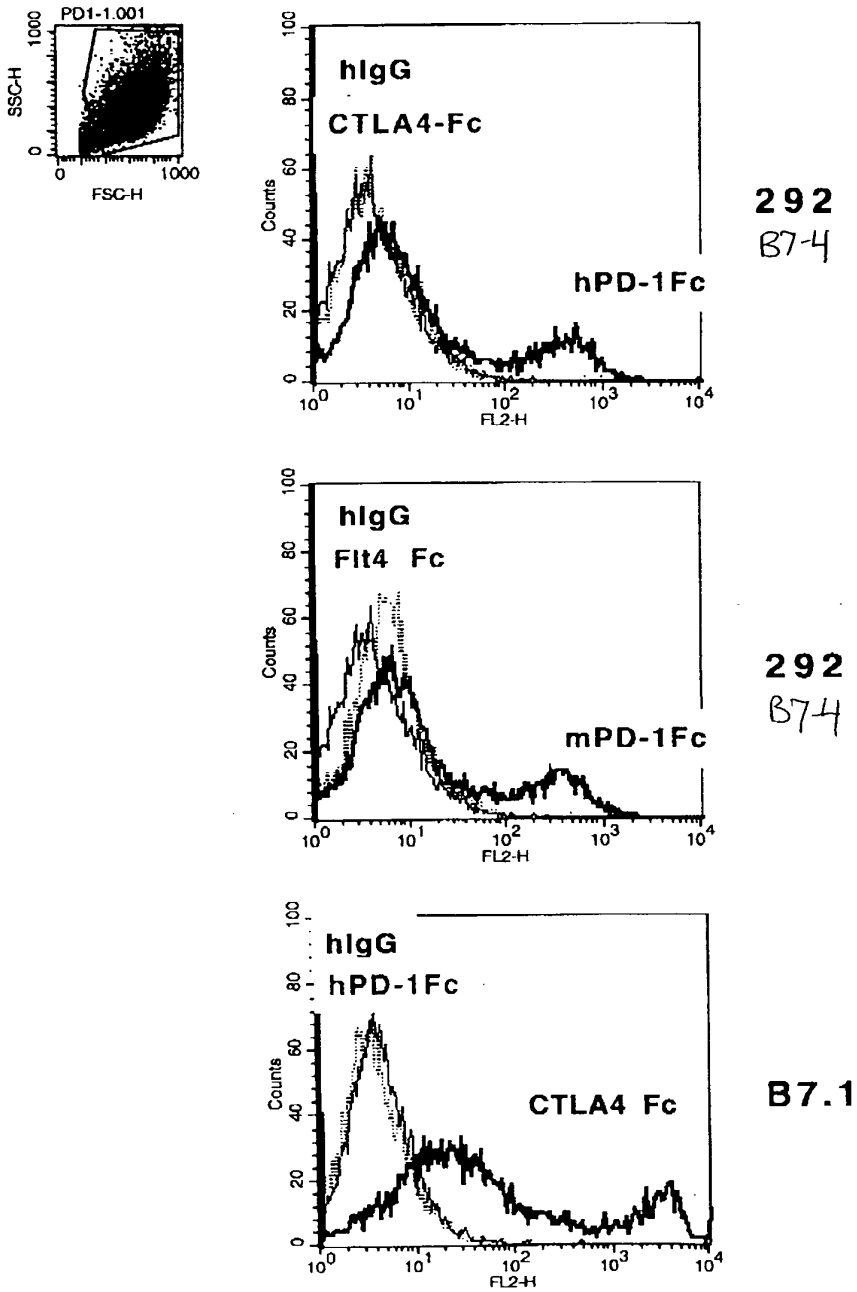

PD1-1.001
SSC-H
FSC-H
hIgG
CTLA4-Fc
hPD-1Fc
Counts
FL2-H
292
B7-4
hIgG
Flt4 Fc
mPD-1Fc
Counts
FL2-H
292
B74
hIgG
hPD-1Fc
CTLA4 Fc
Counts
FL2-H
B7.1

Figure 13

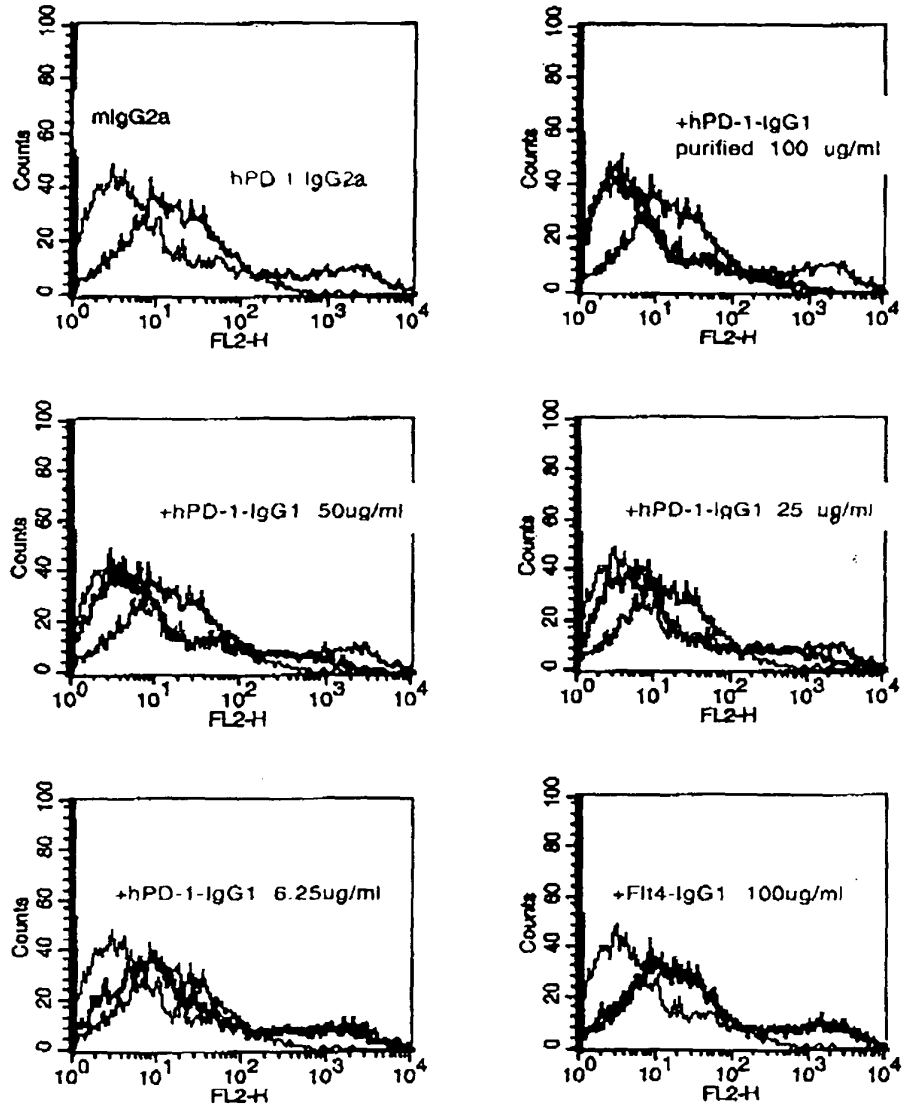

mIgG2a
hPD 1 IgG2a
+hPD-1-IgG1
purified 100 ug/ml
+hPD-1-IgG1 50ug/ml
+hPD-1-IgG1 25 ug/ml
+hPD-1-IgG1 6.25ug/ml
+Flt4-IgG1 100ug/ml
Counts
FL2-H

Figure 14

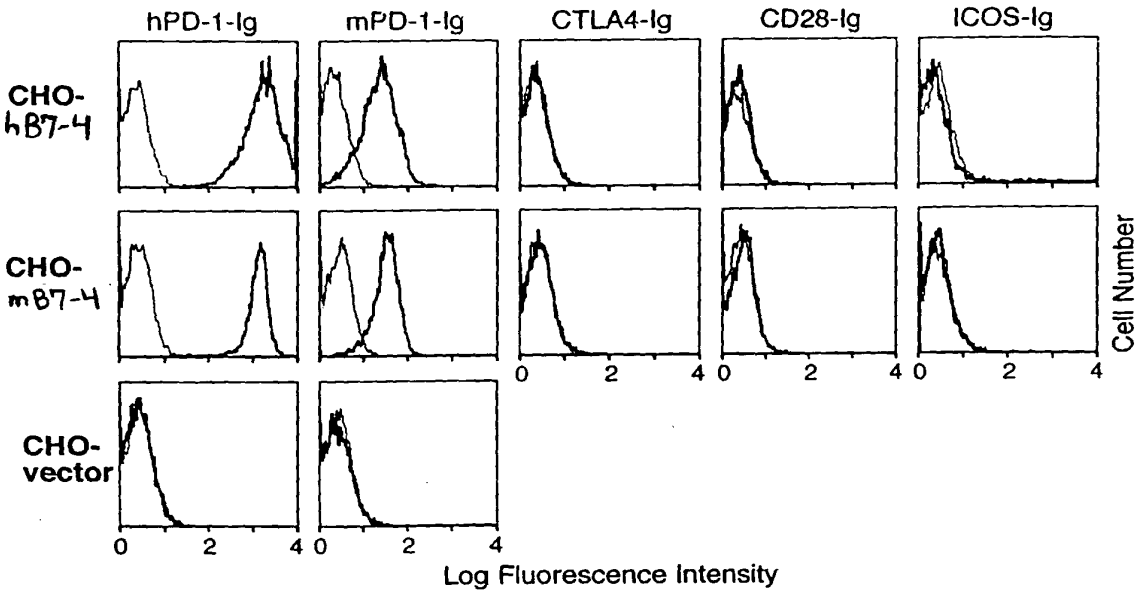

hPD-1-Ig
mPD-1-Ig
CTLA4-Ig
CD28-Ig
ICOS-Ig
CHO-hB7-4
CHO-mB7-4
CHO-vector
Cell Number
0
2
4
Log Fluorescence Intensity

Figure 15

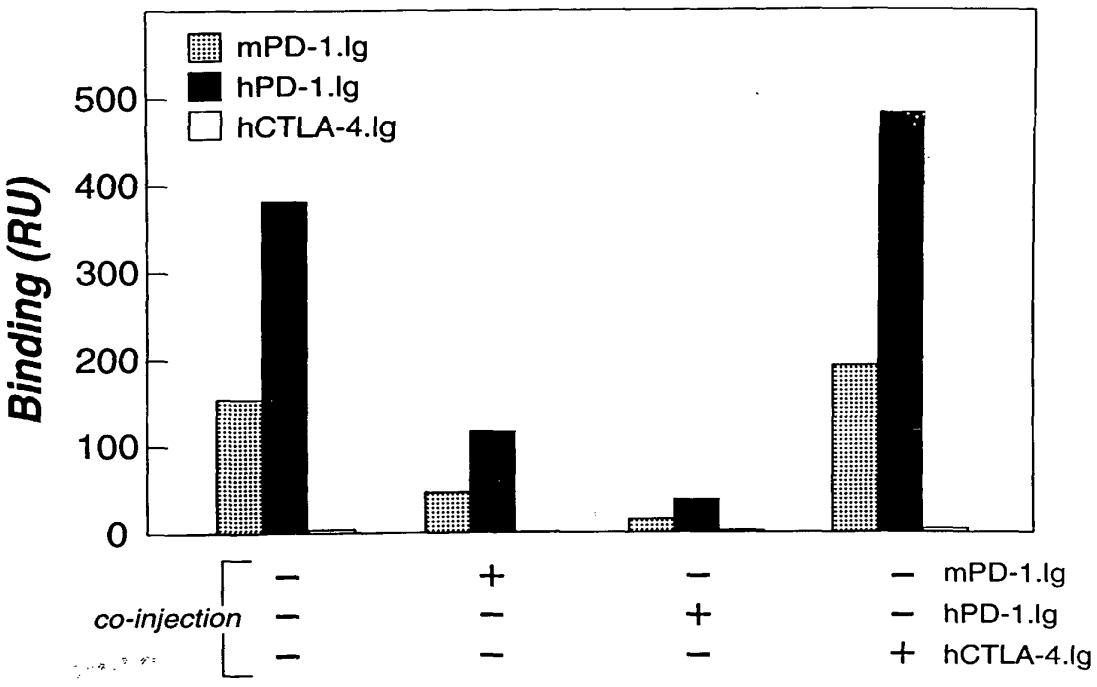

mPD-1.lg
hPD-1.lg
hCTLA-4.lg
Binding (RU)
500
400
300
200
100
0
co-injection
– + – – mPD-1.lg
– – + – hPD-1.lg
– – – + hCTLA-4.lg

Figure 16

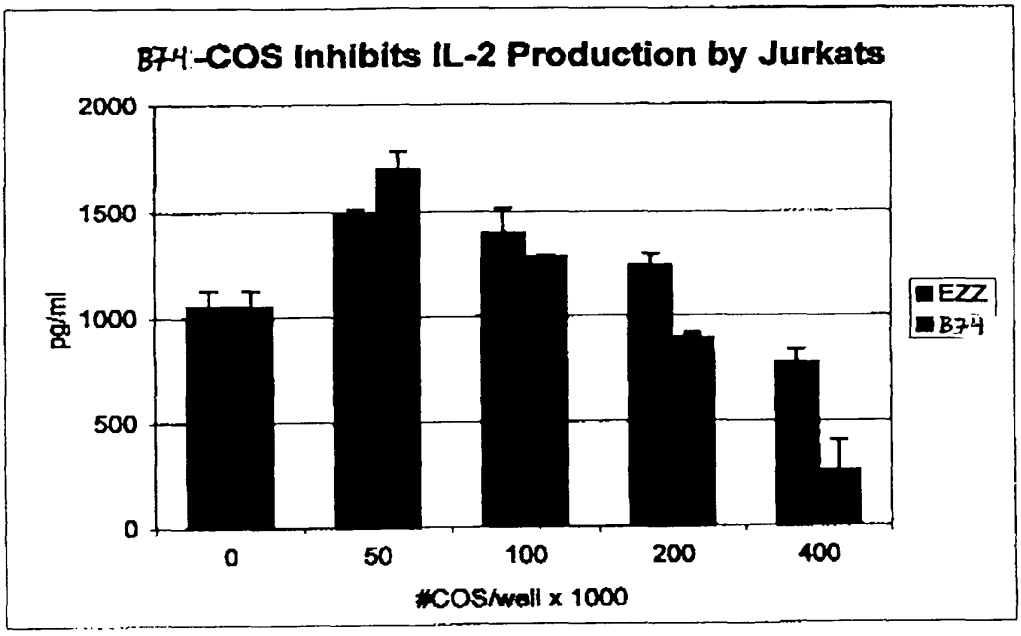

B7-4-COS Inhibits IL-2 Production by Jurkats
2000
1500
1000
500
0
pg/ml
0
50
100
200
400
#COS/well x 1000
EZZ
B7-4

Figure 17

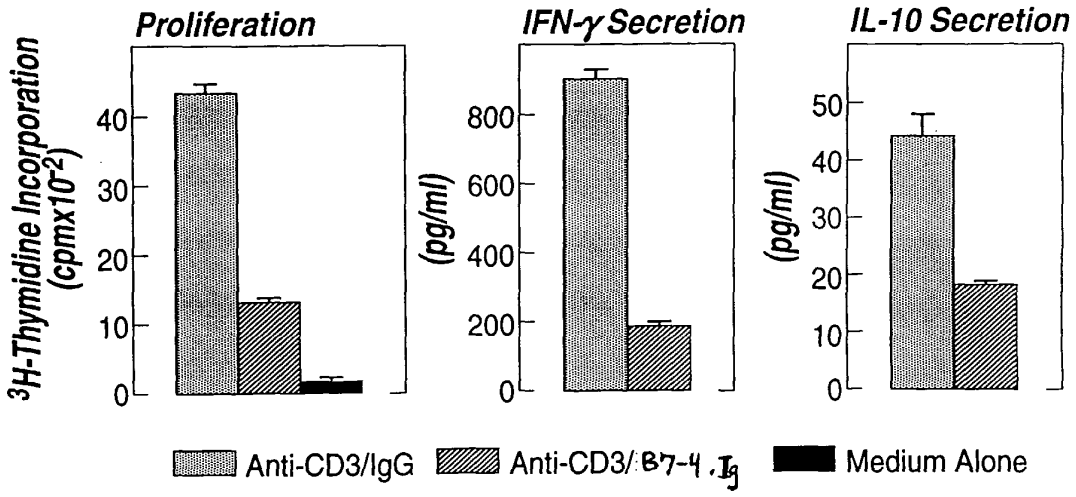

Proliferation
IFN-γ Secretion
IL-10 Secretion
^{3}H-Thymidine Incorporation (cpmx10^{-2})
(pg/ml)
(pg/ml)
0
10
20
30
40
200
400
600
800
50
Anti-CD3/IgG
Anti-CD3/B7-4.Ig
Medium Alone

Figure 18

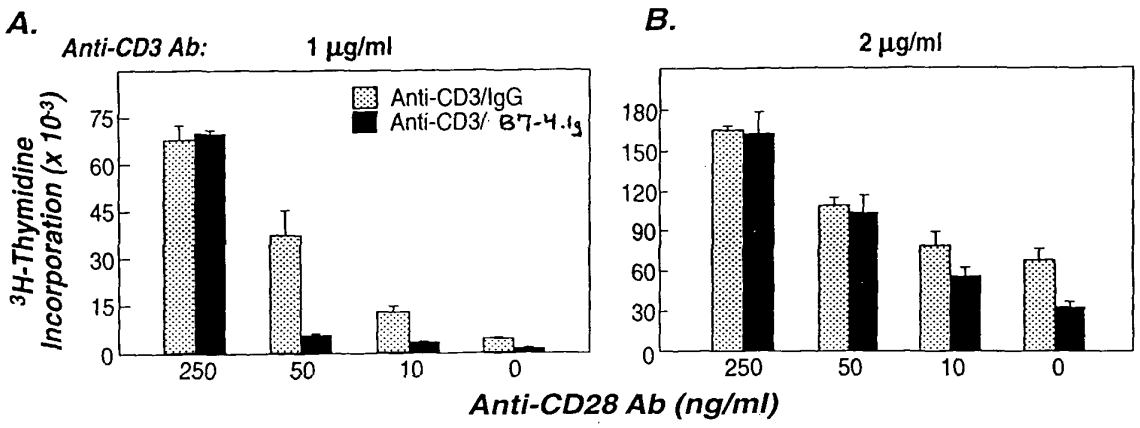

A.
Anti-CD3 Ab:
1 µg/ml
Anti-CD3/IgG
Anti-CD3/ B7-4.1g
^{3}H-Thymidine Incorporation (x 10^3)
75
60
45
30
15
0
250
50
10
0
B.
2 µg/ml
180
150
120
90
60
30
0
250
50
10
0
Anti-CD28 Ab (ng/ml)

Figure 19

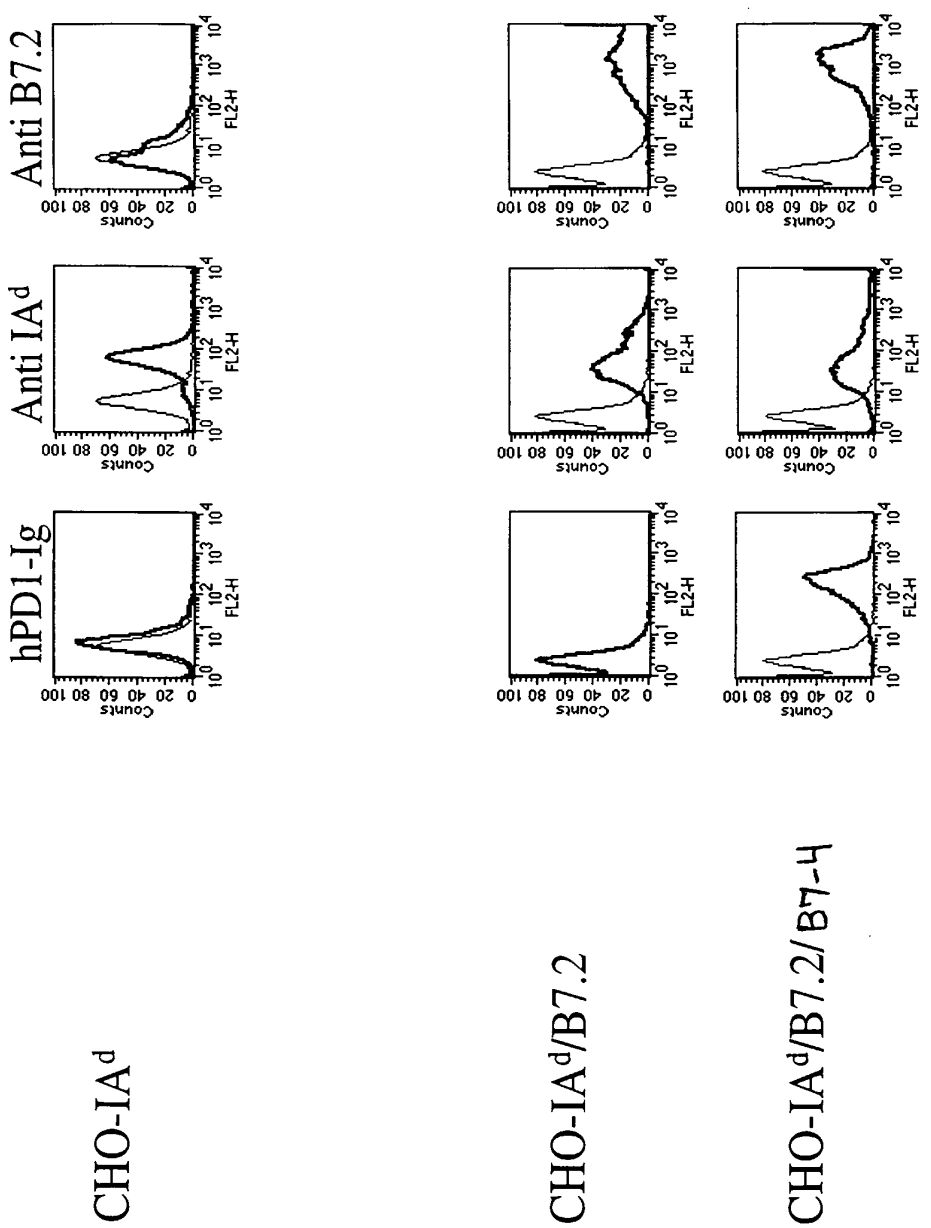

Anti B7.2
Anti IA^d
hPD1-Ig
CHO-IA^d
CHO-IA^d/B7.2
CHO-IA^d/B7.2/B7-4
Cell Number
Log Flourescence
Counts
FL2-H

FIGURE 20

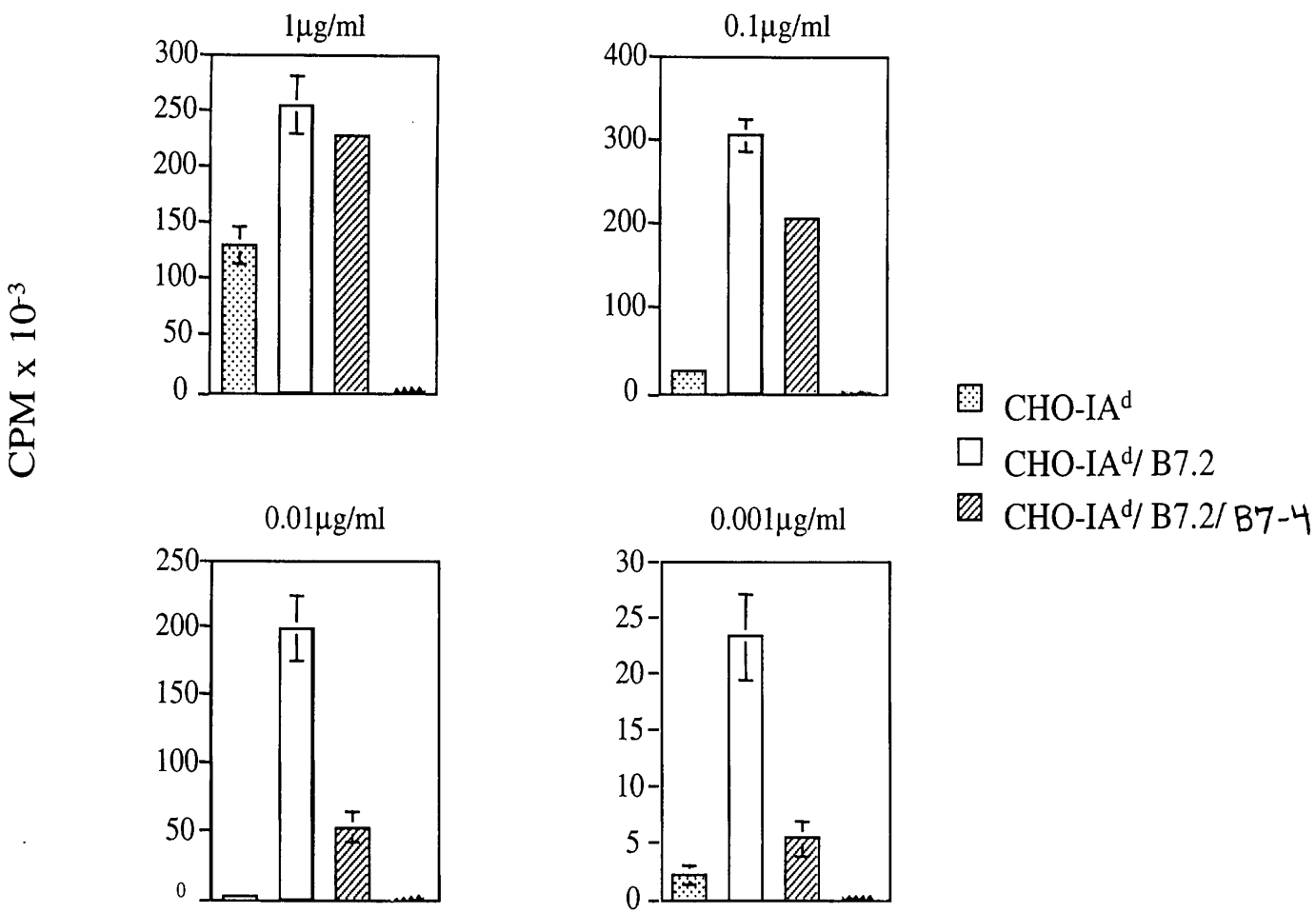


FIGURE 21

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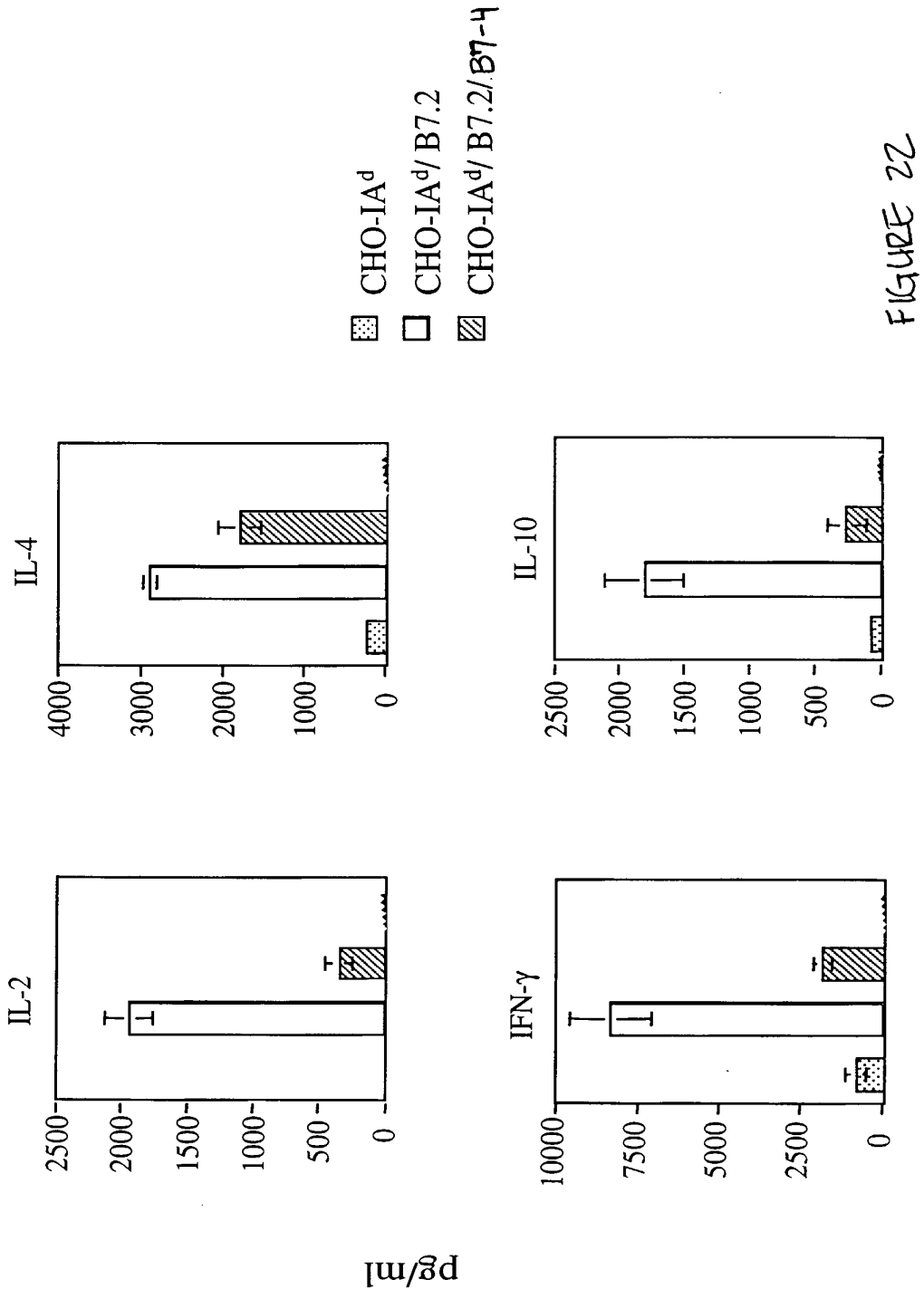

CHO-IA^d
CHO-IA^d/ B7.2
CHO-IA^d/ B7.2/B7-4
IL-2
IL-4
IL-10
IFN-γ
pg/ml
0
500
1000
1500
2000
2500
1000
2000
3000
4000
2500
5000
7500
10000

FIGURE 22

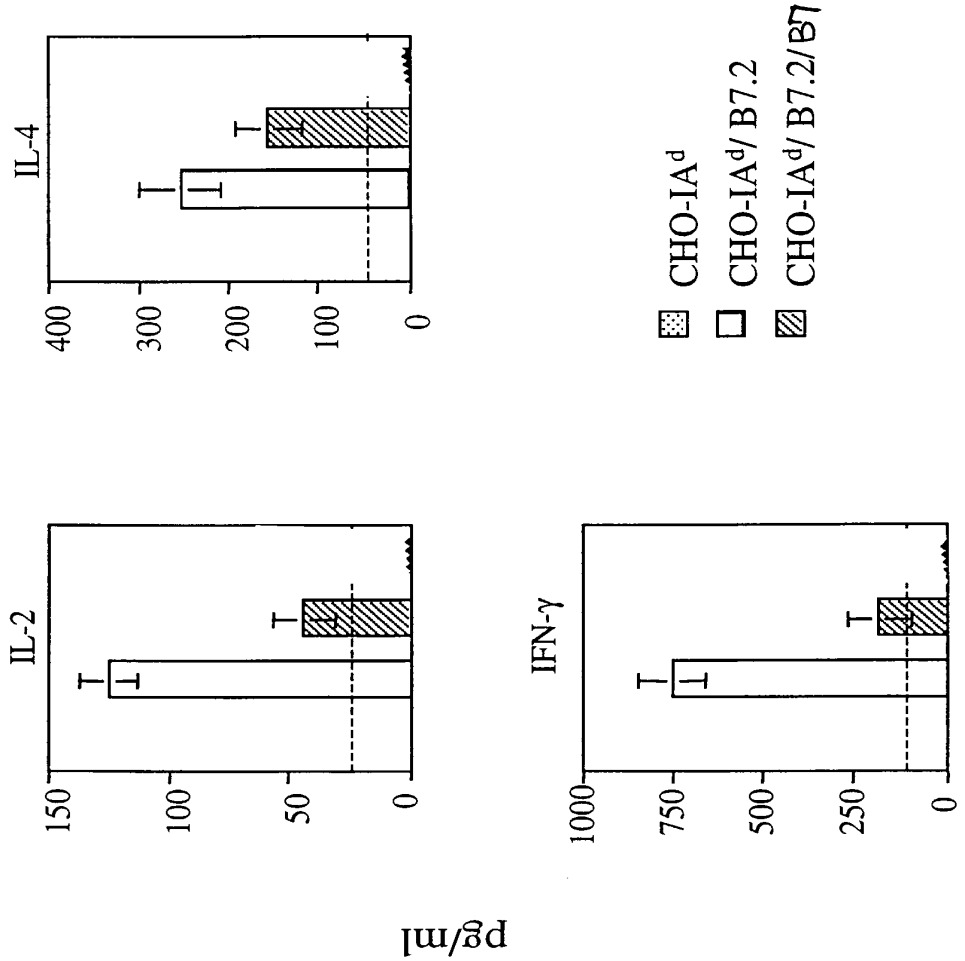

IL-4
IL-2
IFN-γ
400
300
200
100
0
150
100
50
0
1000
750
500
250
0
pg/ml
CHO-IA^d
CHO-IA^d/ B7.2
CHO-IA^d/ B7.2/B7-4

FIGURE 23

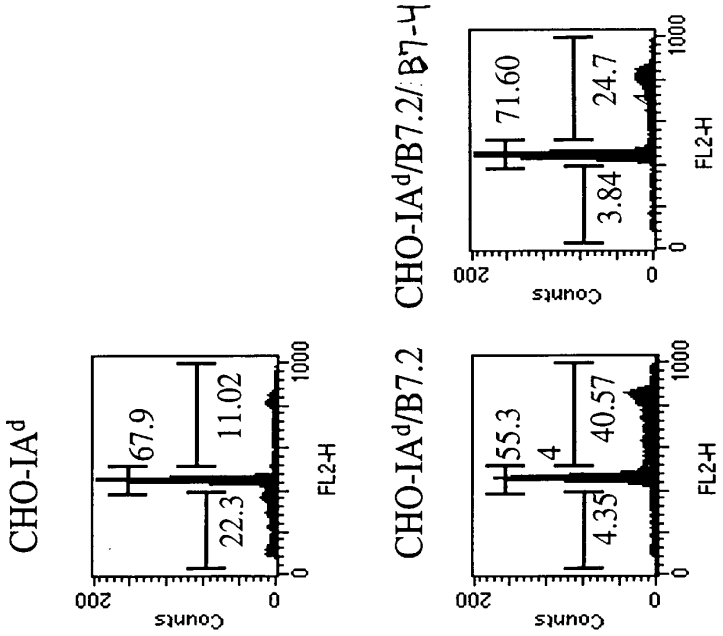
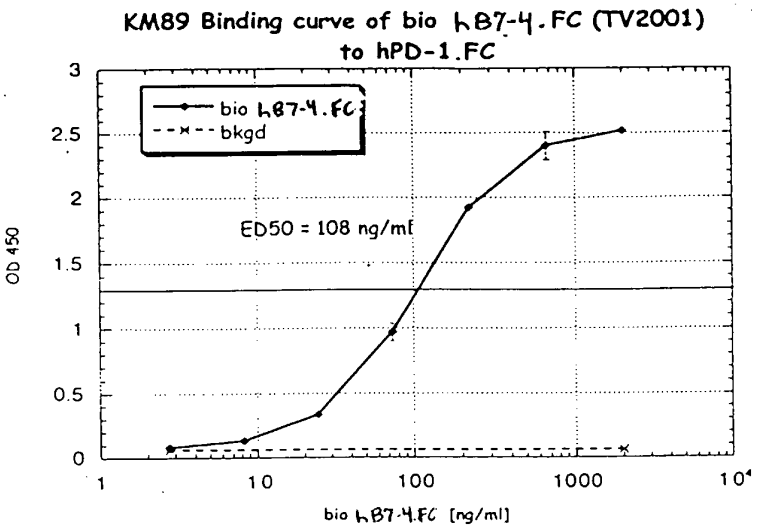
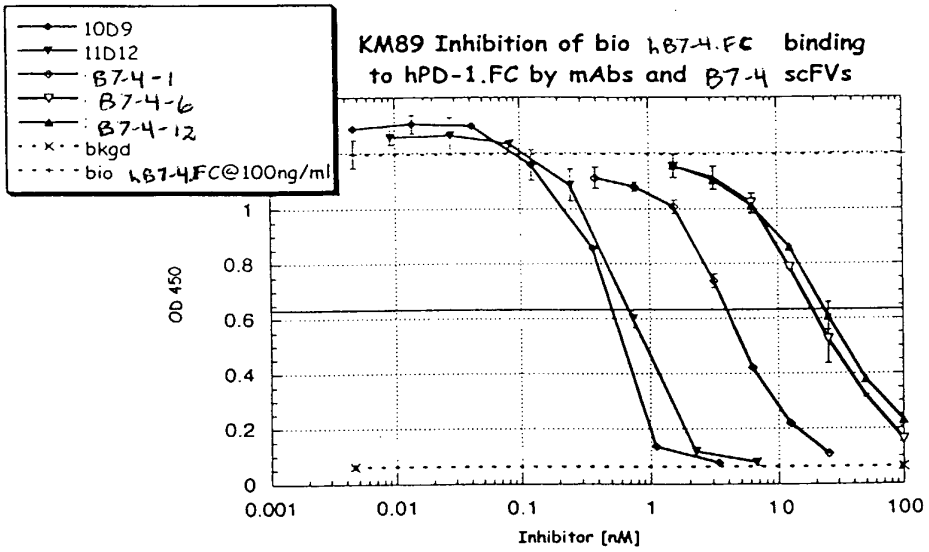


FIGURE 24

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Inhibitor	IC50
10D9	0.5
11D12	0.7
B7-4-1	4
B7-4-6	19
B7-4-12	24

Figure 25

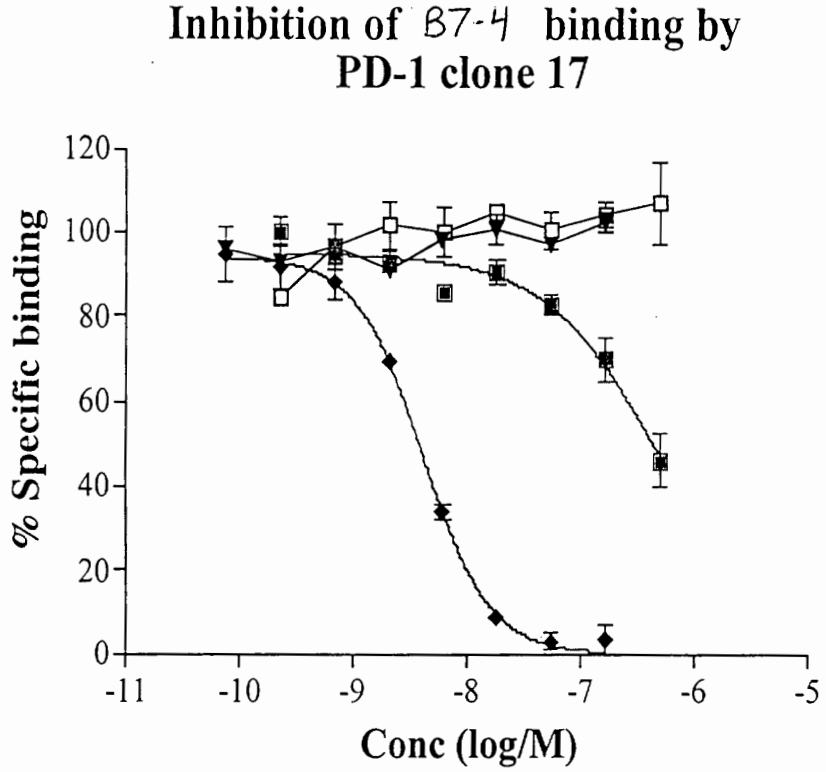
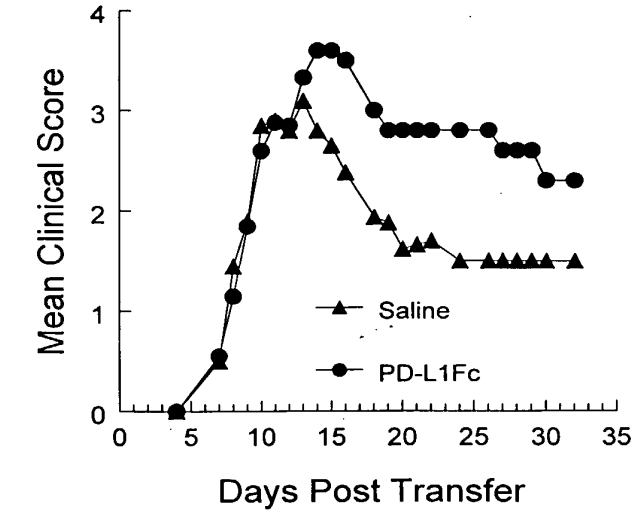


FIGURE 2c

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Mean Clinical Score
4
3
2
1
0
0
5
10
15
20
25
30
35
Saline
PD-L1Fc
Days Post Transfer

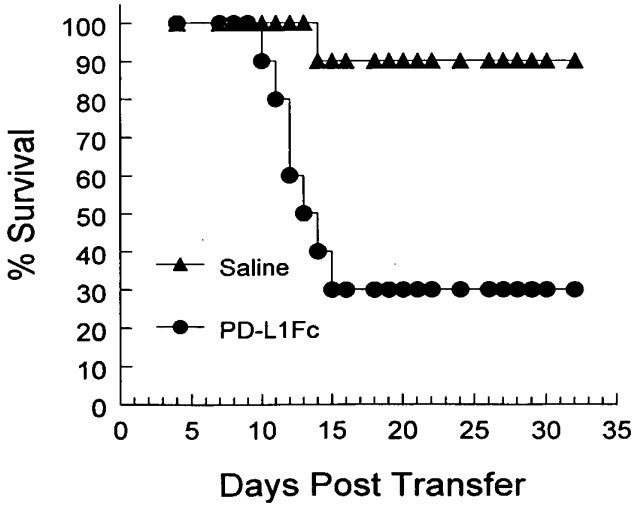

% Survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Saline
PD-L1Fc
Days Post Transfer

Figure 27

INTERNATIONAL SEARCH REPORT

Interna Application No
PCT/US 00/23347

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/705 A61K38/17 A61K39/395 G01N33/50
C07K16/28

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 7 C12N C07K A61K G01N

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)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS 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 5 698 520 A (SHINOHARA TAKASHI ET AL) 16 December 1997 (1997-12-16) column 6, line 25 -column 6, line 48 ---	1,9,10, 19,22
A	ISHIDA Y ET AL: "INDUCED EXPRESSION OF PD-1, A NOVEL MEMBER OF THE IMMUNOGLOBULIN GENE SUPERFAMILY, UPON PROGRAMMED CELL DEATH" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 11, no. 11, 1992, pages 3887-3895, XP002070368 ISSN: 0261-4189 cited in the application --- -/--	

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

[X] Patent family members are listed in annex.

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"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

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"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
8 January 2001	16/01/2001

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	NISHIMURA HIROYUKI ET AL: "Immunological studies on PD-1-deficient mice: Implication of PD-1 as a negative regulator for B cell responses." INTERNATIONAL IMMUNOLOGY, vol. 10, no. 10, October 1998 (1998-10), pages 1563-1572, XP000971789 ISSN: 0953-8178 cited in the application	
T	FREEMAN GORDON J ET AL: "Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 192, no. 7, 2 October 2000 (2000-10-02), pages 1027-1034, XP000971650 ISSN: 0022-1007	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5698520 A	16-12-1997	CA 2143491 A	02-09-1995
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(54) Title: PD-1, A RECEPTOR FOR B7-4, AND USES THEREFOR

(57) Abstract: Assays for identifying compounds which modulate signaling via PD-1 are disclosed.

PD-1, A RECEPTOR FOR B7-4, AND USES THEREFOR

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Related Applications

This claims priority to U.S.S.N. 60/281,064 filed on April 2, 2001. This application is incorporated herein in its entirety by this reference.

Background of the Invention

In order for T cells to respond to foreign proteins, two signals must be provided by antigen-presenting cells (APCs) to resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165:302-319; Mueller, D. L. *et al.* (1990) *J. Immunol.* 144:3701-3709). The first signal, which confers specificity to the immune response, is transduced via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Lenschow *et al.* (1996) *Annu. Rev. Immunol.* 14:233). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M. K. *et al.* (1988) *J. Immunol.* 140:3324-3330; Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 173:721-730; Gimmi, C. D. *et al.* 1991 *Proc. Natl. Acad. Sci. USA* 88:6575-6579; Young, J. W. *et al.* (1992) *J. Clin. Invest.* 90:229-237; Koulova, L. *et al.* (1991) *J. Exp. Med.* 173:759-762; Reiser, H. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:271-275; van-Seventer, G. A. *et al.* (1990) *J. Immunol.* 144:4579-4586; LaSalle, J. M. *et al.* (1991) *J. Immunol.* 147:774-80; Dustin, M. I. *et al.* (1989) *J. Exp. Med.* 169:503; Armitage, R. J. *et al.* (1992) *Nature* 357:80-82; Liu, Y. *et al.* (1992) *J. Exp. Med.* 175:437-445).

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The CD80 (B7-1) and CD86 (B7-2) proteins, expressed on APCs, are critical costimulatory molecules (Freeman *et al.* (1991) *J. Exp. Med.* 174:625; Freeman *et al.* (1989) *J. Immunol.* 143:2714; Azuma *et al.* (1993) *Nature* 366:76; Freeman *et al.* (1993) *Science* 262:909). B7-2 appears to play a predominant role during primary immune responses, while B7-1, which is upregulated later in the course of an immune response, may be important in prolonging primary T cell responses or costimulating secondary T cell responses (Bluestone (1995) *Immunity* 2:555).

One receptor to which B7-1 and B7-2 bind, CD28, is constitutively expressed on resting T cells and increases in expression after activation. After signaling through the T cell receptor, ligation of CD28 and transduction of a costimulatory signal induces T cells to proliferate and secrete IL-2 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 173:721-730; Gimmi, C. D. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:6575-6579; June, C. H. *et al.* (1990) *Immunol. Today.* 11:211-6; Harding, F. A. *et al.* (1992) *Nature* 356:607-609). A second receptor, termed CTLA4 (CD152) is homologous to CD28 but is not expressed on resting T cells and appears following T cell activation (Brunet, J. F. *et al.* (1987) *Nature* 328:267-270). CTLA4 appears to be critical in negative regulation of T cell responses (Waterhouse *et al.* (1995) *Science* 270:985). Blockade of CTLA4 has been found to remove inhibitory signals, while aggregation of CTLA4 has been found to provide inhibitory signals that downregulate T cell responses (Allison and Krummel (1995) *Science* 270:932). The B7 molecules have a higher affinity for CTLA4 than for CD28 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 174:561-569) and B7-1 and B7-2 have been found to bind to distinct regions of the CTLA4 molecule and have different kinetics of binding to CTLA4 (Linsley *et al.* (1994) *Immunity* 1:793). A new molecule related to CD28 and CTLA4, ICOS, has been identified and seems to be important in IL-10 production (Hutloff *et al.* (1999) *Nature* 397:263; WO 98/38216), as has its ligand, which is a new B7 family member (Aicher A. *et al.* (2000) *J. Immunol.* 164:4689-96; Mages H.W. *et al.* (2000) *Eur. J. Immunol.* 30:1040-7; Brodie D. *et al.* (2000) *Curr. Biol.* 10:333-6; Ling V. *et al.* (2000) *J. Immunol.* 164:1653-7; Yoshinaga S.K. *et al.* (1999) *Nature* 402:827-32). If T cells are only stimulated through the T cell receptor, without receiving an additional costimulatory signal, they become nonresponsive, anergic, or die, resulting in downmodulation of the immune response.

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The importance of the B7:CD28/CTLA4 costimulatory pathway has been demonstrated *in vitro* and in several *in vivo* model systems. Blockade of this costimulatory pathway results in the development of antigen specific tolerance in murine and human systems (Harding, F. A. *et al.* (1992) *Nature* 356:607-609; Lenschow, D. J. *et al.* (1992) *Science* 257:789-792; Turka, L. A. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:11102-11105; Gimmi, C. D. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6586-6590; Boussiotis, V. *et al.* (1993) *J. Exp. Med.* 178:1753-1763). Conversely, expression of B7 by B7 negative murine tumor cells induces T-cell mediated specific immunity accompanied by tumor rejection and long lasting protection to tumor challenge (Chen, L. *et al.* (1992) *Cell* 71:1093-1102; Townsend, S. E. and Allison, J. P. (1993) *Science* 259:368-370; Baskar, S. *et al.* (1993) *Proc. Natl. Acad. Sci.* 90:5687-5690.). Therefore, manipulation of the costimulatory pathways offers great potential to stimulate or suppress immune responses in humans.

Summary of the Invention

The present invention is based, at least in part, on the discovery that PD-1 is a receptor for B7-4 molecules expressed on antigen presenting cells. PD-1 transmits a negative signal to immune cells, similar to CTLA4. B7-4 molecules are expressed on the surface of antigen presenting cells and provide a costimulatory signal to immune cells and can transmit downmodulatory signals to immune cells, depending upon the molecule to which they bind. Thus, modulation of PD-1, B7-4, and/or the interaction between B7-4 and PD-1 results in modulation of the immune response.

Accordingly, in one aspect, the invention relates to a method for modulating an immune response comprising contacting an immune cell with an agent that modulates signaling via PD-1 to thereby modulate the immune response. The immune cell may be, for instance, a T cell, a B cell, or a myeloid cell. In one embodiment, the step of contacting occurs *in vivo*. In another embodiment, the step of contacting occurs *in vitro*.

In one embodiment, the immune response is downregulated. This may occur, for instance by stimulating signaling via PD-1 using an agent selected from the group consisting of: an activating antibody that recognizes PD-1, a form of B7-4 that binds to an inhibitory receptor, and a small molecule that binds to PD-1.

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In one embodiment, the stimulation of signaling via PD-1 induces anergy in the immune cell.

The method may further comprise contacting the immune cell with an additional agent that downregulates an immune response.

In yet another embodiment, the immune response is upregulated by inhibiton of signaling via PD-1. Signaling via PD-1 may be inhibited using an agent selected from the group consisting of: a blocking antibody that recognizes PD-1, a non-activating form of B7-4, an antibody that recognizes B7-4, and a soluble form of PD-1.

In another aspect, the invention pertains to a method for modulating the interaction of B7-4 with an inhibitory receptor on an immune cell comprising contacting an antigen presenting cell which expresses B7-4 with an agent selected from the group consisting of: a form of B7-4, a form of PD-1, or an agent that modulates the interaction of B7-4 and PD-1 such that the interaction of B7-4 with an inhibitory receptor on an immune cell is modulated. The immune cell may be, for instance, a T cell, a B cell, or a myeloid cell. In one embodiment, the step of contacting is performed *in vitro*. In another embodiment, the step of contacting is performed *in vivo*. The method may further comprise contacting the immune cell or the antigen presenting cell with an additional agent that modulates an immune response.

In another aspect, the invention pertains to a method for inhibiting activation of an immune cell via a non-apoptotic mechanism comprising increasing the activity or expression of PD-1 in the immune cell such that immune cell activation is inhibited.

In another aspect, the invention pertains to a vaccine comprising an antigen and an agent that inhibits signaling via PD-1 in an immune cell.

In another aspect, the invention pertains to a composition comprising an antigen and an agent that promotes signaling via PD-1 in an immune cell.

In another aspect, the invention pertains to a method for treating a subject having a condition that would benefit from upregulation of an immune response comprising administering an agent that inhibits signaling via PD-1 in a immune cell of the subject such that a condition that would benefit from upregulation of an immune response is treated. The condition may be, for instance, a tumor, a neurological disease or an immunosuppressive disease. In one embodiment, the agent that inhibits signaling via

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PD-1 comprises a soluble form of PD-1 or B7-4. In one embodiment, the method further comprises administering a second agent that upregulates an immune response to the subject.

In another aspect, the invention pertains to a method for treating a subject having a condition that would benefit from downregulation of an immune response comprising administering an agent that stimulates signaling via PD-1 in a immune cell of the subject such that a condition that would benefit from downregulation of an immune response is treated. The condition may be, for instance, a transplant, an allergy, or an autoimmune disorder. In one embodiment, the agent that stimulates signaling via PD-1 is an antibody that stimulates signaling via PD-1, a bispecific antibody, or soluble B7-4. The method may further comprise administering a second agent to the subject that downregulates an immune response.

In another aspect, the invention pertains to a cell-based assay for screening for compounds which modulate the activity of B7-4 or PD-1 comprising contacting a cell expressing a B7-4 target molecule or PD-1 target molecule with a test compound and determining the ability of the test compound to modulate the activity of the B7-4 or PD-1 target molecule.

In yet another aspect, the invention pertains to a cell-free assay for screening for compounds which modulate the binding of B7-4 or PD-1 to a target molecule comprising contacting a B7-4 or PD-1 protein or biologically active portion thereof with a test compound and determining the ability of the test compound to bind to the B7-4 or PD-1 protein or biologically active portion thereof.

Brief Description of the Drawings

Figure 1 depicts the nucleotide sequence encoding a human secreted B7-4, B7-4S (SEQ ID NO: 1).

Figure 2 depicts the nucleotide sequence encoding a human B7-4, B7-4M (SEQ ID NO: 3).

Figure 3 depicts the amino acid sequence of human B7-4S (SEQ ID NO: 2) and illustrates the signal, IgV, IgC, and hydrophilic tail domains.

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Figure 4 depicts the amino acid sequence of human B7-4M (SEQ ID NO: 4) and illustrates the signal, IgV, IgC, and transmembrane and cytoplasmic domains.

Figures 5A-5B depict the nucleotide sequence of murine B7-4 (SEQ ID NO: 22).

Figure 6 depicts the amino acid sequence of murine B7-4 (SEQ ID NO: 23).

Figure 7 depicts an alignment of the human B7-4M and murine B7-4 amino acid sequences (SEQ ID NO: 4 and 23 respectively).

Figure 8 illustrates the results of FACS analysis of binding of CD28Ig, CTLA4-Ig, and control Ig by B7-4M-transfected COS cells.

Figure 9 illustrates the results of FACS analysis of binding of IgG and murine ICOS-his fusion protein by B7-4M-transfected COS cells.

Figure 10 illustrates the results of FACS analysis of binding of IgM, BB1 and 133 antibodies to B7-4M- transfected COS cells.

Figure 11 illustrates that COS cells transfected with B7-4M (292) can costimulate T cell proliferation.

Figure 12 illustrates that COS cells transfected with a B7-4M (292) can costimulate T cell proliferation.

Figures 13A-13D illustrate the binding of PD-1 to B7-4M transfected COS cells.

Figures 14A-14F illustrate the ability of added PD-1 and not Flt4 to compete for the binding of PD-1 to B7-4M transfected COS cells.

Figures 15A-15L illustrate the ability of PD-1 to bind to B7-4 transfected CHO cells, as determined by flow cytometry.

Figure 16 illustrates the ability of PD-1 to bind to B7-4 transfected CHO cells, as determined by BIACORE analysis.

Figure 17 illustrates the ability of B7-4M to transmit a negative signal to T cells.

Figures 18A-18C illustrate the inhibition of T cell proliferation and cytokine production in human T cell stimulated in the presence of B7-4.

Figures 19A-19B illustrate that T cell receptor/B7-4 activation in the presence of CD28 costimulation results in inhibition of T cell proliferation.

Figures 20A-20I illustrate the binding of PD-1 to CHO cells expressing B7-4.

Figures 21A-21D illustrate the action of B7-4 in the inhibition of CD28 signals.

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Figures 22A-22D illustrate the inhibition of cytokine production by the PD-1:B7-4 pathway, as measured by cytokine ELISA.

Figures 23A-23C illustrate the inhibition of cytokine production by the PD-1:B7-4 pathway, as measured by cytokine mRNA levels.

Figures 24A-24C illustrate that the mechanism of action of the PD-1:B7-4 pathway is cell-cycle arrest.

Figures 25A-25B illustrate the ability of antibodies to B7-4 to inhibit the interaction between B7-4 and PD-1.

Figure 26 illustrates the ability of antibodies to PD-1 to inhibit the interaction between B7-4 and PD-1.

Figure 27 illustrates the ability of soluble B7-4Fc to exacerbate disease in a murine model of experimental autoimmune encephalomyelitis.

Figures 28A-28B illustrate the effect of PD-1:B7-4 interaction on mitotic cell division. T cells were labeled with CSFE and stimulated with ctrl.Fc or mB7-4.Fc beads. At the indicated time points, FACS analysis was done. Live-gated events are depicted. Figure 28A: CD4+ T cells. Figure28B: CD8+ T cells. PD-1:B7-4 interaction results in decreased mitotic divisions of both CD4+ and CD8+ T cells.

Figures 29A-29C illustrate the inhibition of both CD4+ and CD8+ T cells by PD-1:B7-4 interaction. Figure 29A illustrates schematically the cell lines and the experimental design. Stable antigen presenting cell (APC) lines were engineered to express GFP or mB7-4/GFP using retroviral technology. $5x10^4$ purified LN T cells from TCR transgenic (Tg) mice were stimulated with APC plus peptide for 2, 2-3, 3, or 4 days. For experiments involving CD4+ T cells, the APC:T cell ratio was 1:10 with 10 μM PCCF peptide. For experiments involving CD8+ T cells, the APC:T cell ratio was 1:1 with 1 mM p2Ca peptide. Figure 29B illustrates the inhibition of proliferation of CD4+ T cells by PD-1:B7-4 interaction. Figure 29C illustrates the inhibition of proliferation of CD8+ T cells by PD-1:B7-4 interaction.

Figures 30A-30B illustrate the ability of costimulation to overcome the inhibition of CD4+ but not CD8+ T cell proliferation by PD-1:B7-4 interaction. Figure 30A: CD4+ T cells. Figure 30B: CD8+ T cells.

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Figures 31A-31B illustrate schematically a screening assay used to identify proteins involved in the PD-1 signaling pathway. Figure 31A depicts a schematic of the steps of the assay. Figure 31B depicts the sequences of a fragment of human PD-1 (SEQ ID NO: 24), a fragment of mouse PD-1 (SEQ ID NO: 25) and the peptides used in the assay. Peptides used in the assay are the ITIM peptides: PD-1_Py1 (SEQ ID NO: 26); PD-1_Y1F (SEQ ID NO: 27); PD-1_Y1 (SEQ ID NO: 28); PD-1_Py2 (SEQ ID NO: 29); PD-1_Y2F (SEQ ID NO: 30); PD-1_Y2 (SEQ ID NO: 31); and Other Peptides: PD-1_K212_ (SEQ ID NO: 32); PD-1_K212D (SEQ ID NO: 33); PD-1_K335 (SEQ ID NO: 34); PD-1_K335D (SEQ ID NO: 35); PD-1_Ctail1 (SEQ ID NO: 36); and PD-1_Ctail2 (SEQ ID NO: 37).

Figure 32 is a photograph of a Western blot containing fractionated cell extracts of human T cells which were unstimulated or stimulated for 5 min., 30 min, or 90 min, in the presence or absence of a PDL1.Ig fusion protein. The blot was probed with an antibody specific for phosphorylated ERK1 and 2.

Figure 33 is a photograph of a Western blot containing fractionated cell extracts of human T cells which were unstimulated, or stimulated for 5 min, 30 min, 90 min. or 2 hr., in the presence (+) or absence (-) of a PDL1.Ig fusion protein. The blot was probed with an antibody specific for phorphorylated PKC-θ to specifically detect phosphorylated PKC-θ (anti-phospho-PKC-θ). The gel was stripped and then probed with an antibody which recognizes both phosphorylated and unphosphorylated PKC-θ (anti-PKC-θ).

Detailed Description of the Invention

In addition to the previously characterized B lymphocyte activation antigens, *e.g.*, B7-1 and B7-2, there are other antigens on the surface of antigen presenting cells which modulate costimulation of immune cells. For example, B7-4 polypeptides have been isolated from keratinocyte and placental cDNA libraries. B7-4 has also been found to costimulate or inhibit T cells. The present invention identifies PD-1 as a receptor for B7-4.

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Immune cells have receptors that transmit activating signals. For example, T cells have T cell receptors and the CD3 complex, B cells have B cell receptors, and myeloid cells have Fc receptors. In addition, immune cells bear receptors that transmit signals that provide costimulatory signals, or receptors that transmit signals that inhibit receptor-mediated signaling. For example, CD28 transmits a costimulatory signal to T cells. After ligation of the T cell receptor, ligation of CD28 results in a costimulatory signal characterized by, *e.g.*, upregulation of IL-2rα, IL-2rβ, and IL-2rγ receptor, increased transcription of IL-2 messenger RNA, and increased expression of cytokine genes (including IL-2, IFN-γ, GM-CSF, and TNF-α). Transmission of a costimulatory signal allows the cell to progress through the cell cycle and, thus, increases T cell proliferation (Greenfield *et al.* (1998) *Crit. Rev. Immunol.* 18:389). Binding of a receptor on a T cell which transmits a costimulatory signal to the cell (*e.g.*, ligation of a costimulatory receptor that leads to cytokine secretion and/or proliferation of the T cell) by a B7 family molecule, such as B7-4, results in costimulation. Thus, inhibition of an interaction between a B7 family molecule, such as B7-4, and a receptor that transmits a costimulatory signal on an immune cell results in a downmodulation of the immune response and/or specific unresponsiveness, termed immune cell anergy. Inhibition of this interaction can be accomplished using, *e.g.*, anti-CD28 Fab fragments, antibodies to B7-1, B7-2 and/or B7-4, or by using a soluble form of a receptor to which a B7 family member molecule can bind as a competitive inhibitor (*e.g.*, CTLA4Ig).

Inhibitory receptors that bind to costimulatory molecules have also been identified on immune cells. Activation of CTLA4, for example, transmits a negative signal to a T cell. Engagement of CTLA4 inhibits IL-2 production and can induce cell cycle arrest (Krummel and Allison (1996) *J. Exp. Med.* 183:2533). In addition, mice that lack CTLA4 develop lymphoproliferative disease (Tivol *et al.* (1995) *Immunity* 3:541; Waterhouse *et al.* (1995) *Science* 270:985). The blockade of CTLA4 with antibodies can block an inhibitory signal, whereas aggregation of CTLA4 with antibody transmits an inhibitory signal. Therefore, depending upon the receptor to which a costimulatory molecule binds (*i.e.*, a costimulatory receptor such as CD28 or an inhibitory receptor such as CTLA4), certain B7 molecules including B7-4 can promote T cell costimulation or inhibition.

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PD-1 is a member of the immunoglobulin family of molecules (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704). PD-1 was previously identified using a subtraction cloning based approach designed to identify modulators of programmed cell death (Ishida *et al.* (1992) *EMBO J.* 11:3887-95; Woronicz *et al.* (1995) *Curr. Top. Microbiol. Immunol.* 200:137). PD-1 is believed to play a role in lymphocyte survival, *e.g.*, during clonal selection (Honjo (1992) *Science* 258:591; Agata *et al.* (1996) *Int. Immunology.* 8:765; Nishimura *et al.* (1996) *Int. Immunology* 8:773). PD-1 was also implicated as a regulator of B cell responses (Nishimura (1998) *Int. Immunology* 10:1563). Unlike CTLA4, which is found only on T cells, PD-1 is also found on B cells and myeloid cells.

The instant discovery that PD-1 binds to B7-4 places PD-1 in a family of inhibitory receptors with CTLA4. While engagement (to produce activation) of a costimulatory receptor results in a costimulatory signal in an immune cell, engagement of an inhibitory receptor, *e.g.*, CTLA4 or PD-1 (for example by crosslinking or by aggregation), leads to the transmission of an inhibitory signal in an immune cell, resulting in downmodulation of immune cell responses and/or in immune cell anergy. While transmission of an inhibitory signal leads to downmodulation in immune cell responses (and a resulting downmodulation in the overall immune response), the prevention of an inhibitory signal (*e.g.*, by using a non-activating antibody against PD-1) in immune cells leads to upmodulation of immune cell responses (and a resulting upmodulation of an immune response).

The instant invention makes available agents useful for modulating the activity and/or expression of PD-1; the interaction between PD-1 and its natural ligand(s), (*e.g.*, B7-4); and agents for modulating the immune response via modulation of the interaction between PD-1 and its natural ligand, *e.g.*, B7-4, also known as PD-L1. Exemplary modulatory agents for use in these methods are described further as follows.

B7-4 and PD-1 Nucleic Acid and Polypeptide Molecules

In one embodiment, a modulatory agent useful for modulating the activity and/or expression of PD-1 is a B7-4 and/or PD-1 nucleic acid molecule, preferably a human B7-4 and/or PD-1 nucleic acid molecule.

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In one embodiment, the isolated nucleic acid molecules of the present invention encode eukaryotic B7-4 or PD-1 polypeptides. The B7-4 family of molecules share a number of conserved regions, including signal domains, IgV domains and the IgC domains. IgV domains and the IgC domains are art recognized Ig superfamily member domains. These domains correspond to structural units that have distinct folding patterns called Ig folds. Ig folds are comprised of a sandwich of two β sheets, each consisting of antiparallel β strands of 5-10 amino acids with a conserved disulfide bond between the two sheets in most, but not all, domains. IgC domains of Ig, TCR, and MHC molecules share the same types of sequence patterns and are called the C1-set within the Ig superfamily. Other IgC domains fall within other sets. IgV domains also share sequence patterns and are called V set domains. IgV domains are longer than C-domains and form an additional pair of β strands.

Two forms of human B7-4 molecules have been identified. One form is a naturally occurring B7-4 soluble polypeptide, *i.e.*, having a short hydrophilic domain and no transmembrane domain, and is referred to herein as B7-4S (shown in SEQ ID NO:2). The second form is a cell-associated polypeptide, *i.e.*, having a transmembrane and cytoplasmic domain, referred to herein as B7-4M (shown in SEQ ID NO:4).

B7-4 proteins comprise a signal sequence, and an IgV domain and an IgC domain. The signal sequence of SEQ ID NO:2 is shown from about amino acid 1 to about amino acid 18. The signal sequence of SEQ ID NO:4 is shown from about amino acid 1 to about amino acid 18. The IgV domain of SEQ ID NO:2 is shown from about amino acid 19 to about amino acid 134 and the IgV domain of SEQ ID NO:4 is shown from about amino acid 19 to about amino acid 134. The IgC domain of SEQ ID NO:2 is shown from about amino acid 135 to about amino acid 227 and the IgC domain of SEQ ID NO:4 is shown from about amino acid 135 to about amino acid 227. The hydrophilic tail of the B7-4 exemplified in SEQ ID NO:2 comprises a hydrophilic tail shown from about amino acid 228 to about amino acid 245. The B7-4 polypeptide exemplified in SEQ ID NO:4 comprises a transmembrane domain shown from about amino acids 239 to about amino acid 259 of SEQ ID NO:4 and a cytoplasmic domain shown from about amino acid 260 to about amino acid 290 of SEQ ID NO:4.

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Murine B7-4 molecules are also identified. The murine cDNA sequence is presented in Figures 5A-5B and the murine B7-4 amino acid sequence is presented in Figure 6. The present invention also pertains to these murine B7-4 molecules.

PD-1 is identified herein as a receptor which binds to B7-4. PD-1 molecules are members of the immunoglobulin gene superfamily. PD-1 (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; U.S. Patent 5,698,520) has an extracellular region containing immunoglobulin superfamily domain, a transmembrane domain, and an intracellular region including an immunoreceptor tyrosine-based inhibitory motif (ITIM). These features also define a larger family of molecules, called the immunoinhibitory receptors, which also includes gp49B, PIR-B, and the killer inhibitory receptors (KIRs) (Vivier and Daeron (1997) *Immunol. Today* 18:286). It is often assumed that the tyrosyl phosphorylated ITIM motif of these receptors interacts with SH2-domain containing phosphatases, which leads to inhibitory signals. A subset of these immunoinhibitory receptors bind to MHC molecules, for example the KIRs, and CTLA4 bind to B7-1 and B7-2. It has been proposed that there is a phylogenetic relationship between the MHC and B7 genes (Henry *et al.* (1999) *Immunol. Today* 20(6):285-8).

The nucleotide sequence of PD-1 is shown in SEQ ID NO:10 and 11 and the amino acid sequence of PD-1 is shown in SEQ ID NO:12 (see also Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; U.S. Patent 5,698,520). PD-1 was previously identified using a subtraction cloning based approach to select for proteins involved in apoptotic cell death. PD-1 is identified herein as a member of the CD28/CTLA-4 family of molecules based on its ability to bind to B7-4. Like CTLA4, PD-1 is rapidly induced on the surface of T-cells in response to anti-CD3 (Agata *et al.* (1996) *Int. Immunol.* 8:765). In contrast to CTLA4, however, PD-1 is also induced on the surface of B-cells (in response to anti-IgM). PD-1 is also expressed on a subset of thymocytes and myeloid cells (Agata *et al.* (1996) *supra*; Nishimura *et al.* (1996) *Int. Immunol.* 8:773). The instant invention identifies B7-4 as a ligand of PD-1.

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Various aspects of the invention are described in further detail in the following subsections:

I. Definitions

As used herein, the term "immune cell" includes cells that are of hematopoietic origin and that play a role in the immune response. Immune cells include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

As used herein, the term "T cell" includes cells bearing a T cell receptor (TCR). Preferably, the term "T cell" inculdes CD4+ T cells and/or CD8+ T cells. The term T cell also includes both T helper 1 type T cells and T helper 2 type T cells. The term "antigen presenting cell" includes professional antigen presenting cells (*e.g.*, B lymphocytes, monocytes, dendritic cells, Langerhans cells) as well as other antigen presenting cells (*e.g.*, keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes).

As used herein, the term "immune response" includes T cell mediated and/or B cell mediated immune responses that are influenced by modulation of T cell costimulation. Exemplary immune responses include T cell responses, *e.g.*, cytokine production, and cellular cytotoxicity. In addition, the term immune response includes immune responses that are indirectly effected by T cell activation, *e.g.*, antibody production (humoral responses) and activation of cytokine responsive cells, *e.g.*, macrophages.

As used herein, the term "costimulatory receptor" includes receptors which transmit a costimulatory signal to a immune cell, *e.g.*, CD28. As used herein, the term "inhibitory receptors" includes receptors which transmit a negative signal to an immune cell (*e.g.*, CTLA4 or PD-1). An inhibitory signal as transduced by an inhibitory receptor can occur even if a costimulatory receptor (such as CD28) is not present on the immune cell and, thus, is not simply a function of competition between inhibitory receptors and costimulatory receptors for binding of costimulatory molecules (Fallarino *et al.* (1998) *J. Exp. Med.* 188:205). Transmission of an inhibitory signal to an immune cell can result in unresponsiveness or anergy or programmed cell death in the immune cell.

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Preferably transmission of an inhibitory signal operates through a mechanism that does not involve apoptosis.

As used herein the term "apoptosis" includes programmed cell death which can be characterized using techniques which are known in the art. Apoptotic cell death can be characterized, *e.g.*, by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation. Cells undergoing apoptosis also display a characteristic pattern of internucleosomal DNA cleavage.

Depending upon the form of the B7-4 molecule that binds to a receptor, either a signal can be transmitted or stimulated (*e.g.*, by a multivalent form of a B7-4 molecule that results in crosslinking of receptor) or a signal can be inhibited (*e.g.*, by a soluble, monovalent form of a B7-4 molecule), *e.g.*, by competing with activating forms of B7-4 molecules for binding to the receptor. However, there are instances in which a soluble molecule can be stimulatory. The effects of the various modulatory agents can be easily demonstrated using routine screening assays as described herein.

As used herein, the term "costimulate" with reference to activated immune cells includes the ability of a costimulatory molecule to provide a second, non-activating receptor mediated signal (a "costimulatory signal") that induces proliferation or effector function. For example, a costimulatory signal can result in cytokine secretion, *e.g.*, in a T cell that has received a T cell-receptor-mediated signal. Immune cells that have received a cell-receptor mediated signal, *e.g.*, via an activating receptor are referred to herein as "activated immune cells."

As used herein, the term "activating receptor" includes immune cell receptors that bind antigen, complexed antigen (*e.g.*, in the context of MHC molecules), or bind to antibodies. Such activating receptors include T cell receptors (TCR), B cell receptors (BCR), cytokine receptors, LPS receptors, complement receptors, and Fc receptors.

For example, T cell receptors are present on T cells and are associated with CD3 molecules. T cell receptors are stimulated by antigen in the context of MHC molecules (as well as by polyclonal T cell activating reagents). T cell activation via the TCR results in numerous changes, *e.g.*, protein phosphorylation, membrane lipid changes, ion fluxes, cyclic nucleotide alterations, RNA transcription changes, protein synthesis changes, and cell volume changes.

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B cell receptors are present on B cells. B cell antigen receptors are a complex between membrane Ig (mIg) and other transmembrane polypeptides (*e.g.*, Igα and Igβ). The signal transduction function of mIg is triggered by crosslinking of receptor molecules by oligomeric or multimeric antigens. B cells can also be activated by anti-immunoglobulin antibodies. Upon BCR activation, numerous changes occur in B cells, including tyrosine phosphorylation.

Fc receptors are found on many cells which participate in immune responses. Fc receptors (FcRs) are cell surface receptors for the Fc portion of immunoglobulin molecules (Igs). Among the human FcRs that have been identified so far are those which recognize IgG (designated Fcγ R), IgE (Fcε R1), IgA (Fcα), and polymerized IgM/A (Fcμα R). FcRs are found in the following cell types: Fcε R I (mast cells), Fcε R.II (many leukocytes), Fcα R (neutrophils), and Fcμα R (glandular epithelium, hepatocytes) (Hogg, N. (1988) *Immunol. Today* 9:185-86). The widely studied FcγRs are central in cellular immune defenses, and are responsible for stimulating the release of mediators of inflammation and hydrolytic enzymes involved in the pathogenesis of autoimmune disease (Unkeless, J. C. *et al.* (1988) *Annu. Rev. Immunol.* 6:251-81). The FcγRs provide a crucial link between effector cells and the lymphocytes that secrete Ig, since the macrophage/monocyte, polymorphonuclear leukocyte, and natural killer (NK) cell FcγRs confer an element of specific recognition mediated by IgG. Human leukocytes have at least three different receptors for IgG: h Fcγ RI (found on monocytes/macrophages), hFcγ RII (on monocytes, neutrophils, eosinophils, platelets, possibly B cells, and the K562 cell line), and Fcγ III (on NK cells, neutrophils, eosinophils, and macrophages).

With respect to T cells, transmission of a costimulatory signal to a T cell involves a signaling pathway that is not inhibited by cyclosporine A. In addition, a costimulatory signal can induce cytokine secretion (*e.g.*, IL-2 and/or IL-10) in a T cell and/or can prevent the induction of unresponsiveness to antigen, the induction of anergy, or the induction of cell death in the T cell.

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As used herein, the term "inhibitory signal" refers to a signal transmitted via an inhibitory receptor (*e.g.*, CTLA4 or PD-1) for a molecule on a immune cell. Such a signal antagonizes a signal produced by an activating receptor (*e.g.*, via a TCR, CD3, BCR, or Fc molecule) and can result in, *e.g.*, inhibition of second messenger generation; an inhibition of proliferation; an inhibition of effector function in the immune cell, *e.g.*, reduced phagocytosis, reduced antibody production, reduced cellular cytotoxicity, the failure of the immune cell to produce mediators, (such as cytokines (*e.g.*, IL-2) and/or mediators of allergic responses); or the development of anergy.

As used herein, the term "unresponsiveness" includes refractivity of immune cells to stimulation, *e.g.*, stimulation via an activating receptor or a cytokine. Unresponsiveness can occur, *e.g.*, because of exposure to immunosuppressants or exposure to high doses of antigen. As used herein, the term "anergy" or "tolerance" includes refractivity to activating receptor-mediated stimulation. Such refractivity is generally antigen-specific and persists after exposure to the tolerizing antigen has ceased. For example, anergy in T cells (as opposed to unresponsiveness) is characterized by lack of cytokine production, *e.g.*, IL-2. T cell anergy occurs when T cells are exposed to antigen and receive a first signal (a T cell receptor or CD-3 mediated signal) in the absence of a second signal (a costimulatory signal). Under these conditions, reexposure of the cells to the same antigen (even if reexposure occurs in the presence of a costimulatory molecule) results in failure to produce cytokines and, thus, failure to proliferate. Anergic T cells can, however, mount responses to unrelated antigens and can proliferate if cultured with cytokines (*e.g.*, IL-2). For example, T cell anergy can also be observed by the lack of IL-2 production by T lymphocytes as measured by ELISA or by a proliferation assay using an indicator cell line. Alternatively, a reporter gene construct can be used. For example, anergic T cells fail to initiate IL-2 gene transcription induced by a heterologous promoter under the control of the 5' IL-2 gene enhancer or by a multimer of the AP1 sequence that can be found within the enhancer (Kang *et al.* (1992) *Science* 257:1134).

The B7-4 protein and nucleic acid molecules comprise a family of molecules having certain conserved structural and functional features. Similarly, the PD-1 protein and nucleic acid molecules are members of a family of molecules having conserved

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structural and functional features. The term "family" when referring to the protein and nucleic acid molecules is intended to mean two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide sequence homology as defined herein. Such family members can be naturally or non-naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or alternatively, can contain homologues of non-human origin. Members of a family may also have common functional characteristics. The B7-4 molecules described herein are members of the B7 family of molecules. The term "B7 family" or "B7 molecules" as used herein includes costimulatory molecules that share sequence homology with B7 polypeptides, *e.g.*, with B7-1, B7-2, B7-3 (recognized by the antibody BB-1), B7h (Swallow *et al.* (1999) *Immunity* 11:423), and/or B7-4. For example, human B7-1 and B7-2 share approximately 26% amino acid sequence identity when compared using the BLAST program at NCBI with the default parameters (Blosum62 matrix with gap penalties set at existence 11 and extension 1 (See http://www.ncbi.nlm.nih.gov)).

Preferred B7 polypeptides are capable of providing costimulatory or inhibitory signals to immune cells to thereby promote or inhibit immune cell responses. For example, when bound to a costimulatory receptor, B7-4 can induce costimulation of immune cells or can inhibit immune cell costimulation, *e.g.*, when present in soluble form. When bound to an inhibitory receptor, B7-4 molecules can transmit an inhibitory signal to an immune cell. Preferred B7 family members include B7-1, B7-2, B7-3 (recognized by the antibody BB-1), B7h, and B7-4 and soluble fragments or derivatives thereof. In one embodiment, B7 family members bind to one or more receptors on an immune cell, *e.g.*, CTLA4, CD28, ICOS, PD-1 and/or other receptors, and, depending on the receptor, have the ability to transmit an inhibitory signal or a costimulatory signal to an immune cell, preferably a T cell.

Preferred PD-1 molecules are capable of transmitting an inhibitory signal to an immune cell to thereby inhibit immune cell effector function or are capable of promoting costimulation (*e.g.*, by competitive inhibition) of immune cells, *e.g.*, when present in soluble, monomeric form. PD-1 induced signaling (also referred to herein as

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signaling via PD-1) is defined herein as one or more cellular events directly or indirectly induced in an immune cell which expresses PD-1 on its cell surface, by the binding if the receptor to a ligand, or other molecule, to activate the PD-1. Activation of PD-1 triggers a secondary signaling event which results in a cellular change. Such a cellular event can be detected , for instance, by inhibition of costimulation in a T cell activation assay, or by increased phosphorylation of the SHP-2 molecule, decreased phosphorylation of the ERK1/2 molecule, or decreased phosphorylation of the PKC-θ molecule. Under the appropriate circumstances the PD-1 induced signaling results in the downmodulation of an immune response by the immune cell.

Preferred PD-1 family members bind to one or more ligands, *e.g.*, B7-1, B7-2, B7-4, and/or other molecules on antigen presenting cells, and share sequence identity with PD-1.

In addition, in one embodiment, proteins that are members of a protein family are bound by antibodies generated against one or more other family member proteins. For example, the anti-BB1 antibody recognizes B7-4 molecules.

As used herein, the term "activity" with respect to a B7-4 or PD-1 polypeptide includes activities which are inherent in the structure of a B7-4 or PD-1 protein. With regard to B7-4, the term "activity" includes the ability to modulate immune cell costimulation, *e.g.*, by modulating a costimulatory signal in an activated immune cell, or to modulate inhibition by modulating an inhibitory signal in an immune cell, *e.g.*, by engaging a natural receptor on a immune cell. When an activating form of the B7-4 molecule binds to a costimulatory receptor, a costimulatory signal is generated in the immune cell. When an activating form of the B7-4 molecule binds to an inhibitory receptor, an inhibitory signal is generated in the immune cell.

Modulation of a costimulatory signal results in modulation of effector function of an immune cell. Thus, the term "B7-4 activity" includes the ability of a B7-4 polypeptide to bind its natural receptor(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

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With respect to PD-1, the term "activity" includes the ability of a PD-1 polypeptide to modulate an inhibitory signal in an activated immune cell, *e.g.*, by engaging a natural ligand on an antigen presenting cell. PD-1 transmits an inhibitory signal to an immune cell in a manner similar to CTLA4. Modulation of an inhibitory signal in an immune cell results in modulation of proliferation of and/or cytokine secretion by the immune cell. PD-1 can also modulate a costimulatory signal by competing with a costimulatory receptor for binding of a B7 molecule. Thus, the term "PD-1 activity" includes the ability of a PD-1 polypeptide to bind its natural ligand(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

As used herein, an "antisense" nucleic acid molecule comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid molecule can hydrogen bond to a sense nucleic acid molecule.

As used herein, the term "coding region" refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the term "noncoding region" refers to regions of a nucleotide sequence that are not translated into amino acids (*e.g.*, 5' and 3' untranslated regions).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the

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genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid molecule of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, a "transgenic animal" refers to a non-human animal, preferably a mammal, more preferably a mouse, in which one or more of the cells of the animal includes a "transgene". The term "transgene" refers to exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, for example directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal.

As used herein, a "homologous recombinant animal" refers to a type of transgenic non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

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As used herein, an "isolated protein" refers to a protein that is substantially free of other proteins, cellular material and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the B7-4 or PD-1 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of B7-4 or PD-1 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of B7-4 or PD-1 protein having less than about 30% (by dry weight) of non-B7-4 or PD-1 protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-B7-4 or PD-1 protein, still more preferably less than about 10% of non-B7-4 or PD-1 protein, and most preferably less than about 5% non-B7-4 or PD-1 protein. When the B7-4 or PD-1 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of B7-4 or PD-1 protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of B7-4 or PD-1 protein having less than about 30% (by dry weight) of chemical precursors or non-B7-4 or PD-1 chemicals, more preferably less than about 20% chemical precursors or non-B7-4 or PD-1 chemicals, still more preferably less than about 10% chemical precursors or non-B7-4 or PD-1 chemicals, and most preferably less than about 5% chemical precursors or non-B7-4 or PD-1 chemicals.

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The term "antibody" as used herein also includes an "antigen-binding portion" of an antibody (or simply "antibody portion"). The term "antigen-binding portion", as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, B7-4). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Osbourn *et al.* (1998) *Nat. Biotechnol.* 16:778). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Any VH and VL sequences of specific scFv can be linked to human immunoglobulin constant region cDNA or genomic sequences, in order to generate expression vectors encoding complete IgG molecules or other isotypes. VH and Vl can also be used in the generation of Fab, Fv or other fragments of immunoglobulins using either protein chemistry or recombinant DNA technology. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R. J. *et al.* (1994) *Structure* 2:1121-1123).

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Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecule, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S.M., *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S.M., *et al.* (1994) *Mol. Immunol.* 31:1047-1058). Antibody portions, such as Fab and $F(ab')_2$ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein.

Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof, *e.g.* humanized, chimeric, etc. Preferably, antibodies of the invention bind specifically or substantially specifically to B7-4 molecules. The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal antibodies" and "polyclonal antibody composition" refer to a population of antibody molecules that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody composition, typically displays a single binding affinity for a particular antigen with which it immunoreacts.

The term "humanized antibody", as used herein, is intended to include antibodies made by a non-human cell having variable and constant regions which have been altered to more closely resemble antibodies that would be made by a human cell. For example, by altering the non-human antibody amino acid sequence to incorporate amino acids found in human germline immunoglobulin sequences. The humanized antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs. The

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term "humanized antibody", as used herein, also includes antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds B7-4 is substantially free of antibodies that specifically bind antigens other than B7-4). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

There is a known and definite correspondence between the amino acid sequence of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite correspondence between the nucleotide sequence of a particular nucleic acid molecule and the amino acid sequence encoded by that nucleic acid molecule, as defined by the genetic code.

GENETIC CODE	
Alanine (Ala, A)	GCA, GCC, GCG, GCT
Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
Asparagine (Asn, N)	AAC, AAT
Aspartic acid (Asp, D)	GAC, GAT
Cysteine (Cys, C)	TGC, TGT
Glutamic acid (Glu, E)	GAA, GAG
Glutamine (Gln, Q)	CAA, CAG
Glycine (Gly, G)	GGA, GGC, GGG, GGT
Histidine (His, H)	CAC, CAT
Isoleucine (Ile, I)	ATA, ATC, ATT
Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
Lysine (Lys, K)	AAA, AAG
Methionine (Met, M)	ATG
Phenylalanine (Phe, F)	TTC, TTT
Proline (Pro, P)	CCA, CCC, CCG, CCT
Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
Threonine (Thr, T)	ACA, ACC, ACG, ACT
Tryptophan (Trp, W)	TGG
Tyrosine (Tyr, Y)	TAC, TAT
Valine (Val, V)	GTA, GTC, GTG, GTT
Termination signal (end)	TAA, TAG, TGA

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An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed (illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA molecule coding for a B7-4 or PD-1 polypeptide of the invention (or any portion thereof) can be used to derive the B7-4 or PD-1 amino acid sequence, using the genetic code to translate the DNA or RNA molecule into an amino acid sequence. Likewise, for any B7-4 or PD-1-amino acid sequence, corresponding nucleotide sequences that can encode B7-4 or PD-1 protein can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure herein of a B7-4 or PD-1 nucleotide sequence should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a B7-4 or PD-1 amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

The term "small molecule" is a term of the art and includes molecules that are less than about 1000 molecular weight or less than about 500 molecular weight. In one embodiment, small molecules do not exclusively comprise peptide bonds. In another embodiment, small molecules are not oligomeric. Exemplary small molecule compounds which can be screened for activity include, but are not limited to, peptides, peptidomimetics, nucleic acids, carbohydrates, small organic molecules (e.g., polyketides) (Cane et al. 1998. Science 282:63), and natural product extract libraries. In

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another embodiment, the compounds are small, organic non-peptidic compounds. In a further embodiment, a small molecule is not biosynthetic.

II. Isolated Nucleic Acid Molecules

In one embodiment, modulating agents for use in the claimed methods comprise isolated nucleic acid molecules that encode B7-4 or PD-1 proteins or biologically active portions thereof. Nucleic acid fragments sufficient for use as hybridization probes to identify B7-4 or PD-1-encoding nucleic acids (*e.g.*, B7-4 or PD-1 mRNA) and fragments for use as PCR primers for the amplification or mutation of B7-4 or PD-1 nucleic acid molecules are also provided. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid molecule (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid molecule is derived. For example, in various embodiments, the isolated B7-4 or PD-1 nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. An "isolated" B7-4 or PD-1 nucleic acid molecule may, however, be linked to other nucleotide sequences that do not normally flank the B7-4 or PD-1 sequences in genomic DNA (*e.g.*, the B7-4 or PD-1 nucleotide sequences may be linked

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to vector sequences). In certain preferred embodiments, an "isolated" nucleic acid molecule, such as a cDNA molecule, also may be free of other cellular material. However, it is not necessary for the B7-4 or PD-1 nucleic acid molecule to be free of other cellular material to be considered "isolated" (*e.g.*, a B7-4 or PD-1 DNA molecule separated from other mammalian DNA and inserted into a bacterial cell would still be considered to be "isolated").

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11 or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, using all or portion of the nucleic acid sequence of SEQ ID NO:1, 3, 10, or 11, as a hybridization probe, B7-4 or PD-1 nucleic acid molecules can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook, J. *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1, 3, 10, or 11 can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence of SEQ ID NO:1, 3, 10, or 11, respectively.

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to B7-4 or PD-1 nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11, or a portion of any of these nucleotide sequences. A nucleic acid molecule which is complementary to the nucleotide sequence

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shown in SEQ ID NO:1, 3, 10, or 11, is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11, respectively, such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11, respectively, thereby forming a stable duplex. An exact complement is 100% homologous to a specified nucleotide sequence.

In still another preferred embodiment, an isolated nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence (*e.g.*, to the entire length of the nucleotide sequence) shown in SEQ ID NO:1, 3, 10, or 11, or a portion of any of these nucleotide sequences.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the nucleic acid sequence of SEQ ID NO:1, 3, 10, or 11, for example a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of a B7-4 or PD-1 protein. The nucleotide sequence determined from the cloning of the B7-4 or PD-1 genes allows for the generation of probes and primers designed for use in identifying and/or cloning other B7-4 or PD-1 family members, as well as B7-4 or PD-1 family homologues from other species. The probe/primer typically comprises a substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12 or 15, preferably about 20 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 consecutive nucleotides of a sense sequence of SEQ ID NO:1, 3, 10, or 11, or of a naturally occurring allelic variant or mutant of SEQ ID NO:1, 3, 10, or 11. In an exemplary embodiment, a nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least 350, 400, 450, 500, 550, 600, 650, 700, 750, or 800 nucleotides in length and hybridizes under stringent hybridization conditions to a nucleic acid molecule of SEQ ID NO:1, 3, 10, or 11.

In another embodiment, a second nucleic acid molecule comprises at least about 500, 600, 700, 800, 900, or 1000 contiguous nucleotides of SEQ ID NO:1, 3, 10, or 11.

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In one embodiment, a nucleic acid molecule of the invention, *e.g.*, for use as a probe, does not include the portion of SEQ ID NO:1 from about nucleotides 815 to about 850 of SEQ ID NO:1 or about nucleotides 320 to 856 of SEQ ID NO:1. In another embodiment, a nucleic acid molecule of the invention does not include the portion of SEQ ID NO:3 from about nucleotides 314 to about 734, or from about nucleotides 835 to about 860, or from about nucleotides 1085 to about 1104 or from about nucleotides 1286 to about 1536 of SEQ ID NO:3.

In one embodiment, a nucleic acid molecule of the invention comprises at least about 500 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3. In a preferred embodiment, a nucleic acid molecule of the invention comprises at least about 600, at least about 700, at least about 800, at least about 900 or at least about 950 contiguous nucleotides of SEQ ID NO:1 or about 1000 contiguous nucleotides of SEQ ID NO:3. In another embodiment, a nucleic acid molecule of the invention comprises at least about 1500 or 1550 nucleotides of SEQ ID NO:3

Preferably, an isolated nucleic acid molecule of the invention comprises at least a portion of the coding region of SEQ ID NO:1 (shown in nucleotides 59-793) or SEQ ID NO:3 (shown in nucleotides 53-922). In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 1 to about nucleotide 319 of SEQ ID NO:1. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 855 to about nucleotide 968 of SEQ ID NO:1. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 1 to about nucleotide 314 of SEQ ID NO:3. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 955 to about nucleotide 1285 of SEQ ID NO:3. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 1535 to about nucleotide 1552 of SEQ ID NO:3.

In other embodiments, a nucleic acid molecule of the invention has at least 70% identity, more preferably 80% identity, and even more preferably 90% identity with a nucleic acid molecule comprising: at least about 500, at least about 600, at least about 700, at least about 800, at least about 900 or at least about 1000 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3.

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Probes based on the B7-4 or PD-1 nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto, *e.g.*, the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissues which misexpress a B7-4 or PD-1 protein, such as by measuring a level of a B7-4 or PD-1-encoding nucleic acid in a sample of cells from a subject *e.g.*, detecting B7-4 or PD-1 mRNA levels or determining whether a genomic B7-4 or PD-1 gene has been mutated or deleted.

A nucleic acid fragment encoding a "biologically active portion of a B7-4 or PD-1 protein" can be prepared by isolating a portion of the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11 which encodes a polypeptide having a B7-4 or PD-1 biological activity (the biological activities of the B7-4 or PD-1 proteins are described herein), expressing the encoded portion of the B7-4 or PD-1 protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the B7-4 or PD-1 protein.

Nucleic acid molecules that differ from SEQ ID NO:1, 3, 10, or 11 due to degeneracy of the genetic code, and thus encode the same B7-4 or PD-1 protein as that encoded by SEQ ID NO:1, 3, 10, or 11, are encompassed by the invention. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO:2, 4, or 12. In another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a B7-4 or PD-1 protein.

In addition to the B7-4 or PD-1 nucleotide sequences shown in SEQ ID NO:1, 3, 10, or 11 it should be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the B7-4or PD-1 proteins may exist within a population (*e.g.*, the human population). Such genetic polymorphism in the B7-4 or PD-1 genes may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which include an open reading frame encoding a B7-4 or PD-1 protein, preferably a mammalian B7-4 or PD-1 protein, and

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can further include non-coding regulatory sequences, and introns. Such natural allelic variations include both functional and non-functional B7-4 or PD-1 proteins and can typically result in 1-5% variance in the nucleotide sequence of a B7-4 or PD-1 gene. Such nucleotide variations and resulting amino acid polymorphisms in B7-4 or PD-1 genes that are the result of natural allelic variation and that do not alter the functional activity of a B7-4 or PD-1 protein are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding other B7-4 or PD-1 family members and, thus, which have a nucleotide sequence which differs from the B7-4 or PD-1 family sequences of SEQ ID NO:1, 3, 10, or 11 are intended to be within the scope of the invention. For example, another B7-4 or PD-1 cDNA can be identified based on the nucleotide sequence of human B7-4 or PD-1. Moreover, nucleic acid molecules encoding B7-4 or PD-1 proteins from different species, and thus which have a nucleotide sequence which differs from the B7-4 or PD-1 sequences of SEQ ID NO:1, 3, 10, or 11 are intended to be within the scope of the invention. For example, a mouse B7-4 or PD-1 cDNA can be identified based on the nucleotide sequence of a human B7-4 or PD-1 molecule.

Nucleic acid molecules corresponding to natural allelic variants and homologues of the B7-4 or PD-1 cDNAs of the invention can be isolated based on their homology to the B7-4 or PD-1 nucleic acids disclosed herein using the cDNAs disclosed herein, or a portion thereof, as a hybridization probe according to standard hybridization techniques. For example, a B7-4 or PD-1 DNA can be isolated from a human genomic DNA library using all or portion of SEQ ID NO:1, 3, 10, or 11 as a hybridization probe and standard hybridization techniques (*e.g.*, as described in Sambrook, J., *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of a B7-4 or PD-1 gene can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO:1, 3, 10, or 11. For example, mRNA can be isolated from cells (*e.g.*, by the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (1979) *Biochemistry* 18:5294-5299) and cDNA can be prepared using reverse transcriptase (*e.g.*, Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from

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Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11. A nucleic acid molecule of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to a B7-4 or PD-1 nucleotide sequence can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In another embodiment, an isolated nucleic acid molecule of the invention is at least 15, 20, 25, 30 or more nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11. In other embodiment, the nucleic acid molecule is at least 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, or 600 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 30%, 40%, 50%, or 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1, 3, 10, or 11 corresponds to a naturally-occurring nucleic acid molecule.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein). In addition to the B7-4 or PD-1 nucleotide sequences shown in SEQ ID NO:1, 3, 10, and 11, it should be appreciated by those skilled in the art that DNA

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sequence polymorphisms that lead to minor changes in the nucleotide or amino acid sequences of a B7-4 or PD-1 may exist within a population. Such genetic polymorphism in a B7-4 or PD-1 gene may exist among individuals within a population due to natural allelic variation. Such natural allelic variations can typically result in 1-2 % variance in the nucleotide sequence of the gene. Such nucleotide variations and resulting amino acid polymorphisms in a B7-4 or PD-1 that are the result of natural allelic variation and that do not alter the functional activity of a B7-4 or PD-1 polypeptide are within the scope of the invention.

In addition to naturally-occurring allelic variants of B7-4 or PD-1 sequences that may exist in the population, the skilled artisan will further appreciate that minor changes may be introduced by mutation into nucleotide sequences, *e.g.*, of SEQ ID NO:1, 3, 10, or 11, thereby leading to changes in the amino acid sequence of the encoded protein, without altering the functional activity of a B7-4 or PD-1 protein. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues may be made in the sequence of SEQ ID NO:1, 3, 10, or 11. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of a B7-4 nucleic acid molecule (*e.g.*, the sequence of SEQ ID NO:1, 3, 10, or 11) without altering the functional activity of a B7-4 or PD-1 molecule. Preferably, residues in the extracellular domain of B7-4 or PD-1 which are found to be required for binding of B7-4 to a receptor or PD-1 to a natural ligand (*e.g.*, identified using an alanine scanning mutagenesis screen or other art recognized screening assay) are not altered. For B7-4 molecules, exemplary residues which are non-essential and, therefore, amenable to substitution, can be identified by one of ordinary skill in the art by performing an amino acid alignment of B7 family members (or of B7-4 family members) and determining residues that are not conserved. Such residues, because they have not been conserved, are more likely amenable to substitution.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding B7-4 or PD-1 proteins that contain changes in amino acid residues that are not essential for a B7-4 or PD-1 activity. Such B7-4 or PD-1 proteins differ in amino acid sequence from SEQ ID NO:2, 4, or 12 yet retain an inherent B7-4 activity or, in the case of PD-1, retain the ability to bind to B7-4. An isolated nucleic acid molecule encoding a

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non-natural variant of a B7-4 or PD-1 protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO:1, 3, 10, or 11 by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in a B7-4 or PD-1 is preferably replaced with another amino acid residue from the same side chain family.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a B7-4 or PD-1 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for their ability to bind to DNA and/or activate transcription, to identify mutants that retain functional activity. Following mutagenesis, the encoded B7-4 or PD-1 mutant protein can be expressed recombinantly in a host cell and the functional activity of the mutant protein can be determined using assays available in the art for assessing a B7-4 or PD-1 activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding B7-4 or PD-1 proteins that contain changes in amino acid residues that are not essential for activity.

Yet another aspect of the invention pertains to isolated nucleic acid molecules encoding a B7-4 or PD-1 fusion proteins. Such nucleic acid molecules, comprising at least a first nucleotide sequence encoding a B7-4 or PD-1 protein, polypeptide or peptide operatively linked to a second nucleotide sequence encoding a non- a B7-4 or PD-1

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protein, polypeptide or peptide, can be prepared by standard recombinant DNA techniques.

In a preferred embodiment, a mutant B7-4 protein can be assayed for the ability to: 1) costimulate (or inhibit the costimulation of, *e.g.*, in soluble form) the proliferation and/or effector function of activated immune cells; 2) bind to an anti-B7 family- or anti B7-4-antibody; and/or 3) bind to a natural receptor(s) of B7-4 (*e.g.*, PD-1).

In a preferred embodiment, a mutant PD-1 protein can be assayed for the ability to: 1) inhibit the costimulation of (*e.g.*, in soluble form) the proliferation and/or effector function of activated immune cells; 2) bind to an anti-PD-1 antibody; and/or 3) bind to a natural ligand(s) of PD-1 (*e.g.*, B7-4).

In addition to the nucleic acid molecules encoding B7-4 or PD-1 proteins described above, isolated nucleic acid molecules which are antisense thereto can be used as modulating agents. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire B7-4 or PD-1 coding strand, or only to a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding B7-4 or PD-1. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding B7-4 or PD-1. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

Given the coding strand sequences encoding B7-4 or PD-1 disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of B7-4 or PD-1 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of

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B7-4 or PD-1 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of B7-4 or PD-1 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid molecule (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid is of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a B7-4 or PD-1 protein to thereby inhibit expression of

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the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids. Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid molecule of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid molecule, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haseloff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave B7-4 or PD-1 mRNA transcripts to thereby inhibit translation of B7-4 or PD-1 mRNA. A ribozyme having specificity for a B7-4 or PD-1-encoding nucleic acid can be designed based upon the nucleotide sequence of a B7-4 or PD-1 cDNA disclosed herein (*i.e.*, SEQ ID NO:1, 3, 10, or 11). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide

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sequence of the active site is complementary to the nucleotide sequence to be cleaved in a B7-4 or PD-1-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, B7-4 or PD-1 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J. W. (1993) *Science* 261:1411-1418.

Alternatively, B7-4 or PD-1 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the B7-4 or PD-1 (*e.g.*, the B7-4 or PD-1 promoter and/or enhancers) to form triple helical structures that prevent transcription of the B7-4 or PD-1 gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L. J. (1992) *Bioessays* 14(12):807-15.

In yet another embodiment, the B7-4 or PD-1 nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety, or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup, B. and Nielsen, P. E. (1996) *Bioorg. Med. Chem.* 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup and Nielsen (1996) *supra* and Perry-O'Keefe *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14670-675.

PNAs of B7-4 or PD-1 nucleic acid molecules can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of B7-4 or PD-1 nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as 'artificial restriction enzymes' when used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup and Nielsen (1996)

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supra)); or as probes or primers for DNA sequencing or hybridization (Hyrup B. and Nielsen (1996) *supra*; Perry-O'Keefe *et al.* (1996) *supra*).

In another embodiment, PNAs of B7-4 or PD-1 can be modified, (*e.g.*, to enhance their stability or cellular uptake), by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of B7-4 or PD-1 nucleic acid molecules can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, (*e.g.*, RNAse H and DNA polymerases), to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup B. and Nielsen (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup B. and Nielsen (1996) *supra* and Finn P. J. *et al.* (1996) *Nucleic Acids Res.* 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry. Modified nucleoside analogs, (*e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite), can be used as a linker between the PNA and the 5' end of DNA (Mag, M. *et al.* (1989) *Nucleic Acids Res.* 17:5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn P. J. *et al.* (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K. H. *et al.* (1975) *Bioorganic Med. Chem. Lett.* 5:1119-11124).

In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.* (1988) *Biotechniques* 6:958-976) or intercalating agents (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, the

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oligonucleotide can be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

III. Isolated B7-4 or PD-1 Proteins and Anti-B7-4 or PD-1 Antibodies

In addition, isolated B7-4 or PD-1 proteins, and biologically active portions thereof, as well as anti-B7-4 or PD-1 antibodies can be used as modulating agents. In one embodiment, native B7-4 or PD-1 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, B7-4 or PD-1 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a B7-4 or PD-1 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

Another aspect of the invention pertains to isolated B7-4 or PD-1 proteins. Preferably, the B7-4 or PD-1 proteins comprise the amino acid sequence encoded by SEQ ID NO:1, 3, 10 or 11. In another preferred embodiment, the protein comprises the amino acid sequence of SEQ ID NO:2, 4, or 12. In other embodiments, the protein has at least 50%, at least 60% amino acid identity, more preferably 70% amino acid identity, more preferably 80%, and even more preferably, 90% or 95% amino acid identity with the amino acid sequence shown in SEQ ID NO:2, 4, or 12.

In other embodiments, the invention provides isolated portions of a B7-4 or PD-1 protein. For example, B7-4 proteins comprise a signal sequence, and an IgV domain and an IgC domain. The signal sequence of SEQ ID NO:2 is shown from about amino acid 1 to about amino acid 18. The signal sequence of SEQ ID NO:4 is shown from about amino acid 1 to about amino acid 18. The IgV domain of SEQ ID NO:2 is shown from about amino acid 19 to about amino acid 134 and the IgV domain of SEQ ID NO:4 is shown from about amino acid 19 to about amino acid 134. The IgC domain of SEQ ID NO:2 is shown from about amino acid 135 to about amino acid 227 and the IgC domain of SEQ ID NO:4 is shown from about amino acid 135 to about amino acid 227. The B7-4 molecule exemplified in SEQ ID NO:2 comprises a hydrophilic tail shown from about amino acid 228 to about amino acid 245. The B7-4 polypeptide exemplified in SEQ ID NO:4 comprises a transmembrane domain shown from about amino acid 239 to about

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amino acid 259 of SEQ ID NO:4 and a cytoplasmic domain shown from about amino acid 260 to about amino acid 290 of SEQ ID NO:4. The PD-1 polypeptide is 288 amino acids in length and its domain structure is known in the art (Shinohara *et al.* (1994) *Genomics* 23:704). The predicted mature form of the protein contains about 268 amino acids and comprises an extracellular domain (147 amino acids), a transmembrane domain (27 amino acids), a transmembrane region (27 amino acids) and a cytoplasmic domain (94 amino acids). Four potential N-glycosylation sites are found in the extracellular domain (U.S. Patent 5,698,520). The 68 amino acid residues between two cysteine residues (cys 54 and cys 123) bear resemblance to a disulfide-linked immunoglobulin domain of the V-set sequences (U.S. Patent 5,698,520).

The invention further pertains to soluble forms of B7-4 or PD-1 proteins. Such forms can be naturally occurring, *e.g.*, as shown in SEQ ID NO:2 or can be engineered and can comprise, *e.g.*, an extracellular domain of a B7-4 or PD-1 protein. Exemplary B7-4 extracellular domains comprise from about amino acids 19-238 of SEQ ID NO:4. Exemplary PD-1 extracellular domains comprise from about amino acids 21-288 of SEQ ID NO:12.

In one embodiment, the extracellular domain of a B7-4 polypeptide comprises the mature form of a B7-4 polypeptide, *e.g.*, the IgV and IgC domains, but not the transmembrane and cytoplasmic domains of a B7-4 polypeptide (*e.g.*, from about amino acid 19 to amino acid 238 of SEQ ID NO:4) or from about amino acid 19 to amino acid 245 of SEQ ID NO:2.

In one embodiment, the extracellular domain of a PD-1 polypeptide comprises the mature form of a PD-1 polypeptide, *e.g.*, immunoglobulin superfamily domains (*e.g.*, V-set sequences), but not the transmembrane and cytoplasmic domains of a PD-1 polypeptide (*e.g.*, from about amino acid 21-288 of SEQ ID NO:12).

Biologically active portions of a B7-4 or PD-1 protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the amino acid sequence of the B7-4 or PD-1 protein, which include fewer amino acids than the full length B7-4 or PD-1 proteins, and exhibit at least one activity of a B7-4 or PD-1 protein, preferably the ability to bind to a natural binding partner. Typically, biologically active portions comprise a domain or motif with at least one activity of the

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B7-4 or PD-1 protein. A biologically active portion of a B7-4 or PD-1 protein can be a polypeptide which is, for example, at least 10, 25, 50, 100, 150, 200 or more amino acids in length.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The residues or nucleic acids at corresponding positions are then compared and when a position in one sequence is occupied by the same residue or nucleic acid as the corresponding position in the other sequence, then the molecules are identical at that position. The percent identity between two sequences, therefore, is a function of the number of identical positions shared by two sequences (*i.e.*, % identity = # of identical positions/total # of positions x 100). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. As used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology".

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the GAP program in the GCG software package, using either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6.

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The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to B7-4 or PD-1 nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to B7-4 or PD-1 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. For example, the nucleotide sequences of the invention were analyzed using the default Blastn matrix 1-3 with gap penalties set at: existence 11 and extension 1. The amino acid sequences of the invention were analyzed using the default settings: the Blosum 62 matrix with gap penalties set at existence 11 and extension 1. (See, e.g., the NCBI web page).

The invention also provides B7-4 or PD-1 chimeric or fusion proteins. As used herein, a B7-4 or PD-1 "chimeric protein" or "fusion protein" comprises a B7-4 or PD-1 polypeptide operatively linked to a non-B7-4 or PD-1 polypeptide. A "B7-4 or PD-1 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to B7-4 or PD-1 polypeptide, whereas a "non-B7-4 or PD-1 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the B7-4 or PD-1 protein, *e.g.*, a protein which is different from the B7-4 or PD-1 protein and which is derived from the same or a different organism. Within a B7-4 or PD-1 fusion protein the B7-4 or PD-1 polypeptide can correspond to all or a portion of a B7-4 or PD-1 protein. In a preferred embodiment, a B7-4 or PD-1 fusion protein comprises at least one biologically active portion of a B7-4 or PD-1 protein, *e.g.*, an extracellular domain of a B7-4 or PD-1 protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the B7-4 or PD-1 polypeptide and the non-B7-4 or PD-1 polypeptide are fused in-frame to each other.

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The non-B7-4 or PD-1 polypeptide can be fused to the N-terminus or C-terminus of the B7-4 or PD-1 polypeptide.

For example, in one embodiment, the fusion protein is a GST-B7-4 or GST-PD-1 fusion protein in which the B7-4 or PD-1 sequences are fused to the C-terminus of the GST sequences. In another embodiment, the fusion protein is a B7-4 or PD-1-HA fusion protein in which the B7-4 or PD-1 nucleotide sequence is inserted in a vector such as pCEP4-HA vector (Herrscher, R. F. *et al.* (1995) *Genes Dev.* 9:3067-3082) such that the B7-4 or PD-1 sequences are fused in frame to an influenza hemagglutinin epitope tag. Such fusion proteins can facilitate the purification of a recombinant B7-4 or PD-1 protein.

A B7-4 or PD-1 fusion protein can be produced by recombinant expression of a nucleotide sequence encoding a first peptide having B7-4 activity, and a nucleotide sequence encoding a second peptide corresponding to a moiety that alters the solubility, affinity, stability or valency of the first peptide, for example, an immunoglobulin constant region. Preferably, the first peptide consists of a portion of the B7-4 polypeptide (*e.g.*, a portion of amino acid residues 1-238 or 19-238 (after cleavage of the signal sequence) of the sequence shown in SEQ ID NO:4 that is sufficient to modulate costimulation or inhibition of activated immune cells). In another preferred embodiment, the first peptide consists of a portion of a PD-1 polypeptide (*e.g.*, a portion of amino acid residues 1-288 (or 21-288 after cleavage of the signal peptide) of the sequence shown in SEQ ID NO:12 that is sufficient to modulate costimulation or inhibition of activated immune cells). The second peptide can include an immunoglobulin constant region, for example, a human Cγ1 domain or Cγ4 domain (*e.g.*, the hinge, CH2 and CH3 regions of human IgCγ1, or human IgCγ4, see *e.g.*, Capon *et al.* US patent 5,116,964; 5,580,756; 5,844,095 and the like, incorporated herein by reference). A resulting fusion protein may have altered B7-4 or PD-1 solubility, binding affinity, stability and/or valency (*i.e.*, the number of binding sites available per molecule) and may increase the efficiency of protein purification. Fusion proteins and peptides produced by recombinant techniques can be secreted and isolated from a mixture of cells and medium containing the protein or peptide. Alternatively, the protein or peptide can be retained cytoplasmically and the cells harvested, lysed and the

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protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. Protein and peptides can be isolated from cell culture media, host cells, or both using techniques known in the art for purifying proteins and peptides. Techniques for transfecting host cells and purifying proteins and peptides are known in the art.

Particularly preferred B7-4 or PD-1 Ig fusion proteins include the extracellular domain portion or variable region-like domain of a human B7-4 or PD-1 coupled to an immunoglobulin constant region (*e.g.*, the Fc region). The immunoglobulin constant region may contain genetic modifications which reduce or eliminate effector activity inherent in the immunoglobulin structure. For example, DNA encoding the extracellular portion of a B7-4 or PD-1 polypeptide can be joined to DNA encoding the hinge, CH2 and CH3 regions of human IgGγ1 and/or IgGγ4 modified by site directed mutagenesis, *e.g.*, as taught in WO 97/28267. Fusion proteins of, *e.g.*, B7-4 and an immunoglobulin fusion protein may be referred to interchangeably herein as "B7-4.Ig" or "B7-4.Fc". Other variations which incorporate the terms "Ig" or "Fc" may also be used.

Preferably, a B7-4 or PD-1 fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide or an HA epitope tag). A B7-4 or PD-1 encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the B7-4 or PD-1 protein.

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In another embodiment, the fusion protein is a B7-4 or PD-1 protein containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of B7-4 or PD-1 can be increased through use of a heterologous signal sequence.

The B7-4 or PD-1 fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. Use of B7-4 or PD-1 fusion proteins is useful for therapeutic modulation of an immune response to, for instance, treatment of immunological disorders, *e.g.*, autoimmune diseases, or in the case of inhibiting rejection of transplants. Moreover, the B7-4 or PD-1-fusion proteins of the invention, (e.g., full length protein or portions thereof) can be used as immunogens to produce anti-B7-4 or PD-1 antibodies in a subject, to purify B7-4 or PD-1 and in screening assays to identify molecules which inhibit the interaction of B7-4 with a B7-4 receptor, *e.g.*, PD-1.

Preferably, a B7-4 or PD-1 chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of nucleic acid (e.g., gene) fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive nucleic acid fragments which can subsequently be annealed and reamplified to generate a chimeric nucleic acid sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A B7-4 or PD-1-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the B7-4 or PD-1 protein.

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The present invention also pertains to variants of the B7-4 or PD-1 proteins which function as either B7-4 or PD-1 agonists (mimetics) or as B7-4 or PD-1 antagonists. Variants of the B7-4 or PD-1 proteins can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of a B7-4 or PD-1 protein. An agonist of the B7-4 or PD-1 proteins can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of a B7-4 or PD-1 protein. An antagonist of a B7-4 or PD-1 protein can inhibit one or more of the activities of the naturally occurring form of the B7-4 or PD-1 protein by, for example, competitively modulating a cellular activity of a B7-4 or PD-1 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the B7-4 or PD-1 protein.

In one embodiment, variants of a B7-4 or PD-1 protein which function as either B7-4 or PD-1 agonists (mimetics) or as B7-4 or PD-1 antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, point mutants or truncation mutants, of a B7-4 or PD-1 protein for B7-4 or PD-1 protein agonist or antagonist activity. In one embodiment, a variegated library of B7-4 or PD-1 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of B7-4 or PD-1 variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential B7-4 or PD-1 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of B7-4 or PD-1 sequences therein. There are a variety of methods which can be used to produce libraries of potential B7-4 or PD-1 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential B7-4 or PD-1 sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S. A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984)

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Annu. Rev. Biochem. 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acids Res.* 11:477.

In addition, libraries of fragments of a B7-4 or PD-1 protein coding sequence can be used to generate a variegated population of B7-4 or PD-1 fragments for screening and subsequent selection of variants of a B7-4 or PD-1 protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a B7-4 or PD-1 coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the B7-4 or PD-1 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of B7-4 or PD-1 proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify B7-4 or PD-1 variants (Arkin and Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delagrave *et al.* (1993) *Protein Eng.* 6(3):327-331).

In one embodiment, cell based assays can be exploited to analyze a variegated B7-4 or PD-1 library. For example, a library of expression vectors can be transfected into a cell line which ordinarily synthesizes and secretes B7-4 or PD-1. The transfected

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cells are then cultured such that B7-4 or PD-1 and a particular mutant B7-4 or PD-1 are secreted and the effect of expression of the mutant on B7-4 or PD-1 activity in cell supernatants can be detected, *e.g.*, by any of a number of functional assays. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of B7-4 or PD-1 activity, and the individual clones further characterized.

In addition to B7-4 or PD-1 polypeptides consisting only of naturally-occurring amino acids, B7-4 or PD-1 peptidomimetics are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) *Adv. Drug Res.* 15:29; Veber and Freidinger (1985) TINS p.392; and Evans *et al.* (1987) *J. Med. Chem.* 30:1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), such as human B7-4 or PD-1, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH2NH-, -CH2S-, -CH2-CH2-, -CH=CH- (cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-, by methods known in the art and further described in the following references: Spatola, A. F. in "*Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*" Weinstein, B., ed., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J. S. (1980) *Trends Pharm. Sci.* pp. 463-468 (general review); Hudson, D. *et al.* (1979) *Int. J. Pept. Prot. Res.* 14:177-185 (-CH2NH-, CH2CH2-); Spatola, A. F. *et al.* (1986) *Life Sci.* 38:1243-1249 (-CH2-S); Hann, M. M. (1982) *J. Chem. Soc. Perkin Trans. I.* 307-314 (-CH-CH-, cis and trans); Almquist, R. G. *et al.* (190) *J. Med. Chem.* 23:1392-1398 (-COCH2-); Jennings-White, C. *et al.* (1982) *Tetrahedron Lett.* 23:2533 (-COCH2-); Szelke, M. *et al.* European Appln. EP 45665 (1982) CA: 97:39405 (1982)(-CH(OH)CH2-); Holladay, M. W. *et al.* (1983) *Tetrahedron Lett.* (1983) 24:4401-4404 (-C(OH)CH2-); and Hruby, V. J. (1982) *Life*

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Sci. (1982) 31:189-199 (-CH2-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH2NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

Systematic substitution of one or more amino acids of a B7-4 or PD-1 amino acid sequence with a D-amino acid of the same type (*e.g.*, D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides comprising a B7-4 or PD-1 amino acid sequence or a substantially identical sequence variation can be generated by methods known in the art (Rizo and Gierasch (1992) *Annu. Rev. Biochem.* 61:387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences of B7-4 or PD-1 polypeptides identified herein will enable those of skill in the art to produce polypeptides corresponding to B7-4 or PD-1 peptide sequences and sequence variants thereof. Such polypeptides can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a B7-4 or PD-1 peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis *et al. Molecular Cloning: A Laboratory Manual* (1989), 2nd Ed.,

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Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) *J. Am. Chem. Soc.* 91:501; Chaiken I. M. (1981) *CRC Crit. Rev. Biochem.* 11: 255; Kaiser *et al.* (1989) *Science* 243:187; Merrifield, B. (1986) *Science* 232:342; Kent, S. B. H. (1988) *Annu. Rev. Biochem.* 57:957; and Offord, R. E. (1980) *Semisynthetic Proteins*, Wiley Publishing, which are incorporated herein by reference).

Peptides can be produced, typically by direct chemical synthesis, and used *e.g.*, as agonists or antagonists of a B7-4/PD-1 interaction. Peptides can be produced as modified peptides, with nonpeptide moieties attached by covalent linkage to the N-terminus and/or C-terminus. In certain preferred embodiments, either the carboxy-terminus or the amino-terminus, or both, are chemically modified. The most common modifications of the terminal amino and carboxyl groups are acetylation and amidation, respectively. Amino-terminal modifications such as acylation (*e.g.*, acetylation) or alkylation (*e.g.*, methylation) and carboxy-terminal-modifications such as amidation, as well as other terminal modifications, including cyclization, can be incorporated into various embodiments of the invention. Certain amino-terminal and/or carboxy-terminal modifications and/or peptide extensions to the core sequence can provide advantageous physical, chemical, biochemical, and pharmacological properties, such as: enhanced stability, increased potency and/or efficacy, resistance to serum proteases, desirable pharmacokinetic properties, and others. Peptides can be used therapeutically to treat disease, *e.g.*, by altering costimulation in a patient.

An isolated B7-4 or PD-1 protein, or a portion or fragment thereof (or a nucleic acid molecule encoding such a polypeptide), can be used as an immunogen to generate antibodies that bind B7-4 or PD-1 using standard techniques for polyclonal and monoclonal antibody preparation. A full-length B7-4 or PD-1 protein can be used, or alternatively, the invention provides antigenic peptide fragments of B7-4 or PD-1 for use as immunogens. The antigenic peptide of B7-4 or PD-1 comprises at least 8 amino acid residues and encompasses an epitope of B7-4 or PD-1 such that an antibody raised against the peptide forms a specific immune complex with B7-4 or PD-1. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least

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15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

Alternatively, an antigenic peptide fragment of a B7-4 or PD-1 polypeptide can be used as the immunogen. An antigenic peptide fragment of a B7-4 or PD-1 polypeptide typically comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2, 4, or 12 and encompasses an epitope of a B7-4 or PD-1 polypeptide such that an antibody raised against the peptide forms an immune complex with a B7-4 or PD-1 molecule. Preferred epitopes encompassed by the antigenic peptide are regions of B7-4 or PD-1 that are located on the surface of the protein, *e.g.*, hydrophilic regions. In one embodiment, an antibody binds substantially specifically to a molecule which contains a portion of a B7-4 or PD-1 molecule (e.g., the extracellular portion). In another embodiment, an antibody binds specifically to a B7-4 or PD-1 polypeptide.

Preferably, the antigenic peptide comprises at least about 10 amino acid residues, more preferably at least about 15 amino acid residues, even more preferably at least about 20 amino acid residues, and most preferably at least about 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of a B7-4 or PD-1 polypeptide that are located on the surface of the protein, *e.g.*, hydrophilic regions, and that are unique to a B7-4 or PD-1 polypeptide. In one embodiment such epitopes can be specific for a B7-4 or PD-1 protein from one species, such as mouse or human (*i.e.*, an antigenic peptide that spans a region of a B7-4 or PD-1 polypeptide that is not conserved across species is used as immunogen; such non conserved residues can be determined using an alignment such as that provided herein). A standard hydrophobicity analysis of the B7-4 or PD-1 protein can be performed to identify hydrophilic regions.

A B7-4 or PD-1 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, a recombinantly expressed B7-4 or PD-1 protein or peptide fragment, or a chemically synthesized B7-4 or PD-1 peptide fragment. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar

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immunostimulatory agent. Immunization of a suitable subject with an immunogenic B7-4 or PD-1 preparation induces a polyclonal anti- B7-4 or PD-1 antibody response.

In another embodiment, nucleic acid vaccines can be administered by a variety of means, for example, by injection (*e.g.*, intramuscular, intradermal, or the biolistic injection of DNA-coated gold particles into the epidermis with a gene gun that uses a particle accelerator or a compressed gas to inject the particles into the skin (Haynes *et al.* (1996) *J. Biotechnol.* 44:37)). Alternatively, nucleic acid vaccines can be administered by non-invasive means. For example, pure or lipid-formulated DNA can be delivered to the respiratory system or targeted elsewhere, *e.g.*, Peyers patches by oral delivery of DNA (Schubbert (1997) *Proc. Natl. Acad. Sci. USA* 94:961). Attenuated microorganisms can be used for delivery to mucosal surfaces (Sizemore *et al.* (1995) *Science* 270:29).

Yet another aspect of the invention pertains to anti-B7-4 or PD-1 antibodies. Such antibodies are, for instance, generated by immunizing an animal with an immunogenic B7-4 or PD-1 protein, or an immunogenic portion thereof which is unique to a B7-4 or PD-1 polypeptide, and then isolating antibodies from the animal that specifically bind to the B7-4 or PD-1 protein, or a fragment thereof.

Polyclonal anti-B7-4 or PD-1 antibodies can be prepared as described above by immunizing a suitable subject with a B7-4 or PD-1 immunogen. The anti-B7-4 or PD-1 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized a B7-4 or PD-1 polypeptide. If desired, the antibody molecules directed against a B7-4 or PD-1 polypeptide can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti- B7-4 or PD-1 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci.* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more

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recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally Kenneth, R. H. in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.* 54:387-402; Gefter, M. L. *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a B7-4 or PD-1 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to a B7-4 or PD-1 polypeptide, preferably specifically.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-B7-4 or PD-1 monoclonal antibody (see, *e.g.*, Galfre, G. *et al.* (1977) *Nature* 266:55052; Gefter *et al.* (1977) *supra*; Lerner (1981) *supra*; Kenneth (1980) *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are

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detected by screening the hybridoma culture supernatants for antibodies that bind a B7-4 or PD-1 molecule, *e.g.*, using a standard ELISA assay.

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-B7-4 or PD-1 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with a B7-4 or PD-1 to thereby isolate immunoglobulin library members that bind a B7-4 or PD-1 polypeptide. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP*^{TM} *Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Biotechnology (NY)* 9:1369-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Biotechnology (NY)* 9:1373-1377; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al.* (1990) *Nature* 348:552-554.

Additionally, recombinant anti-B7-4 or PD-1 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira *et al.* European Patent Application 184,187; Taniguchi, M. European Patent Application 171,496;

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Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *Biotechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyen *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

In addition, humanized antibodies can be made according to standard protocols such as those disclosed in US patent 5,565,332. In another embodiment, antibody chains or specific binding pair members can be produced by recombination between vectors comprising nucleic acid molecules encoding a fusion of a polypeptide chain of a specific binding pair member and a component of a replicable genetic display package and vectors containing nucleic acid molecules encoding a second polypeptide chain of a single binding pair member using techniques known in the art, *e.g.*, as described in US patents 5,565,332, 5,871,907, or 5,733,743. The use of intracellular antibodies to inhibit protein function in a cell is also known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T. M. *et al.* (1990) *FEBS Lett.* 274:193-198; Carlson, J. R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W. A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Biotechnology (NY)* 12:396-399; Chen, S-Y. *et al.* (1994) *Hum. Gene Ther.* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R. R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R. R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J. H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

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In one embodiment, an antibody for use in the instant invention is a bispecific antibody. A bispecific antibody has binding sites for two different antigens within a single antibody molecule. Antigen binding may be simultaneous or sequential. Triomas and hybrid hybridomas are two examples of cell lines that can secrete bispecific antibodies. Examples of bispecific antibodies produced by a hybrid hybridoma or a trioma are disclosed in U.S. Pat. 4,474,893. Bispecific antibodies have been constructed by chemical means (Staerz *et al.* (1985) *Nature* 314:628, and Perez *et al.* (1985) *Nature* 316:354) and hybridoma technology (Staerz and Bevan (1986) *Proc. Natl. Acad. Sci. USA*, 83:1453, and Staerz and Bevan (1986) *Immunol. Today* 7:241). Bispecific antibodies are also described in U.S. Patent 5,959,084. Fragments of bispecific antibodies are described in US patent 5,798,229.

Bispecific agents can also be generated by making heterohybridomas by fusing hybridomas or other cells making different antibodies, followed by identification of clones producing and co-assembling both antibodies. They can also be generated by chemical or genetic conjugation of complete immunoglobulin chains or portions thereof such as Fab and Fv sequences. The antibody component can bind to PD-1 or B7-4.

An anti-B7-4 or PD-1 antibody (*e.g.*, monoclonal antibody) can be used to isolate a B7-4 or PD-1 polypeptide by standard techniques, such as affinity chromatography or immunoprecipitation. Anti-B7-4 or PD-1 antibodies can facilitate the purification of natural B7-4 or PD-1 polypeptides from cells and of recombinantly produced B7-4 or PD-1 polypeptides expressed in host cells. Moreover, an anti-B7-4 or PD-1 antibody can be used to detect a B7-4 or PD-1 protein (*e.g.*, in a cellular lysate or cell supernatant). Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Accordingly, in one embodiment, an anti-B7-4 or PD-1 antibody of the invention is labeled with a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein,

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dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S, and ^{3}H.

IV. Recombinant Expression Vectors and Host Cells

Nucleic acid molecules encoding a B7-4 or PD-1 family protein (or a portion thereof) can be contained in vectors, preferably expression vectors. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

Recombinant expression vectors can comprise a nucleic acid molecule of the invention in a form suitable for expression, *e.g.*, constitutive or inducible expression, of a PD-1 or B7-4 molecule in the indicator cell(s) of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression

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of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel (1990) *Methods Enzymol.* 185:3-7. Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It should be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, B7-4 or PD-1 family proteins, mutant forms of B7-4 or PD-1 proteins, fusion proteins, and the like).

The recombinant expression vectors of the invention can be designed for expression of B7-4 or PD-1 proteins in prokaryotic or eukaryotic cells. For example, B7-4 or PD-1 proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel (1990) *supra*. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and

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their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D. B. and Johnson, K. S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Purified fusion proteins can be utilized in B7-4 or PD-1 activity assays, (*e.g.*, direct assays or competitive assays described in detail below), or to generate antibodies specific for B7-4 or PD-1 proteins, for example.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.* (1990) *Methods Enzymol.* 185:60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S. (1990) *Methods Enzymol.*185:119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.* (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the B7-4 or PD-1 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.* (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.* (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (Invitrogen Corp, San Diego, CA).

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Alternatively, a B7-4 or PD-1 polypeptide can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V. A. and Summers, M. D. (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pMex-NeoI, pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J. *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

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Moreover, inducible regulatory systems for use in mammalian cells are known in the art, for example systems in which gene expression is regulated by heavy metal ions (see *e.g.*, Mayo *et al.* (1982) *Cell* 29:99-108; Brinster *et al.* (1982) *Nature* 296:39-42; Searle *et al.* (1985) *Mol. Cell. Biol.* 5:1480-1489), heat shock (see *e.g.*, Nouer *et al.* (1991) in *Heat Shock Response*, ed. Nouer, L., CRC, Boca Raton, FL, pp167-220), hormones (see *e.g.*, Lee *et al.* (1981) *Nature* 294:228-232; Hynes *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock *et al.* (1987) *Nature* 329:734-736; Israel and Kaufman (1989) *Nucleic. Acids Res.* 17:2589-2604; and PCT Publication No. WO 93/23431), FK506-related molecules (see *e.g.*, PCT Publication No. WO 94/18317) or tetracyclines (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313). Accordingly, in another embodiment, the invention provides a recombinant expression vector in which a B7-4 or PD-1 DNA is operatively linked to an inducible eukaryotic promoter, thereby allowing for inducible expression of a B7-4 or PD-1 protein in eukaryotic cells.

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to B7-4 or PD-1 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.* (1986) "Antisense RNA as a molecular tool for genetic analysis" *Reviews - Trends in Genetics*, Vol. 1(1).

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The invention further pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, a B7-4 or PD-1 protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a B7-4 or PD-1 protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

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A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a B7-4 or PD-1 protein. Accordingly, the invention further provides methods for producing a B7-4 or PD-1 protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell (into which a recombinant expression vector encoding a B7-4 or PD-1 protein has been introduced) in a suitable medium such that a B7-4 or PD-1 protein is produced. In another embodiment, the method further comprises isolating a B7-4 or PD-1 protein from the medium or the host cell.

Certain host cells can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell is a fertilized oocyte or an embryonic stem cell into which B7-4 or PD-1-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous B7-4 or PD-1 sequences have been introduced into their genome or homologous recombinant animals in which endogenous B7-4 or PD-1 sequences have been altered. Such animals are useful for studying the function and/or activity of a B7-4 or PD-1 polypeptide and for identifying and/or evaluating modulators of B7-4 or PD-1 activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, and the like. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous B7-4 or PD-1 gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal can be created by introducing a B7-4 or PD-1-encoding nucleic acid molecule into the male pronucleus of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to develop in a

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pseudopregnant female foster animal. The B7-4 or PD-1 cDNA sequence of SEQ ID NO:1, 3, 10, or 11 can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homologue of a human B7-4 or PD-1 gene, such as a mouse or rat B7-4 or PD-1 gene, can be used as a transgene. Alternatively, a B7-4 or PD-1 gene homologue, such as another B7-4 or PD-1 family member, can be isolated based on hybridization to the B7-4 or PD-1 family cDNA sequences of SEQ ID NO:1, 3, 10, or 11 (described further in subsection I above) and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to a B7-4 or PD-1 transgene to direct expression of a B7-4 or PD-1 protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of a B7-4 or PD-1 transgene in its genome and/or expression of B7-4 or PD-1 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding a B7-4 or PD-1 protein can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a B7-4 or PD-1 gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the B7-4 or PD-1 gene. The B7-4 or PD-1 gene can be a human gene (*e.g.*, the SEQ ID NO:1, 3, 10, or 11), but more preferably, is a non-human homologue of a human B7-4 or PD-1 gene (*e.g.*, a cDNA isolated by stringent hybridization with the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11). For example, a mouse B7-4 or PD-1 gene can be used to construct a homologous recombination vector suitable for altering an endogenous B7-4 or PD-1 gene in the mouse genome. In a preferred embodiment, the vector is designed such that,

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upon homologous recombination, the endogenous B7-4 or PD-1 gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous B7-4 or PD-1 gene is mutated or otherwise altered but still encodes a functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous B7-4 or PD-1 protein). In the homologous recombination vector, the altered portion of the B7-4 or PD-1 gene is flanked at its 5' and 3' ends by additional nucleic acid sequence of the B7-4 or PD-1 gene to allow for homologous recombination to occur between the exogenous B7-4 or PD-1 gene carried by the vector and an endogenous B7-4 or PD-1 gene in an embryonic stem cell. The additional flanking B7-4 or PD-1 nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K. R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced B7-4 or PD-1 gene has homologously recombined with the endogenous B7-4 or PD-1 gene are selected (see, *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, E. J., ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Curr. Opin. Biotechnol.* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

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In addition to the foregoing, the skilled artisan will appreciate that other approaches known in the art for homologous recombination can be applied to the instant invention. Enzyme-assisted site-specific integration systems are known in the art and can be applied to integrate a DNA molecule at a predetermined location in a second target DNA molecule. Examples of such enzyme-assisted integration systems include the Cre recombinase-lox target system (*e.g.*, as described in Baubonis, W. and Sauer, B. (1993) *Nucleic. Acids Res.* 21:2025-2029; and Fukushige, S. and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA* 89:7905-7909) and the FLP recombinase-FRT target system (*e.g.*, as described in Dang, D. T. and Perrimon, N. (1992) *Dev. Genet.* 13:367-375; and Fiering, S. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:8469-8473). Tetracycline-regulated inducible homologous recombination systems, such as described in PCT Publication No. WO 94/29442 and PCT Publication No. WO 96/01313, also can be used.

For example, in another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. *et al.* (1997) *Nature* 385:810-813 and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G_0 phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species

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from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

V. Pharmaceutical Compositions

B7-4 or PD-1 modulators (*e.g.*, B7-4 or PD-1 inhibitory or stimulatory agents, including B7-4 or PD-1 nucleic acid molecules, proteins, antibodies described above, or compounds identified as modulators of a B7-4 or PD-1 activity and/or expression or modulators of the interaction between B7-4 and PD-1) can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "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. 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 the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases,

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such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a B7-4 or PD-1 protein or anti-B7-4 or PD-1 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a

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powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

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In one embodiment, modulatory agents are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations should be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

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The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

VI. Uses and Methods of the Invention

The B7-4 and/or PD-1 modulatory agents, *e.g.*, the nucleic acid molecules, proteins, protein homologues, and antibodies described herein, can be used, for example, in one or more of the following methods: a) methods of treatment, *e.g.*, by up- or down-modulating the immune response; b) screening assays; c) predictive medicine (*e.g.*, diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenetics).

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The isolated nucleic acid molecules of the invention can be used, for example, to express B7-4 or PD-1 protein (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect B7-4 or PD-1 mRNA (*e.g.*, in a biological sample) or a genetic alteration in a B7-4 or PD-1 gene, and to modulate B7-4 or PD-1 activity, as described further below. The B7-4 or PD-1 proteins can be used to treat disorders characterized by insufficient or excessive production of B7-4 or PD-1 inhibitors. In addition, the B7-4 or PD-1 proteins can be used to screen for naturally occurring B7-4 or PD-1 binding partners, to screen for drugs or compounds which modulate B7-4 or PD-1 activity, as well as to treat disorders characterized by insufficient or excessive production of B7-4 or PD-1 protein or production of B7-4 or PD-1 protein forms which have decreased or aberrant activity compared to B7-4 or PD-1 wild type protein. Moreover, the anti-B7-4 or PD-1 antibodies of the invention can be used to detect and isolate B7-4 or PD-1 proteins, regulate the bioavailability of B7-4 or PD-1 proteins, and modulate B7-4 or PD-1 activity *e.g.*, by modulating the interaction of B7-4 and PD-1.

A. Methods of Treatment:

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant B7-4 or PD-1 expression or activity.

1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant B7-4 or PD-1 expression or activity, by administering to the subject a B7-4 or PD-1 polypeptide or an agent which modulates B7-4 or PD-1 polypeptide expression or at least one B7-4 or PD-1 activity. Subjects at risk for a disease which is caused or contributed to by aberrant B7-4 or PD-1 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of B7-4 or PD-1 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of B7-4 or PD-1 aberrancy or condition, for example, a B7-4 or

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PD-1 polypeptide, B7-4 or PD-1 agonist or B7-4 or PD-1 antagonist agent can be used for treating the subject. The appropriate agent can be determined based on clinical indications and can be identified, *e.g.*, using screening assays described herein.

2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating B7-4 or PD-1 expression or activity for therapeutic purposes. B7-4 has been demonstrated to inhibit the costimulation and proliferation of activated immune cells and to transmit an inhibitory signal to immune cells via PD-1. Accordingly, the activity and/or expression of B7-4 or PD-1 as well as the interaction between B7-4 and PD-1 can be modulated in order to modulate the immune response. It should be understood that in embodiments where B7-4 binds to a costimulatory receptor, upregulation of B7-4 activity results in upregulation of immune responses, whereas downregulation of B7-4 activity results in downregulation of immune responses. In embodiments where B7-4 binds to inhibitory receptors, upregulation of B7-4 activity results in downregulation of immune responses, whereas downregulation of B7-4 activity results in upregulation of immune responses. In a preferred embodiment, B7-4 binds to inhibitory receptors. In a particularly preferred embodiment, B7-4 binds to PD-1.

Modulatory methods of the invention involve contacting a cell with a modulator of a B7-4 or a PD-1 polypeptide, *e.g.*, an agent that modulates expression or activity of B7-4 and/or PD-1, or an agent that modulates the interaction of B7-4 and PD-1.

An agent that modulates B7-4 or PD-1 protein activity is an agent as described herein, such as a nucleic acid or a a protein molecule, a naturally-occurring target molecule of a B7-4 or PD-1 protein (*e.g.*, PD-1 in the case of B7-4 or B7-4 in the case of PD-1), a B7-4 or PD-1 antibody, a B7-4 or PD-1 agonist or antagonist, a peptidomimetic of a B7-4 or PD-1 agonist or antagonist, or other small molecule.

An agent that modulates the expression of B7-4 or PD-1 is, *e.g.*, an antisense nucleic acid molecule, triplex oligonucleotide, a ribozyme, or a recombinant vector for expression of a B7-4 or PD-1 protein. For example, an oligonucleotide complementary to the area around a B7-4 or PD-1 polypeptide translation initiation site, can be synthesized and used. One or more antisense oligonucleotides can be added to cell

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media, typically at 200 μg/ml, or administered to a patient to prevent the synthesis of a B7-4 or PD-1 polypeptide. The antisense oligonucleotide is taken up by cells and hybridizes to a B7-4 or PD-1 mRNA to prevent translation. Alternatively, an oligonucleotide which binds double-stranded DNA to form a triplex construct to prevent DNA unwinding and transcription can be used. As a result of either, synthesis of a B7-4 or PD-1 polypeptide is blocked. When PD-1 expression is modulated, preferably, such modulation occurs by a means other than by knocking out the PD-1 gene.

Agents which modulate expression, by virtue of the fact that they control the amount of PD-1 or B7-4 in a cell, also modulate the total amount of PD-1 or B7-4 activity in a cell.

In one embodiment, an agent that stimulates an inhibitory activity of B7-4 or an inhibitory activity of PD-1 is an agonist of B7-4 or PD-1. Examples of such agents include active B7-4 or PD-1 protein and an expressible nucleic acid molecule encoding B7-4 or PD-1 polypeptide that has been introduced into the cell.

In another embodiment, the agent inhibits the costimulatory or inhibitory activity of B7-4 or inhibitory activity of PD-1 and is an antagonist of B7-4 or PD-1. Examples of such agents include antisense B7-4 or PD-1 nucleic acid molecules, anti-B7-4 or PD-1 antibodies (*e.g.*, non-activating antibodies), soluble, nonactivating forms of B7-4 or PD-1 molecules, and B7-4 or PD-1 inhibitors.

These modulatory agents can be administered *in vitro* (*e.g.*, by contacting the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder that would benefit from modulation of a B7-4 or PD-1 protein, *e.g.*, a disorder which would benefit from up or downmodulation of the immune response, or which is characterized by aberrant expression or activity of a B7-4 or PD-1 protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) B7-4 or PD-1 expression or activity. In another embodiment, the method involves administering a B7-4 or PD-1 protein or nucleic acid molecule as therapy to compensate for reduced or aberrant B7-4 or PD-1 expression or activity.

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Stimulation of B7-4 or PD-1 activity is desirable in situations in which B7-4 or PD-1 is abnormally downregulated and/or in which increased B7-4 or PD-1 activity is likely to have a beneficial effect. Likewise, inhibition of B7-4 or PD-1 activity is desirable in situations in which B7-4 or PD-1 is abnormally upregulated and/or in which decreased B7-4 or PD-1 activity is likely to have a beneficial effect. One of ordinary skill in the art should recognize that in embodiments where B7-4 binds to a costimulatory receptor, stimulation of B7-4 and stimulation of PD-1 have opposite effects on immune cell costimulation, and therefore, on the immune response. In such an instance, when stimulation of the activity of one molecule is desirable, suppression of the activity of the other molecule is desirable.

Exemplary agents for use in downmodulating B7-4 activity (B7-4 antagonists) include (for example): antisense molecules, antibodies that recognize B7-4, compounds that block interaction of B7-4 and one of its naturally occurring receptors on a immune cell (*e.g.*, soluble, monovalent B7-4 molecules, and soluble forms of B7-4 ligands or compounds identified in the screening assays described herein). Exemplary agents for use in downmodulating PD-1 (PD-1 antagonists) include (for example): antisense molecules, antibodies that bind to PD-1, but do not transduce an inhibitory signal to the immune cell ("non-activating antibodies"), and soluble forms of PD-1.

Exemplary agents for use in upmodulating B7-4 activity (B7-4 agonists) include (for example): nucleic acid molecules encoding B7-4 polypeptides, multivalent forms of B7-4, compounds that increase the expression of B7-4, and cells that express B7-4, etc. Exemplary agents for use in upmodulating PD-1 (PD-1 agonists) include (for example): antibodies that transmit an inhibitory signal via PD-1 by binding to and activating (e.g., crosslinking) the PD-1 molecule, compounds that enhance the expression of PD-1, nucleic acid molecules encoding PD-1, and forms of B7-4 that transduce a signal via PD-1 (especially bivalent forms of B7-4).

3. Downregulation of Immune Responses by Modulation of B7-4 or PD-1

There are numerous embodiments of the invention for upregulating the inhibitory function or downregulating the costimulatory function of a B7-4 polypeptide to thereby downregulate immune responses. Downregulation can be in the form of inhibiting or

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blocking an immune response already in progress or may involve preventing the induction of an immune response.

The functions of activated immune cells can be inhibited by down-regulating immune cell responses or by inducing specific anergy in immune cells, or both.

For example, anti-B7-4 antibodies or B7-4 polypeptides (*e.g.*, soluble, monomeric forms of a B7-4 polypeptide such as B7-4-Ig), and/or anti-B7-4 antibodies that block the interaction of B7-4 with a costimulatory receptor can be used to inhibit a costimulatory signal and, thus, downmodulate the immune response.

In addition, in embodiments where B7-4 binds to an inhibitory receptor, forms of B7-4 that bind to the inhibitory receptor and activate it, *e.g.*, multivalent B7-4 on a cell surface, can be used to downmodulate the immune response.

Likewise, the PD-1 pathway can also be stimulated by the use of an agent to thereby downmodulate the immune response. This is accomplished via inhibition of the interaction of B7-4 with a stimulatory receptor on an immune cell (*e.g.*, by using a soluble form of PD-1 and/or CTLA4) or activation of PD-1 (*e.g.*, using an activating antibody which cross-links PD-1) to provide negative signals to immune cells.

In one embodiment of the invention, an activating antibody used to stimulate PD-1 activity is a bispecific antibody. For example, such an antibody can comprise a PD-1 binding site and another binding site which targets a cell surface receptor on an immune cell, *e.g.*, on a T cell, a B cell, or a myeloid cell. In one embodiment, such an antibody, in addition to comprising a PD-1 binding site can further comprise a binding site which binds to a molecule which is in proximity to an activating or inhibitory receptor, *e.g.*, B-cell antigen receptor, a T-cell antigen receptor, or an Fc receptor in order to target the molecule to a specific cell population. For example, a CD3 antigen, a T-cell receptor chain, LFA-1, CD2, CTLA-4, immunoglobulin, B cell receptor, Ig alpha, Ig beta, CD22, or Fc receptor could be used. Such antibodies (or other bispecific agents) are art recognized and can be produced, *e.g.*, as described herein. Selection of this second antigen for the bispecific antibody provides flexibility in selection of cell population to be targeted for inhibition.

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In another embodiment, the co-ligation of PD-1 and an activating or inhibitory receptor on a cell can enhance the generation of a negative signal via PD-1. Such co-ligation can be accomplished *e.g.*, by use of a bispecific agent, *e.g.*, a bispecific antibody as described herein having specificity for both PD-1 and a molecule associated with a receptor. In another embodiment, the use of a multivalent form of an agent that transmits a negative signal via PD-1 can be used to enhance the transmission of a negative signal via PD-1, *e.g.*, an agent presented on a bead or on a surface. In another embodiment, such a multivalent agent can comprise two specificities to achieve co-ligation of PD-1 and a receptor or a receptor associated molecule (*e.g.*, a bead comprising anti CD3 and B7-4).

Agents that block or inhibit interaction of B7-4 with a costimulatory receptor (*e.g.*, soluble forms of B7-4 or blocking antibodies to B7-4) as well as agents that promote a B7-4-mediated inhibitory signal or agonists of PD-1 which activate PD-1 (*e.g.*, PD-1 activating antibodies or PD-1 activating small molecules) can be identified by their ability to inhibit immune cell proliferation and/or effector function or to induce anergy when added to an *in vitro* assay. For example, cells can be cultured in the presence of an agent that stimulates signal transduction via an activating receptor. A number of art recognized readouts of cell activation can be employed to measure the inhibition, *e.g.*, cell proliferation or effector function (*e.g.*, antibody production, cytokine production, phagocytosis) in the presence of the activating agent. The ability of a test agent to block this activation can be readily determined by measuring the ability of the agent to affect a decrease in proliferation or effector function being measured.

In one embodiment of the invention, tolerance is induced against specific antigens by co-administering an antigen with a PD-1 agonist. For example, tolerance can be induced to specific proteins. In one embodiment, immune responses to allergens or foreign proteins to which an immune response is undesirable can be inhibited. For example, patients that receive Factor VIII frequently generate antibodies against this clotting factor. Co-administration of an agent that blocks a B7-4-mediated costimulatory signal or an agent that stimulates a PD-1 mediated inhibitory signal in combination with recombinant factor VIII (or physically linked to Factor VIII, *e.g.*, by cross-linking) can result in downmodulation.

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In one embodiment, fusion proteins comprising a B7-4 first peptide fused to a second peptide having an activity of another B lymphocyte antigen (*e.g.*, B7-1 or B7-2), can be used to block interaction of B7-4 with a costimulatory receptor on a immune cell to downmodulate immune responses. Alternatively, two separate peptides (for example, a B7-4 polypeptide with B7-2 and/or B7-1), or a combination of blocking antibodies (*e.g.*, antibodies against a B7-4 polypeptide with anti-B7-2 and/or anti-B7-1 monoclonal antibodies) can be combined as a single composition or administered separately (simultaneously or sequentially) to downregulate immune cell mediated immune responses in a subject. Furthermore, a therapeutically active amount of one or more peptides having a B7-4 polypeptide activity, with B7-1 and/or B7-2 activity can be used in conjunction with other downmodulating reagents to influence immune responses. Examples of other immunomodulating reagents include antibodies that block a costimulatory signal, (*e.g.*, against CD28, ICOS), antibodies that activate an inhibitory signal via CTLA4, and/or antibodies against other immune cell markers (*e.g.*, against CD40, against CD40 ligand, or against cytokines), fusion proteins (*e.g.*, CTLA4-Fc, PD-1-Fc), and immunosuppressive drugs, (*e.g.*, rapamycin, cyclosporine A or FK506).

The B7-4 and/or PD-1 peptides may also be useful in the construction of therapeutic agents which block immune cell function by destruction of cells. For example, portions of a B7-4 or PD-1 polypeptide can be linked to a toxin to make a cytotoxic agent capable of triggering the destruction of cells to which it binds.

For making cytotoxic agents, polypeptides of the invention may be linked, or operatively attached, to toxins using techniques that are known in the art, *e.g.*, crosslinking or via recombinant DNA techniques. The preparation of immunotoxins is, in general, well known in the art (see, *e.g.*, U.S. Pat. Nos. 4,340,535, and EP 44167, both incorporated herein by reference). Numerous types of disulfide-bond containing linkers are known which can successfully be employed to conjugate the toxin moiety with a polypeptide. In one embodiment, linkers that contain a disulfide bond that is sterically "hindered" are to be preferred, due to their greater stability *in vivo*, thus preventing release of the toxin moiety prior to binding at the site of action.

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A wide variety of toxins are known that may be conjugated to polypeptides or antibodies of the invention. Examples include: numerous useful plant-, fungus- or even bacteria-derived toxins, which, by way of example, include various A chain toxins, particularly ricin A chain, ribosome inactivating proteins such as saporin or gelonin, .alpha.-sarcin, aspergillin, restrictocin, ribonucleases such as placental ribonuclease, angiogenic, diphtheria toxin, and pseudomonas exotoxin, etc. A preferred toxin moiety for use in connection with the invention is toxin A chain which has been treated to modify or remove carbohydrate residues, deglycosylated A chain. (U.S. Patent 5,776,427).

Infusion of one or a combination of such cytotoxic agents, (*e.g.*, B7-4 ricin (alone or in combination with B7-2-ricin or B7-1-ricin), into a patient may result in the death of immune cells, particularly in light of the fact that activated immune cells that express higher amounts of B7-4 ligands. For example, because PD-1 is induced on the surface of activated lymphocytes, an antibody against PD-1 can be used to target the depletion of these specific cells by Fc-R dependent mechanisms or by ablation by conjugating a cytotoxic drug (*e.g.*, ricin, saporin, or calicheamicin) to the antibody. In one embodiment, the antibody toxin can be a bispecific antibody. Such bispecific antibodies are useful for targeting a specific cell population, *e.g.*, using a marker found only on a certain type of cell, *e.g.*, a TCR, BCR, or FcR molecule.

Downregulating or preventing B7-4 polypeptide costimulatory functions or activating a B7-4 or a PD-1 inhibitory function (*e.g.*, by stimulation of the negative signaling function of PD-1) is useful to downmodulate the immune response, *e.g.*, in situations of tissue, skin and organ transplantation, in graft-versus-host disease (GVHD), or in autoimmune diseases such as systemic lupus erythematosus, and multiple sclerosis. For example, blockage of immune cell function results in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by immune cells, followed by an immune reaction that destroys the transplant. The administration of a molecule which inhibits or blocks interaction of a B7 molecule with a costimulatory receptor(s) on immune cells (such as a soluble, monomeric form of a B7-4 or PD-1 polypeptide) alone or in conjunction with another downmodulatory agent prior to or at the time of transplantation

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can inhibit the generation of a costimulatory signal. Moreover, inhibition of B7-4 costimulatory signals, or promotion of a B7-4 or PD-1 inhibitory signal may also be sufficient to anergize the immune cells, thereby inducing tolerance in a subject. Induction of long-term tolerance by blocking a B7-4 mediated costimulatory signal may avoid the necessity of repeated administration of these blocking reagents.

To achieve sufficient immunosuppression or tolerance in a subject, it may also be desirable to block the costimulatory function of other molecules. For example, it may be desirable to block the function of B7-1 and B7-4, B7-2 and B7-4, or B7-1 and B7-2 and B7-4 by administering a soluble form of a combination of peptides having an activity of each of these antigens or blocking antibodies against these antigens (separately or together in a single composition) prior to or at the time of transplantation. Alternatively, it may be desirable to promote inhibitory activity of B7-4 or PD-1 and inhibit a costimulatory activity of B7-1 and/or B7-2. Other downmodulatory agents that can be used in connection with the downmodulatory methods of the invention include, for example, agents that transmit an inhibitory signal via CTLA4, soluble forms of CTLA4, antibodies that activate an inhibitory signal via CTLA4, blocking antibodies against other immune cell markers or soluble forms of other receptor ligand pairs (*e.g.*, agents that disrupt the interaction between CD40 and CD40 ligand (*e.g.*, anti CD40 ligand antibodies)), antibodies against cytokines, or immunosuppressive drugs. In another embodiment, a combination of at least two different B7-4 antibodies can be administered to achieve optimal blocking activity.

Blocking B7-4 polypeptide costimulation or activating a B7-4 or PD-1 inhibitory function is also useful in treating autoimmune disease. Many autoimmune disorders are the result of inappropriate activation of immune cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing or downmodulating the activation of autoreactive immune cells may reduce or eliminate disease symptoms. Administration of reagents which block costimulation of immune cells by disrupting receptor:ligand interactions of B7 molecules with costimulatory receptors is useful to inhibit immune cell activation and prevent production of autoantibodies or cytokines which are involved in the disease process. Additionally, agents that promote an inhibitory function of B7-4 or PD-1 may

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induce antigen-specific tolerance of autoreactive immune cells which could lead to long-term relief from the disease. The efficacy of reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythematosus in MRL/*lpr/lpr* mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856).

Inhibition of immune cell activation is useful therapeutically in the treatment of allergy and allergic reactions, *e.g.*, by inhibiting IgE production. An agent that promotes a B7-4 or PD-1 inhibitory function can be administered to an allergic subject to inhibit immune cell mediated allergic responses in the subject. Activating a PD-1 molecule is also useful in treating allergies. Inhibition of B7-4 costimulation of immune cells or stimulation of a B7-4 or PD-1 inhibitory pathway can be accompanied by exposure to allergen in conjunction with appropriate MHC molecules. Allergic reactions can be systemic or local in nature, depending on the route of entry of the allergen and the pattern of deposition of IgE on mast cells or basophils. Thus, inhibition of immune cell mediated allergic responses can be local or systemic by administration (e.g., either local or systemic, respectively) of an inhibitory form of an agent that inhibits the interaction of B7-4 with a costimulatory receptor or an agent that promotes an inhibitory function of B7-4 or PD-1.

Inhibition of immune cell activation through blockage of a B7-4 costimulatory activity or stimulation of PD-1 inhibitory activity is also important therapeutically in viral infections of immune cells. For example, in the acquired immune deficiency syndrome (AIDS), viral replication is stimulated by immune cell activation. Blocking a B7-4/costimulatory receptor interaction or stimulation of B7-4 or PD-1 inhibitory function may result in inhibition of viral replication to thereby ameliorate the course of AIDS. Downregulation of an immune response via stimulation of B7-4 activity or B7-4 interaction with its natural binding partner(s), *e.g.*, PD-1, is also be useful in promoting the maintenance of pregnancy. B7-4 is normally highly expressed in placental trophoblasts, the layer of cells that forms the interface between mother and fetus and is

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thought to play a role in preventing maternal rejection of the fetus. Females at risk for spontaneous abortion (*e.g.*, those identified by screening for B7-4 activity, as described in the "Prognostic Assays" section, those who have previously had a spontaneous abortion or those who have had difficulty conceiving) because of immunologic rejection of the embryo or fetus can be treated with agents that stimulate the activity of B7-4 or its interaction with its natural binding partner(s), *e.g.*, PD-1.

Downregulation of an immune response via stimulation of B7-4 activity or B7-4 interaction with its natural binding partner(s), *e.g.*, PD-1, is also useful in treating an autoimmune attack of autologous tissues. For example, B7-4 is normally highly expressed in the heart and protects the heart from autoimmune attack. This is evidenced by the fact that the Balb/c PD-1 knockout mouse exhibits massive autoimmune attack on the heart with thrombosis. Thus, conditions that are caused or exacerbated by autoimmune attack (*e.g.*, in this example, heart disease, myocardial infarction or atherosclerosis) may be ameliorated or improved by increasing B7-4 activity or B7-4 biding to its natural binding partner, *e.g.*, PD-1. It is therefore within the scope of the invention to modulate conditions exacerbated by autoimmune attack, such as autoimmune disorders (as well as conditions such as heart disease, myocardial infarction, and atherosclerosis) by stimulating B7-4 activity or B7-4 interaction with PD-1.

4. Upregulation of Immune Responses

Upregulation of B7-4 costimulatory activity or inhibition of the inhibitory activity of PD-1 or B7-4, as a means of upregulating immune responses, is also useful in therapy. Upregulation of immune responses can be in the form of enhancing an existing immune response or eliciting an initial immune response. For example, enhancing an immune response through stimulating B7-4 costimulatory activity or inhibition of B7-4 or PD-1 inhibitory activity is useful in cases of infections with microbes, *e.g.*, bacteria, viruses, or parasites. For example, in one embodiment, a form of B7-4 that promotes a costimulatory signal in an immune cell (*e.g.*, a B7-4 peptide in a multi-valent form (*e.g.*, a soluble multivalent form or a form expressed on a cell surface)) or an agent that inhibits the interaction of B7-4 with an inhibitory receptor or an agent that inhibits

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transduction of an inhibitory signal via PD-1, *e.g.*, a non-activating antibody against PD-1 or a small molecule (e.g., peptidomimetic) PD-1 antagonist, is therapeutically useful in situations where upregulation of antibody and cell-mediated responses, resulting in more rapid or thorough clearance of microbes is beneficial. These situations include, without limitation, viral skin diseases such as Herpes or shingles, in which case such an agent may optimally be delivered topically to the skin. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by the administration of such agents systemically.

In certain instances, it may be desirable to further administer other agents that upregulate immune responses, for example, forms other B7 family members that transduce signals via costimulatory receptors, in order further augment the immune response.

Alternatively, immune responses can be enhanced in an infected patient by an *ex vivo* approach, for instance removing immune cells from the patient, contacting immune cells *in vitro* with a form of B7-4 that promotes a costimulatory signal in an immune cell or an agent that inhibits the interaction of B7-4 with an inhibitory receptor, or an agent that inhibits transduction of an inhibitory signal via PD-1, and reintroducing the *in vitro* stimulated immune cells into the patient. In another embodiment, a method of enhancing immune responses involves isolating infected cells from a patient (*e.g.*, virally infected cells) transfecting them with a nucleic acid molecule encoding a form of B7-4 that binds to a costimulatory receptor such that the cells express all or a portion of the B7-4 molecule on their surface, and reintroducing the transfected cells into the patient. The transfected cells are capable of delivering a costimulatory signal to, and thereby activating, immune cells *in vivo*.

Forms of B7-4 that promote a costimulatory signal in an immune cell, or an agent that inhibits the interaction of B7-4 with an inhibitory receptor, or an agent that inhibits transduction of an inhibitory signal via PD-1 can be used prophylactically in vaccines against various polypeptides, *e.g.*, polypeptides derived from pathogens. Immunity against a pathogen (*e.g.*, a virus or bacteria) can be induced by vaccinating with a pathogen protein along with a form of B7-4 that promotes a costimulatory signal in an immune cell, or an agent that inhibits the interaction of B7-4 with an inhibitory

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receptor, or an agent that inhibits transduction of an inhibitory signal via PD-1, in an appropriate adjuvant. Alternately, a vector comprising genes which encode for both a pathogenic antigen and a form of B7-4 that binds to costimulatory receptors can be used for vaccination. Nucleic acid vaccines can be administered by a variety of means, for example, by injection (*e.g.*, intramuscular, intradermal, or the biolistic injection of DNA-coated gold particles into the epidermis with a gene gun that uses a particle accelerator or a compressed gas to inject the particles into the skin (Haynes *et al.* (1996) *J. Biotechnol.* 44:37)). Alternatively, nucleic acid vaccines can be administered by non-invasive means. For example, pure or lipid-formulated DNA can be delivered to the respiratory system or targeted elsewhere, *e.g.*, Peyers patches by oral delivery of DNA (Schubbert (1997) *Proc. Natl. Acad. Sci. USA* 94:961). Attenuated microorganisms can be used for delivery to mucosal surfaces (Sizemore *et al.* (1995) *Science* 270:29).

In one embodiment, a form of a B7-4 polypeptide which transmits a costimulatory signal can be administered with class I MHC proteins by, for example, a cell transfected to coexpress a B7-4 polypeptide and MHC class I α chain protein and β_2 microglobulin to result in activation of T cells and provide immunity from infection. Pathogens for which vaccines are useful include, without limitation, hepatitis B, hepatitis C, Epstein-Barr virus, cytomegalovirus, HIV-1, HIV-2, tuberculosis, malaria and schistosomiasis.

In another application, upregulation or enhancement of a B7-4 costimulatory function is useful in the induction of tumor immunity. Endogenous expression of wild-type B7-4 on tumor cells inhibits the immune response against tumor cells. Accordingly, inhibition of the the interaction between B7-4 on tumor cells and PD-1 is useful in inducing tumor immunity. In one embodiment, a PD-1 antagonist (e.g., a non-activating antibody against PD-1 or B7-4 or a small molecule PD-1 or B7-4 antagonist) is administered to a subject having or at risk for a tumor.

In another embodiment, tumor cells (*e.g.*, sarcoma, melanoma, lymphoma, leukemia, neuroblastoma, carcinoma) transfected with a nucleic acid molecule encoding a B7-4 antigen are administered to a subject to overcome tumor-specific tolerance in the subject. If desired, the tumor cell can be transfected to express a combination of B7 polypeptides (*e.g.*, B7-1, B7-2, B7-4 in any combination). For example, tumor cells

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obtained from a patient can be transfected *ex vivo* with an expression vector directing the expression of a B7-4 polypeptide alone, or in conjunction with a peptide having B7-1 activity and/or B7-2 activity. The transfected tumor cells are returned to the patient to result in expression of the peptides on the surface of the transfected cell. Alternatively, gene therapy techniques can be used to target a tumor cell for transfection *in vivo*.

In addition, tumor cells which lack MHC class I or MHC class II molecules, or which fail to express sufficient amounts of MHC class I or MHC class II molecules, can be transfected with nucleic acid encoding all or a portion of (*e.g.*, a cytoplasmic-domain truncated portion) of an MHC class I α chain protein and β_2 microglobulin protein or an MHC class II α chain protein and an MHC class II β chain protein to thereby express MHC class I or MHC class II proteins on the cell surface. Expression of the appropriate class I or class II MHC in conjunction with a peptide having the activity of a B lymphocyte antigen (*e.g.*, B7-1, and/or B7-2, and/or B7-4) induces a T cell mediated immune response against the transfected tumor cell. Optionally, a gene encoding an antisense construct which blocks expression of an MHC class II associated protein, such as the invariant chain, can also be cotransfected with a DNA encoding a B7-4 polypeptide to promote presentation of tumor associated antigens and induce tumor specific immunity (for instance as taught in U.S. Patent No. 5,919,639, the contents of which are icorporated herein by reference). Expression of B7-1 by B7 negative murine tumor cells has been shown to induce T cell mediated specific immunity accompanied by tumor rejection and prolonged protection to tumor challenge in mice (Chen, L., *et al.* (1992) *Cell* 71:1093-1102; Townsend, S. E. and Allison, J. P. (1993) *Science* 259:368-370; Baskar, S. *et al.* (1993) *Proc. Natl. Acad. Sci.* 90:5687-5690). Thus, the induction of a immune cell mediated immune response in a human subject can be sufficient to overcome tumor-specific tolerance in the subject.

In another embodiment, the immune response can be stimulated by the transmission of a signal via a costimulatory receptor that binds to B7-4 or by inhibiting signaling via an inhibitory receptor that binds to B7-4, *e.g.*,PD-1, such that preexisting tolerance is overcome. For example, immune responses against antigens to which a subject cannot mount a significant immune response, *e.g.*, to an autologous antigen, such as a tumor specific antigen, can be induced by administering an agent that inhibits the

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inhibitory activity of PD-1 or the ability of B7-4 to bind to an inhibitory ligand. For example, in one embodiment, soluble PD-1 or soluble B7-4 can be used (*e.g.*, PD-1Fc or B7-4 Fc) to enhance an immune response, *e.g.*, to a tumor cell. In one embodiment, an autologous antigen, such as a tumor-specific antigen can be coadministered with an agent that inhibits the inhibitory activity of PD-1 or the ability of B7-4 to bind to an inhibitory ligand. In another embodiment, an immune response can be stimulated against an antigen (*e.g.*, an autologous antigen) to treat a neurological disorder. In another embodiment, PD-1 antagonists can be used as adjuvants to boost responses to foreign antigens in the process of active immunization.

In yet another embodiment, the cellular production of a form of B7-4 that binds to an inhibitory receptor or that competes with the binding of B7-4 to a costimulatory receptor (*e.g.*, a form of B7-4 that binds to PD-1 or a naturally occurring soluble molecule) can be inhibited, *e.g.*, using antisense RNA, in order to upregulate the immune response. For example, in one embodiment, the production of inhibitory B7-4 molecules by a tumor cell can be inhibited in order to increase anti-tumor immunity.

In one embodiment, immune cells are obtained from a subject and cultured *ex vivo* in the presence of a form of B7-4 that binds a costimulatory molecule or in the presence of an agent that inhibits a B7-4 or PD-1 inhibitory signal, to expand the population of immune cells. In a further embodiment the immune cells are then administered to a subject. Immune cells can be stimulated to proliferate *in vitro* by, for example, providing to the immune cells a primary activation signal and a costimulatory signal, as is known in the art. Various forms of B7-4 proteins or agents that bind a costimulatory receptor or that inhibit signaling via PD-1 can also be used to costimulate proliferation of immune cells. In one embodiment immune cells are cultured *ex vivo* according to the method described in PCT Application No. WO 94/29436. The costimulatory molecule can be soluble, attached to a cell membrane, or attached to a solid surface, such as a bead.

B. Identification of Cytokines Modulated by Modulation of B7-4 and/or PD-1

The B7-4 and PD-1 molecules described herein can be used to identify cytokines which are produced by or whose production is enhanced or inhibited in immune cells in

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response to modulation of B7-4 and/or PD-1 activity. Immune cells expressing PD-1 can be suboptimally stimulated *in vitro* with a primary activation signal, for example, T cells can be stimulated with phorbol ester, anti-CD3 antibody or preferably antigen in association with an MHC class II molecule, and given a costimulatory signal, *e.g.*, by a stimulatory form of B7 family antigen, for instance by a cell transfected with nucleic acid encoding a B7 polypeptide and expressing the peptide on its surface or by a soluble, stimulatory form of the peptide. Known cytokines released into the media can be identified by ELISA or by the ability of an antibody which blocks the cytokine to inhibit immune cell proliferation or proliferation of other cell types that is induced by the cytokine. For example, an IL-4 ELISA kit is available from Genzyme (Cambridge MA), as is an IL-7 blocking antibody. Blocking antibodies against IL-9 and IL-12 are available from Genetics Institute (Cambridge, MA). The effect of stimulating or blocking the interaction of B7-4 with PD-1 on the cytokine profile can then be determined.

An *in vitro* immune cell costimulation assay as described above can also be used in a method for identifying novel cytokines which can be modulated by modulation of B7-4 and or PD-1. For example, where stimulation of the CD28/CTLA4 pathway seems to enhance IL-2 secretion, stimulation of the ICOS pathway seems to enhance IL-10 secretion (Hutloff *et al.* 199. Nature 397:263). If a particular activity induced upon costimulation, *e.g.*, immune cell proliferation, cannot be inhibited by addition of blocking antibodies to known cytokines, the activity may result from the action of an unknown cytokine. Following costimulation, this cytokine can be purified from the media by conventional methods and its activity measured by its ability to induce immune cell proliferation.

To identify cytokines which may play a role the induction of tolerance, an *in vitro* T cell costimulation assay as described above can be used. In this case, T cells would be given the primary activation signal and contacted with a selected cytokine, but would not be given the costimulatory signal. After washing and resting the immune cells, the cells would be rechallenged with both a primary activation signal and a costimulatory signal. If the immune cells do not respond (*e.g.*, proliferate or produce cytokines) they have become tolerized and the cytokine has not prevented the induction

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of tolerance. However, if the immune cells respond, induction of tolerance has been prevented by the cytokine. Those cytokines which are capable of preventing the induction of tolerance can be targeted for blockage *in vivo* in conjunction with reagents which block B lymphocyte antigens as a more efficient means to induce tolerance in transplant recipients or subjects with autoimmune diseases. For example, one could administer a cytokine blocking antibody to a subject along with an agent that promotes a B7-4 or a PD-1 inhibitory activity.

C. Identification of Molecules which Modulate Expression of a B7-4 or PD-1 Polypeptide

The antibodies produced using the proteins and peptides of the current invention can be used, e.g., in a screening assay for molecules which modulate the expression of B7-4 or PD-1 polypeptide on cells. For example, molecules which modulate intracellular signaling pathways that culminate in changes in expression of B7-4 or PD-1 polypeptides (*e.g.*, in response to activation signals), can be identified by assaying expression of one or more B7-4 or PD-1 polypeptides on the cell surface. Reduced immunofluorescent staining by an appropriate antibody in the presence of the molecule would indicate that the molecule inhibits intracellular signals. Molecules which upregulate B7-4 or PD-1 polypeptide expression result in an increased immunofluorescent staining. Alternatively, the effect of a molecule on expression of a polypeptide can be determined by detecting cellular mRNA levels using a probe of the invention. For example, a cell which expresses a B7-4 or PD-1 polypeptide can be contacted with a molecule to be tested, and an increase or decrease in mRNA levels in the cell detected by standard techniques, such as Northern hybridization analysis or conventional dot blot of mRNA or total poly(A^+)RNAs using a cDNA probe labeled with a detectable marker. Molecules which modulate expression of a B7-4 or PD-1 polypeptide are useful therapeutically for either upregulating or downregulating immune responses alone or in conjunction with soluble blocking or stimulating reagents as described above. For instance, a molecule which inhibits expression of B7-4 can be administered together with a second agent (*e.g.*, an immunosuppressant) or a molecule which inhibits expression of PD-1 can be given with an immunostimulant (*e.g.*, an

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adjuvant). Exemplary molecules which can be tested for their ability to modulate B7-4 or PD-1 include cytokines such as IL-4, γINF, IL-10, IL-12, GM-CSF and prostagladins.

D. Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules, or other drugs) which bind to B7-4 or PD-1 proteins, have a stimulatory or inhibitory effect on, for example, B7-4 or PD-1 expression, or B7-4 or PD-1 activity.

One such assay for identifying a compound which modulates the activity of B7-4 or PD-1 is a cell-based assay. In one embodiment of the assay, a cell expressing PD-1 is contacted with a test compound. The cell is also contacted with an activator of PD-1 (e.g., B7-4 in soluble form or expressed on a cell) and then is assayed for PD-1 activity. Increased or decreased activity of the PD-1 of the cell in the presence of the test compound, as compared to the activity of the PD-1 of the cell in the absence of the test compound indicates that the compound modulates the activity of the PD-1, and thus modulates signaling of PD-1. A similar assay can be performed for identifying a modulator of B7-4 activity. For instance, an activating form of B7-4 (in soluble form or expressed on a cell) can be added to a cell expressing a receptor known to be bound and activated by B7-4, in the presence and absence of a test compound, and the B7-4 activity then assayed to determine if the test compound has modulatory activity.

In one embodiment, the invention provides assays for screening candidate or test compounds which bind to, or modulate the activity of, a B7-4 or PD-1 protein or polypeptide or biologically active portion thereof, *e.g.*, which modulate the ability of B7-4 or PD-1 polypeptide to interact with its cognate binding partner or an interactor molecule, such as an intracellular interactor molecule.

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library

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method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K. S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a B7-4 target molecule (an intracellular interactor molecule or a PD-1 receptor) or PD-1 target molecule (*e.g.*, a B7-4 ligand or intracellular interactor molecule) with a test compound and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the B7-4 or PD-1 target molecule. Determining the ability of the test compound to modulate the activity of a B7-4 or PD-1 target molecule can be accomplished, for example, by determining the ability of the B7-4 or PD-1 protein to bind to or interact with the B7-4 or PD-1 target molecule, as molecules which bind to or interact with the target molecule are more likely to modulate activity. Determining the ability of the B7-4 or PD-1 protein to bind to or interact with its binding partner can be accomplished, *e.g.*, by measuring direct binding.

In a direct binding assay, the B7-4 or PD-1 protein (or their respective target molecules) can be coupled with a radioisotope or enzymatic label such that binding of the B7-4 or PD-1 protein to a B7-4 or PD-1 target molecule can be determined by

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detecting the labeled protein in a complex. For example, B7-4 or PD-1 molecules, *e.g.*, B7-4 or PD-1 proteins, can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, B7-4 or PD-1 molecules can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a compound to modulate the interaction between B7-4 or PD-1 and its target molecule, without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of B7-4 or PD-1 with its target molecule without the labeling of either B7-4 or PD-1 or the target molecule (McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912). As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between compound and receptor.

In a preferred embodiment, determining the ability of the B7-4 or PD-1 protein to bind to or interact with a B7-4 or PD-1 target molecule can be accomplished by determining the activity of the B7-4, PD-1 or the appropriate target molecule. For example, the activity of B7-4, PD-1 or the appropriate target molecule can be determined by detecting induction or inhibition of induction of a cellular second messenger (*e.g.*, serine/threonine or tyrosine kinase activity), detecting catalytic/enzymatic activity of an appropriate substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, chloramphenicol acetyl transferase), or detecting a cellular response regulated by B7-4, PD-1 or the appropriate target molecule. For example, determining the ability of the B7-4 or PD-1 protein to bind to or interact with a B7-4 or PD-1 target molecule can be accomplished, for example, by measuring the ability of a compound to modulate immune cell costimulation or inhibition in a proliferation assay, or by interfering with the ability of a B7-4 or PD-1 polypeptide to bind to antibodies that recognize a portion of the B7-4 or PD-1 polypeptide.

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In another example, the activation of PD-1 (via ligation of the receptor) results in the phosphorylation of the SHP-2 molecule. SHP-2 (also known as Syp, SHPTP2, PTP2C, PTPN11, PTP1D and BPTP3) is a member of the family of non-membrane tyrosine phosphatases (U.S. Patent No. 5,589,375, and U.S. Patent No. 5,831,009, the contents of which are incorporated herein by reference). It is ubiquitously expressed in all tissues examined with the highest levels in the heart and brain (Ahmad et al., *Proc. Natl. Acad. Sci. U.S.A., 90*: 2197-2201(1993); Bastien et al., *Biochem. Biophys. Res. Commun. 196*: 124-133 (1993); Freeman et al., *Proc. Natl. Acad. Sci. U.S.A., 89*: 11239-11243 (1992)).

The SHP-2 protein contains two src homology 2 (SH2) domains, conserved regions of approximately 100 amino acids originally identified in Src protein tyrosine kinases, that promote protein-protein interactions through phosphotyrosyl residue binding (Neel, Semin. *Cell. Biol. 4*: 419-432 (1993)). These two domains have been shown to display differential functions in the regulation of the SHP-2 phosphatase and consequently affect different signaling pathways. The N-terminal SH2 domain serves as a regulatory and recruiting domain, producing an autoinhibitory effect through intramolecular interactions with the internal catalytic phosphatase domain. While the C-terminal SH2 domain acts merely to recruit other proteins for intermolecular interactions necessary for signal transduction (Pei et al., *Proc. Natl. Acad. Sci. U.S.A. 93*: 1141-1145 (1996)). The phosphorylation state of the SHP-2 molecule regulates its phosphatase activity.

Protein-tyrosine phosphatases, including SH2-containing phosphatases, are highly conserved among eukaryotes from such diverse species as mammals, including humans, to yeast and Xenopus. Mutations and altered expression of protein-tyrosine phosphatases have been implicated in a number of pathologic conditions, such as neoplastic diseases and developmental abnormalities (Tonks, N.K., et al., *Cell 87*:365-368 (1996); Neel, B. G., et al., *Curr. Op. Cell Biol. 9*:193-204 (1997); Li, J. et al., *Science275*: 1943-1947 (1997)). SHP-2 has been shown to play a critical role in aberrant immunological responses (e.g., in the allergic response. (Pazdrak et al., *J. Exp. Med. 186*: 561-568 (1997)).

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Phosphorylation of the SHP-2 molecule is an indication of signaling via PD-1. SHP-2 phosphorylation is easily detectable by methods known in the art, including, without limitation, the detection of altered mobility of the SHP-2 molecule on a PAGE gel, phosphorylation assays, and assays which measure the activity of the SHP-2 molecule. Detection of SHP-2 phosphorylation may be direct, or alternatively may be indirect, e.g.,. detection of a downstream activity or event.

In another example, the activation of PD-1 (via ligation of the receptor) results in the inhibition of phosphorylation of the extracellular signal-regulated kinase 1 and 2 (ERK 1 and 2), induced by T cell receptor activation. ERK1 and ERK2 (also known as MAPK1 and MAPK2) are themselves kinases (Boulton, et al., *Cell 65*: 663-675, (1991)). Activation of the ERK molecule is via serine/threonine phosphorylation or tyrosine phosphorylation. Thus, the inhibition of phosphorylation of ERK1 and 2 by PD-1 signaling in an otherwise activated T cell, indicates inhibition of ERK 1 and 2 activation by PD-1. Inhibition of ERK 1 and 2 activation may result from inhibition of upstream phosphorylation of the ERK 1 and 2 molecules, or may result from the activation of a phosphatase which dephosphorylates ERK1 and 2, to reduce activity.

The ERK proteins are known to be activated by phosphorylation by the MEK molecule, a dual-specificity kinase. Upon activation, ERK1 and ERK2 translocate to the nucleus where they can directly phosphorylate and activate a variety of transcription factors including c-Myc, C/EBPβ, p62,TCF /Elk-1, ATF-2 and c-Jun.

The phosphorylation state/activation state of the ERK1 and 2 molecules upon T cell activation is an indication of signaling via PD-1. The phosphorylation state/state of activation of ERK 1 and 2 can readily be determined by the skilled practitioner using assays readily available in the art. For instance, the phosphorylation state of the ERK1 and 2 molecules can be determined using an antibody specific for the phosphorylated form of the p42/44 ERK1/2 proteins (phospho-Thr202/Tyr204 specific), several of which are commercially available. Alternatively, the phosphorylation state of the ERK1 and 2 molecules can be determined by their mobility on a gel, using an antibody which recognizes ERK1 and 2, regardless of the phosphorylation state) for identification. Alternatively, the activation state of ERK1 and 2 can be determined by assaying kinase activity of the ERK1 and 2 molecules. Determination of the ERK1 and 2

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phosphorylation/activation state may also be by indirect methods, e.g.,. detection of a downstream activity or event.

In another example, the activation of PD-1 (via ligation of the receptor) results in the inhibition of phosphorylation of the protein kinase C-θ (PKC-θ) molecule induced by T cell receptor activation. The PKC isoenzymes play an important role in many cell signaling events. PKC-θ (also known as PKC-θ, PKCT, PRKCT, nPKC-θ and PRKCQ) is a calcium-independent isoform of the PKC family of serine-threonine kinases. PKC-θ, one of the novel serine/threonine protein kinase C isoforms (nPKC), is expressed ubiquitously in tissues with the highest levels found in hematopoietic cell lines, including T-cells and thymocytes. (Baier et al., *J. Biol. Chem. 268*: 4997-5004 (1993); Keenan et al., *Immunology 90*: 557-563 (1997); Meller et al., *Cell. Immunol. 193*: 185-193 (1999); Wang et al., *Biochem.Biophys. Res. Commun. 91*: 240-246 (1993)). Transient overexpression of the PKC-θ protein in murine thymoma cells resulted in transcriptional activation of an interleukin-2 promoter-driven construct (Baier et al., *Eur. J. Biochem. 225*: 195-203(1994)), indicating a role for PKC-θ in T-cell signaling pathways. More recently, PKC-θ has been shown to be activated in the course of T cell receptor mediated T cell activation, and this activation correlates with translocation of the PKC-θ molecule to the plasma membrane at the site of APC contact (U.S. Patent No. 6,040,152, the contents of which are incorporated herein by reference). PKC-θ has also been implicated in other cellular processes including apoptosis (Datta et al., *J. Biol. Chem. 272*: 20317-20320 (1997)), cytoskeletal arrangement (Pietromonaco et al., *J. Biol. Chem. 273*: 7594-7603 (1998); Simons et al., *Biochem. Biophys. Res. Commun. 253*: 561-565 (1998)), proliferation (Passalacqua et al., *Biochem. J. 337*: 113-118 (1999)), and angiogenesis and wound repair (Tang et al., *J. Biol. Chem. 272:* 28704-28711 (1997)).

The phosphorylation/activation state of the PKC-θ molecule upon T cell activation is an indication of signaling via PD-1. The phosphorylation state reflects the activation state of the PKC-θ molecule, with phosphorylation indicating activation. The phosphorylation state/state of activation or PKC-θ can readily be determined by the skilled practitioner using assays readily available in the art. For instance, the phosphorylation state of the PKC-θ molecule can be determined using an antibody

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specific for the phosphorylated form of the PKC-θ protein (e.g., anti-phospho T_{538}), which is commercially available. Alternatively, the phosphorylation state of the PKC-θ molecule can be determined by its mobility on a gel, using an antibody which recognizes PKC-θ (regardless of the phosphorylation state) for detection. Alternatively, the activation state of PKC-θ can be determined by assaying kinase activity of the PKC-θ molecule (Kupfer et al., U.S. Patent No. 6,040,152), or by assaying for translocation to the membrane at the point of APC contact (Kupfer et al., U.S. Patent No. 6,040,152). Determination of the PKC-θ phosphorylation/activation state may also be by indirect methods, e.g.,. detection of a downstream activity or event.

In yet another embodiment, an assay of the present invention is a cell-free assay in which a B7-4 or PD-1 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the B7-4 or PD-1 protein or biologically active portion thereof is determined. Binding of the test compound to the B7-4 or PD-1 protein can be determined either directly or indirectly as described above. In a preferred embodiment, the assay includes contacting the B7-4 or PD-1 protein or biologically active portion thereof with a known compound which binds B7-4 or PD-1 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a B7-4 or PD-1 protein, wherein determining the ability of the test compound to interact with a B7-4 or PD-1 protein comprises determining the ability of the test compound to preferentially bind to B7-4 or PD-1 polypeptide or biologically active portion thereof as compared to the known compound.

In another embodiment, the assay is a cell-free assay in which a B7-4 or PD-1 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the B7-4 or PD-1 protein or biologically active portion thereof is determined. Determining the ability of the test compound to modulate the activity of a B7-4 or PD-1 protein can be accomplished, for example, by determining the ability of the B7-4 or PD-1 protein to bind to a B7-4 or PD-1 target molecule by one of the methods described above for determining direct binding. Determining the ability of the B7-4 or PD-1 protein to bind to a B7-4 or PD-1 target molecule can also be accomplished using a technology such as

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real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another embodiment, the cell-free assay involves contacting a B7-4 or PD-1 protein or biologically active portion thereof with a known compound which binds the B7-4 or PD-1 protein to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the B7-4 or PD-1 protein, wherein determining the ability of the test compound to interact with the B7-4 or PD-1 protein comprises determining the ability of the B7-4 or PD-1 protein to preferentially bind to or modulate the activity of a B7-4 or PD-1 target molecule.

The cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of proteins (*e.g.*, B7-4 or PD-1 proteins or biologically active portions thereof, or binding partners to which B7-4 or PD-1 binds). In the case of cell-free assays in which a membrane-bound form of a protein is used (*e.g.*, a cell surface B7-4 or PD-1 receptor) it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either B7-4 or PD-1 or an appropriate target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to a B7-4 or PD-1 protein, or interaction of a B7-4 or PD-1 protein with a target molecule in the presence and absence of a candidate compound, can be

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accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/B7-4 or PD-1 fusion proteins, or glutathione-S-transferase/target fusion proteins, can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or B7-4 or PD-1 protein, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of B7-4 or PD-1 binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either a B7-4 or PD-1 protein or a B7-4 or PD-1 target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated B7-4 or PD-1 protein or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with B7-4 or PD-1 protein or target molecules but which do not interfere with binding of the B7-4 or PD-1 protein to its target molecule can be derivatized to the wells of the plate, and unbound target or B7-4 or PD-1 protein trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the B7-4 or PD-1 protein or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the B7-4 or PD-1 protein or target molecule.

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In an alternative embodiment, determining the ability of the test compound to modulate the activity of a B7-4 or PD-1 protein can be accomplished by determining the ability of the test compound to modulate the activity of a molecule that functions downstream of B7-4, *e.g.*, a molecule that interacts with B7-4, or a molecule that functions downstream of PD-1, *e.g.*, by interacting with the cytoplasmic domain of PD-1. For example, levels of second messengers can be determined, the activity of the interactor molecule on an appropriate target can be determined, or the binding of the interactor to an appropriate target can be determined as previously described.

In another embodiment, modulators of B7-4 or PD-1 expression are identified in a method wherein a cell is contacted with a candidate or test compound and the expression of B7-4 or PD-1 mRNA or protein in the cell is determined. The level of expression of B7-4 or PD-1 mRNA or protein in the presence of the candidate compound is compared to the level of expression of B7-4 or PD-1 mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of B7-4 or PD-1 expression based on this comparison. For example, when expression of B7-4 or PD-1 mRNA or protein is greater (*e.g.*, reproducibly and statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of B7-4 or PD-1 mRNA or protein expression. Alternatively, when expression of B7-4 or PD-1 mRNA or protein is less (*e.g.*, reproducibly and statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of B7-4 or PD-1 mRNA or protein expression. The level of B7-4 or PD-1 mRNA or protein expression in the cells can be determined by methods described herein for detecting B7-4 or PD-1 mRNA or protein.

In another embodiment of the invention, peptides and/or proteins which are involved in PD-1 signaling pathways (e.g., proteins which can act as PD-1:B7-4 agonists or antagonists, proteins which are phosphorylated in response to PD-1 signaling, and/or proteins which bind PD-1) can be identified using a peptide binding/immunoprecipitation and mass spectrometry assay. For example, T cells (e.g., Jurkat T cells) can be activated in the presence or absence of B7-4, and in the presence or absence of pervanadate. Proteins can then be immunoprecipitated from lysates of the

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T cells using, *e.g.*, anti-PD-1 antibodies, anti-phosphotyrosine (Ptyr), or PD-1 peptides. The patterns of immunoprecipitated proteins and the changes in phosphorylation can then be compared using, e.g., SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Proteins can then be analyzed and identified using ESI MS/MS (mass spectrometry). A schematic depicting the above described screening assay is shown in Figure 31A.

Proteins identified using the above-described screening assay (*e.g.*, in an assay using a Jurkat T cell) can then be used in analysis of human T cells. Such proteins can then be used in signaling-based screening assays to identify PD-1 agonists or antagonists, *e.g.*, small molecule agonists or antagonists.

In yet another aspect of the invention, the B7-4 or PD-1 proteins, preferably in membrane bound form, can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins ("B7-4 or PD-1 binding proteins" or "B7-4 or PD-1 bp"), which bind to or interact with B7-4 or PD-1 and are involved in B7-4 or PD-1 activity. Such B7-4 or PD-1 binding proteins are also likely to be involved in the propagation of signals by the B7-4 or PD-1 proteins or B7-4 or PD-1 targets as, for example, upstream or downstream elements of a B7-4 or PD-1 mediated signaling pathway. Alternatively, such B7-4 or PD-1 binding proteins may be B7-4 or PD-1 inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for a B7-4 or PD-1 protein is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming a B7-4-dependent complex, the DNA-binding and activation domains of the transcription factor are

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brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the B7-4 or PD-1 protein.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a B7-4 or PD-1 modulating agent, an antisense B7-4 or PD-1 nucleic acid molecule, a B7-4-or PD-1 specific antibody, or a B7-4 or PD-1 binding partner) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

E. Detection Assays

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) map their respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

1. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. This process is called chromosome mapping. Accordingly, portions or fragments of B7-4 nucleotide sequences, described herein, can be used to map the location of B7-4 genes on a

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chromosome. The mapping of B7-4 sequences to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, B7-4 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from B7-4 nucleotide sequences. Computer analysis of B7-4 sequences can be used to predict primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to B7-4 sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (*e.g.*, human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes (D'Eustachio, P. *et al.* (1983) *Science* 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using B7-4 nucleotide sequences to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a sequence to its chromosome include *in situ* hybridization (described in Fan, Y. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

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Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical such as colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York 1988).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data (Such data are found, for example, in McKusick, V., Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between a gene and a disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. *et al.* (1987) *Nature* 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the B7-4 gene can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for

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structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

2. Tissue Typing

The B7-4 sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the B7-4 nucleotide sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The B7-4 nucleotide sequences of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared

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for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1 or 3 can comfortably provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences are used, a more appropriate number of primers for positive individual identification would be 500-2,000.

If a panel of reagents from B7-4 nucleotide sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

3. Use of Partial B7-4 Sequences in Forensic Biology

DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, *e.g.*, hair or skin, or body fluids, *e.g.*, blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, *e.g.*, PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (*i.e.*, another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of

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polynucleotide reagents include the B7-4 nucleotide sequences or portions thereof having a length of at least 20 bases, preferably at least 30 bases.

The B7-4 nucleotide sequences described herein can further be used to provide polynucleotide reagents, *e.g.*, labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, *e.g.*, brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such B7-4 probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, *e.g.*, B7-4 primers or probes can be used to screen tissue culture for contamination (*i.e.*, screen for the presence of a mixture of different types of cells in a culture).

F. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining B7-4 or PD-1 protein and/or nucleic acid expression as well as B7-4 or PD-1 activity, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant B7-4 or PD-1 expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with B7-4 or PD-1 protein, nucleic acid expression or activity. For example, mutations in a B7-4 or PD-1 gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with B7-4 or PD-1 protein, nucleic acid expression or activity. The assays described herein, such as the preceding diagnostic assays or the following assays, can also be used to detect a tendency to have spontaneous abortions.

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Another aspect of the invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of B7-4 or PD-1 in clinical trials.

These and other agents are described in further detail in the following sections.

1. Diagnostic Assays

An exemplary method for detecting the presence or absence of B7-4 or PD-1 protein or nucleic acid in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting B7-4 or PD-1 protein or nucleic acid (*e.g.*, mRNA, genomic DNA) that encodes B7-4 or PD-1 protein such that the presence of B7-4 or PD-1 protein or nucleic acid is detected in the biological sample. A preferred agent for detecting B7-4 or PD-1 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to B7-4 or PD-1 mRNA or genomic DNA. The nucleic acid probe can be, for example, a human B7-4 or PD-1 nucleic acid, such as the nucleic acid of SEQ ID NO:1, 3, 10, or 11 or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to B7-4 or PD-1 mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

A preferred agent for detecting B7-4 or PD-1 protein is an antibody capable of binding to B7-4 or PD-1 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or $F(ab')_2$) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect B7-4 or PD-1 mRNA,

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protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of B7-4 or PD-1 mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of B7-4 or PD-1 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of B7-4 or PD-1 genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of B7-4 or PD-1 protein include introducing into a subject a labeled anti-B7-4 or PD-1 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A preferred biological sample is a serum sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting B7-4 or PD-1 protein, mRNA, or genomic DNA, such that the presence of B7-4 or PD-1 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of B7-4 or PD-1 protein, mRNA or genomic DNA in the control sample with the presence of B7-4 or PD-1 protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of B7-4 or PD-1 in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting B7-4 or PD-1 protein or mRNA in a biological sample; means for determining the amount of B7-4 or PD-1 in the sample; and means for comparing the amount of B7-4 or PD-1 in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect B7-4 or PD-1 protein or nucleic acid.

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2. Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant B7-4 or PD-1 expression or activity. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with B7-4 or PD-1 protein, expression or activity. Thus, the present invention provides a method for identifying a disease or disorder associated with aberrant B7-4 or PD-1 expression or activity in which a test sample is obtained from a subject and B7-4 or PD-1 protein or nucleic acid (*e.g.*, mRNA, genomic DNA) is detected, wherein the presence of B7-4 or PD-1 protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant B7-4 or PD-1 expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue.

Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant B7-4 or PD-1 expression or activity. Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant B7-4 or PD-1 expression or activity in which a test sample is obtained and B7-4 or PD-1 protein or nucleic acid expression or activity is detected (*e.g.*, wherein the abundance of B7-4 or PD-1 protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant B7-4 or PD-1 expression or activity).

The methods of the invention can also be used to detect genetic alterations in a B7-4 or PD-1 gene, thereby determining if a subject with the altered gene is at risk for a disorder associated with the B7-4 or PD-1 gene. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding a B7-4 or PD-1 protein, or the mis-expression of the B7-4 or PD-1 gene.

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For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a B7-4 or PD-1 gene; 2) an addition of one or more nucleotides to a B7-4 or PD-1 gene; 3) a substitution of one or more nucleotides of a B7-4 or PD-1 gene, 4) a chromosomal rearrangement of a B7-4 or PD-1 gene; 5) an alteration in the level of a messenger RNA transcript of a B7-4 or PD-1 gene, 6) aberrant modification of a B7-4 or PD-1 gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a B7-4 or PD-1 gene, 8) a non-wild type level of a B7-4 or PD-1 protein, 9) allelic loss of a B7-4 or PD-1 gene, and 10) inappropriate post-translational modification of a B7-4 or PD-1 protein. As described herein, there are a large number of assay techniques known in the art which can be used for detecting alterations in a B7-4 or PD-1 gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject, *e.g.*, a cardiac tissue sample.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the B7-4 or PD-1 gene (see Abravaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (*e.g.*, genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a B7-4 or PD-1 gene under conditions such that hybridization and amplification of the B7-4 or PD-1 gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

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Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. *et al.*, (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P. M. *et al.* (1988) *Biotechnology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a B7-4 or PD-1 gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in B7-4 or PD-1 can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M. T. *et al.* (1996) *Hum. Mutat.* 7:244-255; Kozal, M. J. *et al.* (1996) *Nat. Med.* 2:753-759). For example, genetic mutations in B7-4 or PD-1 can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M. T. *et al.* (1996) *supra.* Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

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In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the B7-4 or PD-1 gene and detect mutations by comparing the sequence of the sample B7-4 or PD-1 with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) *Proc. Natl. Acad. Sci. USA* 74:560) or Sanger ((1977) *Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the B7-4 or PD-1 gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type B7-4 or PD-1 sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4397; Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point

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mutations in B7-4 or PD-1 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a B7-4 sequence, *e.g.*, a wild-type B7-4 or PD-1 sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility can be used to identify mutations in B7-4 or PD-1 genes. For example, single strand conformation polymorphism (SSCP) can be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci. USA*: 86:2766, see also Cotton (1993) *Mutat. Res.* 285:125-144; and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control B7-4 or PD-1 nucleic acids can be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments can be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet.* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA can be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys. Chem.* 265:12753).

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Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification can be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner *et al.* (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci. USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein can be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which can be conveniently used, *e.g.,* in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a B7-4 or PD-1 gene.

Furthermore, any cell type or tissue in which B7-4 or PD-1 is expressed can be utilized in the prognostic assays described herein.

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VII. Administration of B7-4 or PD-1 Modulating Agents

B7-4 or PD-1 modulating agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to either enhance or suppress immune cell mediated immune responses. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term subject is intended to include living organisms in which an immune response can be elicited, *e.g.*, mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of an agent as described herein can be in any pharmacological form including a therapeutically active amount of an agent alone or in combination with a pharmaceutically acceptable carrier.

Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a B7-4 or PD-1 polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The B7-4 or PD-1 modulating agent (*e.g.*, a peptide, a nucleic acid molecule, antibody, peptidomimetic, or small molecule) can be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound can be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. For example, to administer B7-4 or PD-1 modulating agent by other than parenteral administration, it may be desirable to coat the peptide with, or co-administer the peptide with, a material to prevent its inactivation.

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A B7-4 or PD-1 modulating agent can be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Sterna *et al.* (1984) *J. Neuroimmunol.* 7:27).

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about

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by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound (*e.g.*, a B7-4 or PD-1 polypeptide) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yields a powder of the active ingredient (*e.g.*, peptide) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the protein can be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. 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 the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

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In one embodiment of the present invention a therapeutically effective amount of an antibody to a B7-4 or PD-1 protein is administered to a subject. As defined herein, a therapeutically effective amount of antibody (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to, the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result from the results of diagnostic assays as described herein.

Monitoring the influence of agents (*e.g.*, drugs or compounds) on the expression or activity of a B7-4 or PD-1 protein can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase B7-4 or PD-1 gene expression, protein levels, or upregulate B7-4 or PD-1 activity, can be monitored in clinical trials of subjects exhibiting decreased B7-4 or PD-1 gene expression, protein levels, or downregulated B7-4 or PD-1 activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease B7-4 or PD-1 gene expression, protein levels, or downregulate B7-4 or PD-1 activity, can be monitored in clinical trials of subjects exhibiting increased B7-4 or PD-1 gene expression, protein levels, or upregulated B7-4 or PD-1 activity. In such clinical trials, the expression or activity of a B7-4 or PD-1 gene, and preferably, other genes that have been implicated in a disorder can be used as a "read out" or markers of the phenotype of a particular cell.

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For example, and not by way of limitation, genes, including B7-4 or PD-1, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) which modulates B7-4 or PD-1 activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on a B7-4 or PD-1 associated disorder, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of B7-4 or PD-1 and other genes implicated in the B7-4 or PD-1 associated disorder, respectively. The levels of gene expression (*i.e.*, a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of B7-4 or PD-1 or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state can be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a B7-4 or PD-1 protein, mRNA, or genomic DNA in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the B7-4 or PD-1 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the B7-4 or PD-1 protein, mRNA, or genomic DNA in the pre-administration sample with the B7-4 or PD-1 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of B7-4 or PD-1 to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of B7-4 or PD-1 to lower levels than detected, *i.e.*, to

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decrease the effectiveness of the agent. According to such an embodiment, B7-4 or PD-1 expression or activity can be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference.

EXAMPLES

Example 1: Isolation of B7-4 cDNA Molecules

The protein sequence of the extracellular domain of human B7-1 was used to search the public databases for nucleic acid molecules encoding homologous polypeptides. Two overlapping sequences in the EST database, AA292201 and AA399416, were identified. These sequences were used to isolate full-length B7-4 cDNAs from human activated keratinocyte and placental cDNA libraries as follows.

Oligonucleotides with the sequence 5'-CAGCTATGGTGGTGCCGACTACAA-3' (SEQ ID NO:5) and 5'-AGGTGCTAGGGGACAGTGTTAGACA-3' (SEQ ID NO:6) from these ESTs were synthesized. These oligonucleotides were used to prime a PCR reaction using as template cDNA prepared by reverse transcription of mRNAs from the spleen of a case of follicular lymphoma, activated B cells, INF-γ activated keratinocytes, normal spleen, and placenta. Conditions were 94°C, 1 min; 94°C, 30 sec, 56°C, 30 sec, 68°C, 1 min for 35 cycles; 68°C, 3 min, hold 4°C. All templates gave a band of the expected size of 389 bp. The 389 bp product from the PCR of INF-γ activated keratinocytes was purified by agarose gel electrophoresis and 0.12 ng was used as a template in a PCR reaction containing 0.05 mM biotin-21-dUTP and the above primers. Conditions were 94°C, 1 min; 94°C, 30 sec, 56°C, 30 sec, 68°C, 2 min for 20 cycles; 68°C, 5 min, hold 4°C. The biotinylated PCR product was purified on a Nucleospin column (Clontech) and used as a probe in the ClonCapture cDNA selection procedure (Clontech). 60 ng of denatured, biotinylated PCR product was incubated with 2 mM

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$CoCl_2$, 1 X RecA buffer, 1 μg of RecA protein, 1X ATP in a final volume of 30 μl. The reaction was incubated at 37°C for 15 min. To that mixture, 0.7 μg of plasmid DNA of an activated keratinocyte cDNA library and 0.4 μg of a human placental cDNA library was added and incubation continued for 20 min. 50 ng of EcoRV digested lambda DNA was added to the reaction and incubated 5 min. 0.6 μl of 10% SDS and 5.6 μg of proteinase K were added and incubated at 37°C for 10 min. Proteinase K was inactivated by adding 1 μl of 0.1 M PMSF. Streptavidin magnetic beads were preincubated with 5 μg of sheared salmon sperm DNA for 10 min and the beads captured with a magnet, the supernatant removed, and the beads resuspended in 30 μl of binding buffer (1 mM EDTA, 1 M NaCl, 10 mM Tris-HCl, pH 7.5). The beads were added to the reaction and the reaction incubated for 30 min at room temperature with gentle mixing. The beads were captured with a magnet and the supernatant removed. The beads were washed with 1 ml of washing buffer (1 mM EDTA, 2 M NaCl, 10 mM Tris-HCl, pH 7.5), beads were captured with a magnet and the supernatant removed. The wash procedure was repeated 3 times. One ml of sterile H_2O was added to the washed beads, incubated 5 min at 37°C, beads were captured on a magnet and the supernatant removed. Captured DNA was eluted by adding 0.1 ml of elution buffer (1 mM EDTA, 0.1 N NaOH), incubating 5 min at room temperature, beads were captured with a magnet and the supernatant removed and saved in a new tube. 22.5 μl of precipitation mix containing carrier and pH neutralizers was added along with 2.5 volumes of ethanol. The plasmid DNA was concentrated by centrifugation and re-dissolved in H_2O. Plasmid DNA was re-introduced into *E. coli* DH10B/P3 by electroporation and selected on LB-agar plates containing 7.5 μg/ml tetracycline and 25 μg/ml ampicillin. Colonies were lifted onto Nytran filters and hybridized with ^{32}P-labeled oligonucleotides with the sequence 5'-CAGCTATGGTGGTGCCGACTACAA-3' (SEQ ID NO:5), 5'-AGGTGCTAGGGGACAGTGTTAGACA-3' (SEQ ID NO:6), and 5'-TCGCTTGTAGTCGGCACCACCATA-3' (SEQ ID NO:9). All oligos are from AA292201 sequence. Final wash conditions were 2 X SSC, 0.1 % SDS at 55° for 20

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min. The two hybridizing colonies were picked and the sequence of the cDNA inserts was determined.

Sequencing revealed two forms of B7-4 molecules. The first form, B7-4 secreted (B7-4S) encodes a protein having a short hydrophilic domain without a membrane anchor. The nucleotide and amino acid sequences of this form are shown in SEQ ID NO:1 and 2, respectively. The second form, B7-4 membrane (B7-4M) encodes a protein having a transmembrane and short cytoplasmic domain. The nucleotide and amino acid sequences of this form are shown in SEQ ID NO:3 and 4, respectively. Both members of the B7-4 family identified have signal, IgV, and IgC domains, as illustrated in Figures 3 and 4. The B7-4M form has approximately 21% amino acid identity to human B7-1 and about 20% amino acid identity to human B7-2 as calculated using the default Blosum62 matrix with gap penalties set at existence 11 and extension 1 (See http://www.ncbi.nlm.nih.gov), under conditions where B7-1 and B7-2 have about 26% identity.

Example 2: Expression of B7-4 mRNA: Northern Blot Analysis

An mRNA of the soluble form of B7-4 is predicted to be about 1.2kb though other sizes are possible. The mRNA of the second form is about 3.8 kb, with minor mRNAs of 1.6 and 6.5 kb.

Expression of B7-4 polypeptides was analyzed. RNA was prepared by guanidine thiocyanate homogenization and cesium chloride centrifugation. Equal amounts of RNA (approximately 2 μg poly(A)+ RNA) were electrophoresed on an agarose gel, blotted, and hybridized to a portion of ^{32}P-labeled B7-4 cDNA common to both the B7-4S and B7-4 M forms. These B7-4 mRNAs are highly expressed in placenta, lung, and heart and are moderately expressed in the thymus. In addition, these B7-4 mRNAs are weakly expressed in skeletal muscle, kidney, pancreas, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. They were also found to be very weakly expressed in liver or brain. B7-4 mRNAs were not expressed in unstimulated monocytes, but were strongly induced by IFN-γ. Similarly, the expression of these polypeptides was found to be induced in keratinocytes by TPA/IFN- γ and in dendritic

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cells by IFN- γ. These B7-4 mRNAs were not expressed in unstimulated B cells, but were induced by Ig crosslinking.

Expression of these B7-4 mRNAs was also examined in a variety of cell lines. They were not found to be expressed in B cell lines such as Raji, Ramos, LBL, Nalm 6, and DHL-4. They were also not expressed in T cell lines, such as Jurkat, Rex, CEM, HPB-ALL, Peer4, and H9 or in HTLV-1 transformed T cell lines such as SPP and MT2 or in the myeloid line U937.

Example 3: Further Characterization of B7-4 mRNA Expression: Northern Blot Analysis

Mouse and human multiple tissue Northern blots (Clontech, Palo Alto, CA) were probed with ^{32}P-dCTP radiolabeled cDNA probes in QuikHyb (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The human B7-4 probe consisted of a 1 kb BamHI/NotI fragment of the cDNA spanning the coding region and 3' untranslated region of SEQ ID NO:1. The mouse B7-4 probe consisted of a 300 bp cDNA fragment from the coding region. Control actin probes were supplied by Clontech. Blots were washed twice at room temperature in 2X SSC, 0.1% SDS, followed by 0.2X SSC, 0.1% SDS at 65°C, and examined by autoradiography.

B7-4 mRNA was expressed at high levels in heart, human placenta, and human fetal liver, and at lower levels in spleen, lymph nodes, thymus, and mouse liver.

B7-4 mRNA was expressed in a variety of transformed mouse cell lines, including PU5-1.8, RAW 264.7, K-Balb, M-MSV-Balb/3T3, Hepa 1-6, R1.1, L1210, P38D1, P815, and NB41A3 cells.

Example 4: Further Characterization of B7-4 mRNA Expression: Quantitative PCR, Genechip Hybridization, and RNA Blot Analysis

B7-4 mRNA expression on antigen presenting cells was examined and compared to the expression of B7-1 and B7-2 on those cells. For quantitative PCR analysis, cellular RNA was deoxyribonuclease-treated, re-extracted and converted to first strand cDNA. FAM (6-caroxyfluorescein)-labeled human B7-4, B7-1, B7-2, and GAPDH probes were purchased from PE Biosystems (B7-4: primers 5'-

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GCCGAAGTCATCTGGACAAG-3' (SEQ ID NO:13) and 5'-TCTCAGTGTGCTGGTCACAT-3' (SEQ ID NO:14), probe 5'-FAM-CACCACCACCAATTCCAAGA-3' (SEQ ID NO:15); B7-1: primers 5'-ACGTGACCAAGGAAGTGAAAGAA-3' (SEQ ID NO:16) and 5'-TGCCAGCTCTTCAACAGAAACAT-3' (SEQ ID NO:17), probe 5'-FAM-TGGCAACGCTGTCCTGTGGTCAC-3' (SEQ ID NO:18); B7-2: primers 5'-GGGCCGCACAAGTTTTGAT-3' (SEQ ID NO:19) and 5'-GCCCTTGTCCTTGATCTGAAGA-3' (SEQ ID NO:20), probe 5'-FAM-CGGACAGTTGGACCCTGAGACTTCACA-3' (SEQ ID NO:21).

PCR reactions were set up in 96-well plates using reagents from the Perkin Elmer TaqMan™ EZ kit, according to the manufacturer's instructions. Standard curves were set up for each of the four genes analyzed. Forty cycles of PCR were run in an ABI Prism 7700 Sequence Detector and GAPDH was used to normalize the B7-4, B7-1, and B7-2 results.

The Affymetrix Mu19KsubA chip was used for Genechip hybridization analysis. The sequence of a portion of murine B7-4 is represented by expressed sequence tag TC17781 of The Institute for Genomic Research on this chip. RNA isolation, chip hybridization and scanning was performed as described in Byrne, M. C. *et al.* (2000) *Curr. Prot. Mol. Biol. Suppl.* 49:22.2.1-22.2.13.

For RNA blot hybridization, the 1.6 kb human and 3.6 kb murine B7-4 cDNAs were excised by digestion with Xba I and labeled by random priming with γ-^{32}P-ATP and the Klenow fragment of DNA polymerase I. RNA blots were hybridized as described in Freeman, G. J. *et al.* (1992) *J. Immunol.* 149:3795-3801.

Human dendritic cells were derived from peripheral blood. Mononuclear cells were isolated after fractionation on a Ficoll gradient. Non-adherent cells were removed and the remaining cells cultured in 150 ng/ml human GM-CSF (R&D Systems) and 100 ng/ml human IL-4 (R&D Systems) for 2 days. The non-adherent dendritic cells were isolated ($CD80^+$ $CD86^+$ HLA-DR^+ $CD54^+$ $CD58^+$ $CD1a^+$) and cultured in GM-CSF alone or activated with GM-CSF, 2.5 μg/ml LPS (Sigma Chemicals), and 10 ng/ml human Interferon-γ. At 4 hours and 20 hours after activation, cells were harvested and RNA isolated using the RNeasy kit (Qiagen).

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Murine bone marrow mononuclear cells were immuno-depleted of granulocytes, lymphocytes and Ia^+ cells by magnetic activated cell sorting and cultured in petri dishes with GM-CSF and IL-4. Dendritic cells were harvested as the non-adherent population after 7 days of culture, and demonstrated to be 75-80% $CD11c^+$, high IA^+ cells. Cells were activated with LPS and human interferon-γ.

Analysis of expression in human blood monocytes by RNA blot hybridization demonstrated that B7-2 is not expressed by unstimulated monocytes, but is rapidly upregulated upon interferon-γ treatment. Treatment of monocytes with another pro-inflammatory cytokine, tumor necrosis factor (TNF)-α led to a low level induction similar to that found with medium alone, presumably as a result of activation by adherence to plastic. In addition to the major 4.2 kb B7-4 mRNA, a minor 1.8 kb B7-4 mRNA species was also observed in interferon-γ treated monocytes. Expression of B7-4 by human B-cells activated by cell surface immunoglobulin cross-linking, but not by the Raji cell line, was also observed. Similarly, B7-1 is not expressed by unstimulated monocytes, but is upregulated in response to interferon-γ with kinetics similar to B7-4 expression. In contrast, B7-2 mRNA is constitutively expressed in monocytes and levels are unaffected by interferon-γ or TNF-α treatment.

B7-4, B7-1, and B7-2 mRNA expression by human dendritic cells was also examined by quantitative PCR. Human peripheral blood-derived dendritic cells were treated with granulocyte-macrophage colony stimulated factor (GM-CSF) alone or activated with GM-CSF, lipopolysaccharide (LPS), and interferon-γ. As a result of activation by LPS and interferon-γ, B7-4 mRNA was rapidly induced with a 16-fold increase at 4 hours and a 34-fold increase at 20 hours, relative to non-induced cells. B7-1 and B7-2 mRNAs were also induced upon activation: B7-1 was induced 21-fold at 4 hours and 22-fold at 20 hours. B7-2 showed little induction at 4 hours; however, expression was induced 5-fold at 20 hours. Expression of B7-4 by murine bone marrow-derived dendritic cells treated with LPS and interferon-γ was examined using Genechip™ hybridization. B7-4 expression in these cells follows a pattern similar to that observed on human dendritic cells: a 5-fold induction of the B7-4 mRNA relative to the uninduced cells at 6 and 20 hours after induction. These data demonstrate that B7-4 is expressed by antigen presenting cells and lymphocytes, and it is induced on

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dendritic cells in a manner similar to B7-1 and B7-2. Treatment of human keratinocytes with phorbol ester and interferon-γ also induced B7-4.

In murine tissues, an approximately 3.7kb B7-4 mRNA transcript was detected by northern blot hybridization. The distribution of the murine B7-4 mRNA closely resembled that of the human B7-4, with high levels in heart, thymus and lung, and low levels in kidney, spleen and liver.

Example 5: Chromosomal Localization of B7-4

The chromosomal localization of the B7-4 genes was determined using a monochromosomal blot kit commercially available from Quantum (Toronto, Canada). The blots were probed with a sequence that recognizes both B7-4S and B7-4M. Using this method, the B7-4 polypeptides have been localized to human chromosome 9, whereas B7-1 and B7-2 have been localized to human chromosome 3. The butyrophilins, which also share limited amino acid sequence identity with the B7-4 family have been localized to the major histocompatibility complex on chromosome 6. The chromosomal location of B7-4 was confirmed using B7-4 specific primers in PCR amplification of monochromosomal somatic cell hybrid DNA templates available from Quantum Technologies (Canada).

Example 6: Binding of B7-4 Molecules to T Cell Ligands or Antibodies

COS cells were transfected with either vector DNA (pcDNAI), or an expression plasmid containing the B7-4M cDNA. After 72 hours, the transfected COS cells were detached by incubation in PBS containing 0.5 mM EDTA for 30 min. at 37°C.

The ability of COS cells expressing B7-4M to bind to various T cell receptors and antibodies was tested. FACS analysis of binding of CD28Ig, CTLA4-Ig, and control Ig by B7-4-transfected COS cells showed that neither CD28Ig nor CTLA4-Ig was bound by B7-4 (Figure 8). The ability of COS cells expressing B7-4M to bind to IgG and murine ICOS-his fusion protein was also tested. No binding of human B7-4 to murine ICOS was detected (Figure 9). As shown in Figure 10, FACS analysis revealed binding of BB1 (anti B7-1 and anti B7-3), but not IgM or 133 (anti-B7) antibodies to B7-4-transfected COS cells.

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Example 7: Costimulation of T Cell Proliferation By B7-4 Molecules

The ability of B7-4 polypeptides to costimulate human T cell proliferation was tested. Human $CD28^+$ T cells were isolated by immunomagnetic bead depletion using monoclonal antibodies directed against B cells, natural killer cells and macrophages as previously described (Gimmi, C.D. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6586-6590). B7-4 and vector transfected COS cells were harvested 72 hours after transfection, incubated with 25μg/ml of mitomycin-C for 1 hour, and then extensively washed. 10^5 naïve T Cells were stimulated with plate bound anti-CD3 mAb plus 20,000 mitomycin-c treated COS cells transfected with the indicated DNA construct.

T cell proliferation was measured by 3H-thymidine (1 μCi) incorporated for the last 12 hours of a 72 hour incubation. As shown in Figures 11 and 12, COS cells expressing B7-4 can costimulate T cell proliferation.

Example 8: Generation of Murine Antibodies to B7-4 and Use in Detecting Cell-Surface Expression of B7-4

Mammalian expression vectors (pEF6 or pcDNA3.1 (Invitrogen)) were prepared comprising the entire murine or human B7-4 cDNA. The cDNA/vector construct was dissolved in 0.9% saline at 1 mg/ml (not TE or PBS).

Before immunization, 78 μl of 1 mg/ml cardiotoxin (Sigma #C-1777) in 0.9% saline was injected into the tibialis anterior muscle of each hind limb of the mouse being immunized. Each mouse was then left alone for 5 days.

After anesthetizing the mice, 50 μl of 1 mg/ml purified B7-4 cDNA/vector construct (in 0.9% saline) was injected into each regenerating tibialis anterior muscle.

Antibody titers were measured approximately six days after immunization using standard methods, for example, in an ELISA assay. The cDNA immunization was repeated every 2-4 weeks for three cycles (until the antibody titre was >1:10,000). Mice were then boosted with CHO cells transfected with B7-4.

Spleen cells isolated from mice having appropriate antibody titers were harvested. The spleen cells were fused to fusion partners SP2-0) to make hybridomas. Hybridomas and antibodies were manipulated using standard methods (see, *e.g.*,

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"Antibodies: A Laboratory Manual", Harlow, E. and Lane, D., Cold Spring Harbor Laboratory (1988), which is incorporated herein by reference).

Antibodies 2A3, 10D9, 5A9, and 11D12 were among those selected in screening assays. These antibodies were found to bind to COS or CHO cells transfected with human B7-4 and not to mock transfected cells or to cells transfected with mouse B7-4. The antibodies were used to detect the presence of B7-4 on various cell populations. B7-4 expression was observed, inter alia, on heart tissue, tumor cells (including some lung tumor cells, some ovarian tumor cells, some breast tumor cells, some epithelial tumor cells, and some squamous cell carcinomas), placenta, and thymic epithelium.

Another antibody, clone 29E.2A3.C6 (mouse IgG2bκ) was used to examine cell surface expression of B7-4. B7-4 is expressed on human breast cancer cell lines MDA-231, SKBR-3, and MCF-7.

Example 9: Generation of Fully Human Antibodies to B7-4

In this example, fully human antibodies against B7-4 or PD-1 are made in mice that are transgenic for human immunoglobulin genes. Transgenic mice are made using standard methods, *e.g.*, according to Hogan, *et al.*, "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory, which is incorporated herein by reference, or are purchased commercially. Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and embryonic stem cells: a practical approach, Robertson, E. J. ed., IRL Press, Washington, D.C., 1987; Zijlstra *et al.* (1989) *Nature* 342:435-438; and Schwartzberg *et al.* (1989) *Science* 246:799-803, each of which is incorporated herein by reference). DNA cloning procedures are carried out according to Sambrook, J. *et al.* in Molecular Cloning: A Laboratory Manual, 2d ed., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference. Oligonucleotides are synthesized, *e.g.*, on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer or are purchased commercially.

Transgenic mice are immunized using a purified or recombinant B7-4 or PD-1 or a fusion protein comprising at least an immunogenic portion of the extracellular domain of B7-4 or PD-1. Approximately four hundred μg of B7-4 or PD-1 in 100 μL of

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phosphate buffered saline (PBS) is injected intraperitoneally into each mouse. Serum samples are collected approximately six days later by retro-orbital sinus bleeding.

Antibody reactivity and specificity for B7-4 or PD-1 are assessed using an indirect enzyme-linked immunosorbent assay (ELISA). Several immunoglobulin superfamily molecules are tested as controls (*e.g.*, CTLA4 and CD28) to analyze the antibody specificity of the antibody for B7-4 or PD-1. Antibodies having human variable regions which bind to B7-4 or PD-1 are detected by enzyme conjugates specific for human IgM and human IgG sub-classes with no cross reactivity to mouse immunoglobulin. Briefly, PVC microtiter plates are coated with B7-4 or PD-1 by coating wells overnight at 37°C with 5 μg/mL B7-4 in PBS. Serum samples are diluted in PBS, 5% serum, 0.5% Tween-20 and are incubated in the wells for 1 hour at room temperature, followed by anti-human IgG Fc and IgG F(ab')-horseradish peroxidase or anti-human IgM Fc-horseradish peroxidase in the same diluent. After 1hour at room temperature enzyme activity is assessed by addition of ABTS substrate (Sigma, St. Louis, Mo.) and read after 30 minutes at 415-490 nm. In pre-immunization serum samples from the same mice, titers of human antibodies to the same target antigens are also tested.

Spleen cells isolated from mice having appropriate antibody titers are harvested. The spleen cells are fused to appropriate fusion partners (*e.g.*, myeloma cells) to make hybridomas. Hybridomas and antibodies are manipulated according to "Antibodies: A Laboratory Manual", Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), which is incorporated herein by reference.

The complementarity determining sequences of the murine VH and VL domains of a murine antibody could be used to graft into the framework of human immunoglobulins in order to generate a humanized antibody against B7-4 or PD-1 (Riechmann *et al.* (1988) *Nature* 332:323; Verhoeyen *et al.* (1988) *Science* 239:1534).

Example 10: Generation of Human Single Chain Fvs Reactive with B7-4 or PD-1

As an alternative to preparing monoclonal antibody-secreting hybridomas, anti B7-4 or anti-PD-1 antibodies (single chain Fv-like portions of antibodies) were identified and isolated by screening a combinatorial library of human immunoglobulin

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sequences displayed on M13 bacteriophage from Cambridge Antibody Technology Ltd., Melbourn, UK (Winter *et al.* (1994) *Annu. Rev. Immunol.* 12:433; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1). PD-1.Fc or B7-4.Fc was used to thereby isolate immunoglobulin library members that bind a B7-4 or PD-1 polypeptide. Kits for generating and screening phage display libraries are commercially available and standard methods were employed to generate the scFv (Helfrich *et al.* (2000) *J. Immunol. Methods* 237:131-45; Cardoso *et al.* (2000) *Scand. J. Immunol.* 51:337-44). PD-1.Fc or B7-4.Fc were immobilized on plastic and phage expressing specific scFv were selected by panning and multiple rounds of enrichment (Griffiths *et al.* (1993) *EMBO J.* 12:725).

Example 11: Identification of a receptor for B7-4

Fusion proteins consisting of the extracellular region of human PD-1 fused to the hinge-CH2-CH3 domains of either human immunoglobulin gamma 1 or murine Ig gamma2a (with mutations blocking FcR and complement interaction) were used to search for a ligand that binds to PD-1. As part of this search, staining of the cell surface of monocytes stimulated with gamma-interferon was found. B7-4 is induced in monocytes after stimulation with gamma-interferon, as observed by northern blot hybridization.

The binding of PD-1-Fc (human Ig gamma1) to the surface of COS cells transiently transfected with a B7-4-expression vector was tested. COS cells were transfected with either B7-4M or B7-1 using LipofectAMINE transfection reagent. After 48 hours, the cells were stained with human PD-1-Fc, murine PD-1-Fc, CTLA4-Fc, Flt4-Fc, or IgG followed by anti-IgG conjugated to phycoerythrin (PE). The cells were then analyzed by flow cytometry. As shown in Figures 13A-13D, COS cells expressing B7-4 bound both human PD-1-Fc and murine PD-1-Fc, but did not bind CTLA4-Fc, Flt4-Fc, or human IgG. As a positive control, it was demonstrated that B7-1 expressing COS cells bound CTLA4-Fc, but not PD-1-Fc or IgG.

In addition, an *in situ* assay of transfected COS cell monolayers was performed. Monolayers were probed with PD-1Fc, CTLA4Fc or human IgG1 and binding was detected with a secondary antibody directed against the Fc portion and conjugated to

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alkaline phosphatase. Binding was visualized with chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium and light microscopy. In parallel, cells transfected with B7-4 were found to bind to PD-1-Fc, and not CTLA4-Fc (human Ig gamma 1) or Flt4-Fc, the extracellular region of murine Flt4 linked to human Ig gamma 1. In parallel, PD-1Fc did not bind the surface of mock-transfected, B7-1 or B7-2 transfected COS cells.

In another experiment, no binding of PD-1-Fc to soluble forms of B7-1 or B7-2, and binding to B7-4, was detected using a BIACORE-based assay. In parallel, hCTLA4 was shown to bind to B7-1 and not to B7-4. PD-1-Fc or CTLA4-Fc was immobilized and conditions were essentially as described by Fitz *et al.* (1997) *Oncogene* 15:613). Concentrated COS cell medium from cells that had been transfected with full length B7-4M or B7-4-Fc was injected and interactions were measured using real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705). Human B7-4 was found to bind human and mouse PD-1 and this binding was inhibited by competition with a coinjected PD-1-Fc, but not CTLA4-Fc. These experiments demonstrate not only the binding of soluble B7-4-Fc fusion protein to immobilized PD-1-Fc, but also demonstrate the presence of a soluble form of B7-4 in the conditioned medium of B7-4M cDNA transfected cells, presumably as a result of shedding.

Figures 14A-14F illustrate the ability of PD-1 and not Flt4 (the receptor for vascular endothelial growth factor C) to competitively inhibit the binding of PD-1 to B7-4. The binding of human PD-1 gamma 2a fusion protein to COS cells expressing B7-4M is shown in Panel A. The binding was detected with anti-gamma 2a specific reagents linked to PE. Human PD-1 linked to IgG1 was added at: 50 μg/ml, 6.25 μg/ml, 100 μg/ml, or 25 μg/ml and was found to compete for binding. As a control, Flt4IgG1 at 100 μg/ml was not found to compete for binding of PD-1 to B7-4.

In yet another experiment, the ability of B7-4 to bind to PD-1 was determined by flow cytometry and BIACORE-binding assays. Human and murine PD-1.Ig fusion proteins bound to both human and murine B7-4 expressed on CHO cells, as detected by flow cytometry (Figures 15A-15F). However, neither human CTLA-4.Ig, human CD28.Ig, nor human ICOS.Ig bound to either B7-4 expressing cell line. The PD-1

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fusion proteins did not bind CHO cells transfected with vector alone. Further confirmation of the PD-1:B7-4 interaction was obtained using surface plasmon resonance with a BIACORE instrument. The human and murine PD-1.Ig proteins and human CTLA-4.Ig were immobilized on the flow cell surfaces of a dextran chip and tested for binding to soluble human B7-4.Ig. B7-4.Ig bound to both human and murine PD-1.Ig, but not to human CTLA-4.Ig (Figure 16). This binding was blocked by competition with co-injected soluble PD-1.Ig, but not CTLA-4.Ig. Soluble forms of human B7-1 and B7-2 did not bind immobilized human PD-1.

BIACORE analysis was also used to analyze PD-1.Fc:B7-4.Fc binding kinetics. A BIA 2000, CM5 sensor chip was used. NHS/EDC immobilization was used to bind 50-150 RU PD-1.Fc to the chip. The surface was conditioned with 20 injections of ionic regeneration buffer (30% 1.83 M $MgCl_2$, 0.46 M KSCN, 0.92 M Urea, 1.83 M guanidine-HCl). The running buffer was PBS (phosphate buffered saline) with 3.4 mM EDTA, 0.005% Tween-20, and 100 μg/ml BSA (bovine serum albumin) at 25°C. The reference surfaces used were (1) blank activation (NHS/EDC) followed by ethanolamine block and (2) mutated mIgG2a. The concentration of B7-4.Fc used (undetectable multimer, determined by SEC) was determined by ELISA and BCA protein assay (Pierce). The range of B7-4.mFc used was 20 nM to 2 μM. The flow rate was 60 μl/minute. Association was for 3-4 minutes with 4 minutes dissociation. Injections were done in triplicate and randominzed. Analysis was done using BIAevaluation software, with 1:1 Langmuir and global analysis. The results of the analysis showed that hPD-1.Fc:hB7-4.Fc binding fit a 1:1 model. The kinetic rate constant (K_D) calculated for hPD-1.Fc:hB7-4.Fc was 186 nM (k_{on} = 6.57 ± 1.2 Xe4 $M^{-1}s^{-1}$; k_{off} = 0.122 ± 0.0007 s^{-1}).

These data demonstrate that PD-1 binds B7-4, and that this interaction may regulate the action of PD-1.

Example 12: B7-4 Can Transmit a Negative Signal to Immune Cells

In this example, $5x10^5$ Jurkat T cells per well were stimulated with anti-CD3 coated beads (at a 1:1 ratio) and soluble anti-CD28. COS cells expressing B7-4 or a negative control, called EZZ, were titrated into the wells. Supernatants were harvested

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at 48 hours and assayed by ELISA for human IL-2. Figure 17 shows that increasing numbers of COS B7-4 cells (bars on the right in the figure) lead to a decrease in IL-2 production.

Using similar assay formats, for example in which human PHA-blasts from PBMCs were stimulated with immobilized anti-CD3 and soluble anti-CD28, a decrease in T-cell proliferation was observed by titrating in COS cells expressing B7-4.

Example 13: The PD-1:B7-4 Interaction Inhibits CD3-mediated T-Cell Proliferation, Cytokine Production, and Expression of T Cell Activation Markers

To examine the functional significance of the PD-1:B7-4 interaction, the functional consequences of B7-4 interaction with its receptor were also examined using human T-cells. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation. $CD4^+$ T cell populations (85-90% purity) were purified by negative selection using a cocktail of monoclonal antibodies and immunomagnetic beads (PerseptiveBiosystems). Anti-CD3, control IgG and fusion protein were covalently attached to polyurethane-coated tosyl activated Dynabeads (Dynal) according to manufacturer's instructions and as described previously (Blair, P. J. *et al.* (1998) *J. Immunol.* 160:12-15). Anti-CD3 antibody (UCHT1, Pharmingen) at the indicated concentration was added to 1 x 10^7 beads/ml 0.1 M phosphate buffer pH 7.4. Control IgG was added to the bead suspension in order to maintain a constant total Ig concentration of 5μg/ml during binding. Similarly, anti-CD3/B7-4.Ig(γ2a) beads were prepared with the indicated anti-CD3 antibody concentration, a constant concentration of either B7-4.Ig representing 40% of the total bound protein (2μg/10^7 beads), and control IgG to make up the remaining total bound protein. 10^5 T cells were cultured in 96 well flat-bottom plates, and beads were added at a 1 bead to 1 cell ratio in the presence or absence of the indicated concentrations of anti-CD28 antibody (CD28.2, Pharmingen). Proliferation was determined by labeling cultures for the last 6 hr of a 4-day assay with 1 μCi ^{3}H-thymidine/well. For analysis by cytokine ELISAs, cultures were set up as described above and supernatants harvested at the indicated times. Interferon-γ, IL-10 and IL-2 concentrations were determined using commercially available ELISA kits (Genzyme).

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Purified $CD4^+$ T-cells obtained from peripheral blood mononuclear cells (PBMC) were activated with beads coated with anti-CD3 mAb and either human B7-4.Ig or a control Ig. Proliferation and cytokine production was assessed 96 hours after stimulation. Cells activated with anti-CD3 mAb/B7-4.Ig coated beads showed a 69% decrease in proliferation relative to anti-CD3 mAb/control Ig activated cells (Figure 18A). Proliferation was inhibited in a dose dependent manner when different concentrations of B7-4.Ig coated beads were used (0.25, 0.5, 1, and 2 μg B7-4.Ig/10^7 beads).

Furthermore, activation of cells in the presence of B7-4 also impaired cytokine secretion. In the presence of B7-4, interferon-γ (Figure 18B) and IL-10 (Figure 18C) secretions were decreased by approximately 80% and 60%, respectively. IL-2 production was below detection under these activation conditions at both 24 and 96 hr. However, activation of T cells using beads as in Example 14 below showed that B7-4 could inhibit IL-2 production at 2, 3, and 4 days of stimualation. B7-4 also inhibited IL-2 production in Jurkat T cells at beads:cell ratios of 1:1, 2:1, and 4:1. Furthermore, under conditions in which costimulation in the form of soluble anti-CD28 was provided, activation of cells in the presence of B7-4 also led to a decrease in IL-2 production. PD-1:B7-4 interaction also led to a decrease in both IL-10 and IFN-γ production. Thus, activation of murine and human T-cells in the presence of B7-4 leads to inhibition of both proliferation and cytokine secretion.

Further experiments showed that activation of T cells in the presence of B7-4 results in decreased expression of T cell activation markers. For example, when T cells were activated in the presence of B7-4, and the expression of the early activation marker CD69 examined by flow cytometry, the results were as follows (% positive for CD69 expression):

	anti-CD3	anti-CD3/B7-4.Fc	Media
% CD69+			
24 hours	26%	26%	1%
48 hours	46%	36%	2%
72 hours	63%	42%	5%

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When T cells were activated in the presence of B7-4, and the expression of IL-2R (CD25) examined by flow cytometry, the results were as follows (% positive for IL-2R expression):

	anti-CD3	anti-CD3/B7-4.Fc	Media
% IL-2R+			
24 hours	17%	12%	2%
48 hours	29%	17%	4%
72 hours	54%	15%	5%

Example 14: PD-1:B7-4 Inhibition Kinetics Correlate With PD-1 Expression

This example describes the correlation between the timing of PD-1 expression during T cell activation and the ability of B7-4 to inhibit T cell proliferation. T cell activation was achieved using tosyl-activated beads (10^7) coated with 3 μg anti-CD3 and 2 μg of either control Fc (ctrl.Fc) or mB7-4.Fc fusion protein. $5x10^4$ purified Balb/c LN T cells were stimulated with $1x10^5$ beads in a 96 well plate format. For proliferation, plates were pulsed for the final ~10 hours of the culture period. For IL-2 ELISAs, supernatents were harvested from parallel wells.

To determine the kinetics of PD-1 expression during T cell activation, purified Balb/c LN T cells were stimulated with anti-CD3/ctrl.Fc beads. At 0, 1, 2, 3, 4, and 5 days of stimulation, cells were harvested, washed, and stained using biotinylated anti-murine PD-1 (made from monoclonal antibody J43, described in Agata, Y. *et al.* (1996) *Int. Immunol.* 8:765-72) or biotinylated hamster control, followed by PE-Streptavidin. Data were calculated for live-gated cells, and the percent positive were calculated relative to isotype control. PD-1 expression increased over the course of time during T cell activation as follows: Day 0 (0%); Day 1 (27%); Day 2 (37%); Day 3 (40%); Day 4 (74%); and Day 5 (83%).

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When proliferation of Balb/c LN T cells activated using either anti-CD3/ctrl.Fc beads or anti-CD3/mB7-4.Fc was compared, the kinetics of the B7-4 inhibition of T cell proliferation correlated with the expression kinetics of PD-1, providing further evidence that B7-4 acts to inhibit T cell activation via its interaction with PD-1.

Example 15: The Outcome of PD-1:B7-4 Interaction Depends on the Strength of T-cell Receptor and CD28 Signals

To examine the relationship between T-cell receptor, CD28 and PD-1 mediated signals, human $CD4^+$ T-cells were stimulated with suboptimal or optimal concentrations of anti-CD3 mAb, a fixed concentration of B7-4.Ig and increasing concentrations of soluble anti-CD28 mAb. Using anti-CD3 mAb-coated beads, the concentrations required for suboptimal and optimal T-cell stimulation were established. Under conditions of suboptimal T-cell receptor engagement (anti-CD3 mAb at 1μg/ml), minimal proliferation was observed in the absence of costimulation (Figure 19A). Addition of increasing concentrations of soluble anti-CD28 mAb led to an up to 30-fold increase in proliferation. Under these conditions, activation of T cells in the presence of B7-4 resulted in an 80% reduction in proliferation (Figure 19A). A maximal level of costimulation (anti-Cd28 at 250 ng/ml) was required to rescue the inhibition of proliferation mediated by B7-4 stimulation. In contrast, under saturating conditions of T-cell receptor activation (anti-CD3 mAb at 2μg/ml), B7-4 mediated inhibition of T-cell proliferation was only observed in the absence of CD28 costimulation (Figure 19B).

To examine the ability of costimulation to rescue the PD-1:B7-4 mediated inhibition of proliferation at different time points during T cell activation, Balb/c LN T cells were stimulated as in Example 14 in the presence or absence of 1 μg/ml soluble anti-CD28. Proliferation was measured on days 2, 3, and 4. Anti-CD28 reverses PD-1:B7-4.Fc mediated inhibition at early (day 2), but not late (days 3 and 4) timepoints of T cell activation. Reversal of inhibition was dose-dependent and seen across a range of anti-CD28 concentrations (160 ng/ml, 800 ng/ml, 1 μg/ml, 4 μg/ml, and 20 μg/ml). Under the same conditions of strong costimulation (1 μg/ml anti-CD28), however, B7-4.Fc inhibits IL-2 production at all time points examined (days 2, 3, and 4). B7-4.Fc also inhibits IL-2 production across a broad range of soluble anti-CD28 costimulation

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(100 ng/ml, 160 ng/ml, 500 ng/ml, 800 ng/ml, 4 μg/ml, 5 μg/ml, 10 μg/ml, and 20 μg/ml).

ICOS mediated costimulation also rescues PD-1:B7-4 medidated inhibition of proliferation.

Example 16: Ability of B7-4 To Inhibit CD28 Signals and Cytokine Production

The inhibitory effects of the PD-1:B7-4 pathway appear to be determined by the strength of signal through the TCR and CD28 (see previous example), whereby weak CD3/CD28-mediated responses are easily downregulated. To study the interaction of the CD28 signal and the PD-1:B7-4 pathway, pre-activated DO11.10 $CD4^+$ T cells were activated with OVA peptide presented by CHO-IA^d/B7.2 or CHO-IA^d/B7.2/B7-4.

For detection of B7-4, 5 x 10^4 CHO transfectants cells were incubated with 5μg/ml of human PD-1Ig (hPD-1-Ig) (Genetics Institute, Cambridge, MA) and developed with goat anti-murine IgG2a-phycoerythrin (PE) (Southern Biotechnology Associates Inc., Birmingham, AL). In addition, cells were stained separately with 5μg/ml anti-IA^d-PE or B7.2-PE (Pharmingen, San Diego, CA). Following each step, cells were washed three times with PBS/1% BSA/0.02% sodium azide. After the final incubation, cells were fixed with 1% paraformaldehyde. Ten thousand events were analyzed on a FACSCalibar (Becton Dickinson, Mountain View, CA). All isotype controls were obtained from Pharmingen.

Splenocytes were prepared from DO11.10 mice and treated with Tris-NH_4Cl to deplete erythrocytes. Cells were cultured with 1μg/ml of OVA peptide for 72 hours (Analytical Biotechnology Services, Boston, MA) in RPMI 1640 (Life Technologies, Grand Island, New York) supplemented with 10% FCS (Sigma, St Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 10 mM HEPES, 50 μM 2-ME (all from Life Technologies) and 15mg/ml of gentamicin (BioWhittaker, Walkersville, MD). $CD4^+$ T cells were purified by positive selection using magnetic-activated cell sorting separation columns (Miltenyi Biotec, Auburn, CA) with resulting purity of >98%. Cells were rested overnight before re-stimulation.

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Proliferation of CHO cells was inhibited by incubation with 50 μg/ml of mitomycin C (Bristol Laboratories, Princeton, NJ) for 16 hours at 37°C. At the end of the incubation period, the cells were harvested with 10 mM EDTA in PBS, washed twice and left on ice for 1 hour. The cells were subsequently washed three times and resuspended in culture medium. 10^5 pre-activated $CD4^+$ T cells were cultured with varying concentrations of OVA peptide and 10^4 mitomycin C-treated CHO transfectants in 96 well plates. To assay proliferation, cultures were incubated for 48 hrs and pulsed with 1μCi/well of [^{3}H] thymidine (New England Nuclear, Boston, MA) for the last 6 hours of the incubation period.

The expression of B7 and IA^d was similar on all CHO transfectants (Figure 20). As expected, introduction of B7.2 led to an increase in proliferative responses by T cells at all antigen concentrations (Figures 21A-21D). However, B7-4 inhibited responses at lower peptide concentrations (0.01μg/ml and 0.001μg/ml) (Figures 21C and 21D, respectively).

To address the capacity of PD-1:B7-4 pathway to inhibit cytokine production, supernatants from DO11.10 $CD4^+$T cells activated with OVA peptide presented by CHO cell transfectants were analyzed. Aliquots of supernatants were harvested at various times after initiation of cultures. IL-2, IL-4, IFN-γ and IL-10 levels were analyzed using mAbs and recombinant cytokine standards from Pharmingen. Detection limits were as follows: IL-2, 20pg/ml, IL-4, 40pg/ml. IFN-γ, 100pg/ml and IL-10, 200pg/ml. Production of IL-2 (Figure 22A), IL-4 (Figure 22B), IFN-γ (Figure 22C), and IL-10 (Figure 22D) was inhibited significantly when DO11.10 $CD4^+$T cells were cultured with 0.1 μg/ml peptide and B7-4. At this concentration there was only a weak inhibition of proliferation. However, B7-4 significantly inhibited cytokine production at 0.01μg/ml peptide, consistent with the inhibition of proliferation (Figures 23A-23C). IL-10 was not detected under these activation conditions. Therefore, PD-1 engagement by B7-4 can downregulate cytokine production even when T cell proliferation is not affected.

To determine whether the diminished cytokine production was due to reduced mRNA levels, and RNase protection assay was utilized. $CD4^+$ T cells were restimulated with various CHO cell transfectants and 0.01μg/ml OVA peptide. After 48 hours, cells were harvested and mRNA was isolated using TRIzol® reagent (Life Technologies).

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5μg mRNA was analyzed for cytokine levels by RNase protection assay using RiboQuant multiprobe kit mCK1 according to the manufacturer's instructions (Pharmingen). Transcript levels of IL-4, IL-10, IL-13, IL-2, IL6 and IFN-γ mRNA were detected in pre-activated DO11-10 $CD4^+$T cells after stimulation with 0.01μg/ml OVA peptide presented by CHO-IA^d/B7.2. However, the introduction of B7-4 significantly reduced cytokine mRNA levels. There was minimal upregulation of mRNA for cytokines in unstimulated T cell cultures or T cells activated with peptide presented by CHO-IA^d. These results further demonstrate the capacity of the PD-1:B7-4 pathway to antagonize a strong B7/CD28 signal at least when antigenic stimulation is weak or limiting, and the inhibition of at least cytokine production in conditions of strong antigenic stimulation.

Example 17: Mechanism of action of the PD-1:B7-4 pathway

Cross-linking of CTLA-4 has been shown to inhibit cell cycle progression in naïve T cells (Krummel, M. F. and Allison, J. P. (1996) *J. Exp. Med.* 183:2533-2540; Walunas, T. L. *et al.* (1996) *J. Exp. Med.* 183:2541-2550). As PD-1 was isolated from murine cell lines undergoing apoptosis, a possible mechanism of action of the PD-1:B7-4 pathway might be to increase programmed cell death. To address this issue, DO11.10 $CD4^+$ T cells were restimulated with 0.01μg/ml peptide and various CHO transfectants and cell cycle progression was analyzed. $CD4^+$ T cells were restimulated with 0.01μg/ml peptide as described previously. After 36 hours of culture, cells were recovered and stained with anti CD4-FITC. Cells were washed in PBS, fixed in 70% ethanol for 1 hour on ice and then resuspended in PBS containing 10 μg/ml RNase (Sigma) and 50 μg/ml propidium iodide (Sigma). Analysis was performed within an hour of staining.

After 48 hours, cells were recovered and stained with CD4-FITC. After permeabilization, cells were incubated with propidium iodide to analyze the G_0/G_1, S/G_2 and sub-diploid populations. $CD4^+$ T cells restimulated with peptide presented by CHO-IA^d have a large proportion of cells in the sub-diploid population, indicative of apoptosis (Figure 24A). In cultures where $CD4^+$ T cells were stimulated by peptide presented by CHO-IA^d/B7-2, there were increased number of cells in the S/G_2 phase, and a decreased

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number in the sub-diploid population (Figure 24B), indicating that cells were in cycle and rescued from apoptosis by B7/CD28 costimulation. The introduction of B7-4 led to an increased number of cells in the G0/G1 phase (Figure 24C). There were comparable levels of apoptosis in the B7-4 cultures to the CHO-IA^d/B7 cultures. This was confirmed by annexin staining. The inhibition of cell progression by the PD-1:B7-4 pathway confirms its role in downregulating T cell activation.

Further experiments using PD-L1.Fc confirmed that PD-1:B7-4 engagment on T cells does not lead to apoptosis. Cells were treated with anti-CD3 or anti-CD3/B7-4.Fc, or irradiated, stained with propidium iodide and annexin, and analyzed by flow cytometry. Apoptotic cells were identified as those that were positive for both propidium iodide and annexin. Controls done in parallel showed that anti-CD3/B7.4.Fc inhibited proliferation at 48 and 72 hours. There results were as follows:

	anti-CD3	anti-CD3/B7-4.Fc	Irradiated
% apoptotic			
24 hours	3%	3%	18%
48 hours	5%	4%	25%
72 hours	10%	12%	42%

Still further experiments analyzing DNA content demonstrated that activation of T cells in the presence of B7-4.Fc results in cell cycle arrest. T cells were stimulated with ctrl.Fc or mB7-4.Fc beads. On day 4, the percentage of dividing cells was determined by propidium iodide staining. T cells stimulated with mB7-4.Fc showed half (11% vs. 21% for the control) the number of cells with the increased DNA content indicative of dividing cells. In two other experiements, the percentages were similar: 14% vs. 28% and 11% vs. 24%.

Analysis of mitosis was also examined. T cells were labeled with CSFE and stimulated with ctrl.Fc or mB7-4.Fc beads as in Example 14. At days 1, 2, 3, and 4, FACS analysis was done. Only live-gated events were analyzed. Percentages are for non-dividing cells. As the following data indicates, treatment with B7-4 inhibits cell division:

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	Day 1	Day 2	Day 3	Day 4
% non-dividing				
Ctrl.Fc	87.4%	32.4%	11.9%	3.8%
mB7-4.Fc	87.7%	46.7%	35.1%	24.5%

Mitosis was also examined (as above) separately for CD4+ T cells and CD8+ T cells. The results are set forth in Figures 28A (CD4+) and 28B (CD8+).

Example 18: IL-2, Induced By Costimulation or Added Exogenously, Can Overcome Proliferative Inhibition Induced By PD-1:B7-4 Interaction

Purified Balb/c LN T cells were stimulated as in Example 14 in the presence or absence of 1 μg/ml soluble anti-CD28 and 10 μg/ml anti-IL-2. Proliferation was measured on day 2. In the presence of costimulation by anti-CD28, addition of anti-IL-2 restores PD-1:B7-4 mediated inhibition, suggesting that anti-CD28 reverses the inhibitory effect by inducing IL-2 production. Addition of anti-IL-2 also restores PD-1:B7-4 mediated inhibition in the presence of costimulation by ICOS ligand.

In another example, Balb/c LN T cells were stimulated as in Example 14 with the addition of exogenous IL-2. When proliferation was measured on day 3, exogenous IL-2 reverses PD-1:B7-4 mediated inhibition of proliferation in a dose-dependent manner (IL-2 concentration range: 3 pg/ml, 10 pg/ml, 30 pg/ml, 100 pg/ml, and 300 pg/ml). Moreover, addition of exogenous IL-2 (at U/ml rhIL-2) reverses PD-1:B7-4 mediated inhibition of proliferation at all time points (proliferation measured on days 2, 3, 4, and 5).

Example 19: CD8+ T Cells Are More Susceptible to Inhibition By B7-4

This example describes the differences between CD4+ and CD8+ T cell activation in response to B7-4.

Stable antigen presenting cell (APC) lines were engineered to express GFP or mB7-4/GFP using retroviral technology. Figure 29A shows a schematic of the cells used. For CD4+ T cell experiments, the APC:T cell ratio was 1:10 with 10 μM PCCF

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peptide. For CD8+ T cell experiments, the APC:T cell ratio was 1:1 with 1 mM p2Ca peptide. $5x10^4$ purified LN T cells from TCR transgenic mice were stimulated with irradiated APC plus peptide for 2, 3, or 4 days. As shown in Figures 29B and 29C, both CD4+ and CD8+ T cells are inhibited by PD-1:B7-4 interactions.

While addition of anti-CD28 can overcome PD-1:B7-4 mediated inhibition of proliferation of CD4+ T cells (Figure 30A), it cannot overcome the inhibition of CD8+ T cells (Figure 30B). Furthermore, either exogenous IL-2 or IL-15 (20 U/ml and 50 ng/ml, respectively, added on day 0) can overcome PD-1:B7-4 mediated inhibition of CD8+ T cells (proliferation measured on day 2). CD8+ T cells may be more susceptible to inhibition because of their intrinsic inability to produce IL-2.

Example 20: Recruitment of Signaling Molecules Through Engagement of the PD-1:B7-4 Signaling Pathway

To further investigate the mechanism of action of the PD-1:B7-4 pathway in T cells, a Jurkat based system was used. Jurkat cells constitutively express low levels of PD-1. The Jurkat T cells were activated with beads coated with anti-CD3/ctrl.Ig or anti-CD3/hB7-4.Ig. Ligation of PD-1 plus CD3 leads to inhibition of IL-2 secretion by Jurkat cells, compared to cells stimulated with anti-CD3 alone. Cells were lysed and subjected to anti-SHP-2 immunoprecipitation, run on a gel, and transferred to a membrane. The membrane was immunoblotted with 4G10-HRP (horseradish peroxidase conjugated anti-phosphotyrosine antibody) and subsequently with anti-SHP-2 to confirm SHP-2 expression in the immunoprecipitates. With anti-CD3 and hB7-4.Ig., ligation of PD-1 plus the TCR rapidly resulted in the conversion of SHP-2 to the phosphorylated state, compared to TCR activation alone. SHP-1 was not phosphorylated under these conditions. This data indicates that recruitment of SHP-2 is a mechanism for downregulating TCR signaling events via the PD-1:B7-4 pathway.

Under similar experimental conditions, anti-Zap70 immunoprecipitation results in inhibition of Zap70 associated p23 pTyr upon coligation of CD3 and PD-1 (using anti CD-3 and B7-4.Ig). CD3ζ immunoprecipitation of Jurkat lysates and anti-pTyr blotting results in inhibition of CD3ζ phosphorylation upon coligation of CD3 and PD-1 (using

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anti CD-3 and B7-4.Ig). Inhibition of CD3ζ in this system persists with CD28 stimulation.

Example 21: Inhibition of binding of biotinylated human B7-4 Fc to human PD-1Fc

Fc fusion proteins were generated by linking the extracellular region of PD-1 or B7-4 to the hinge-CH2-CH3 domains of murine Igγ2a. Recombinant proteins were produced in COS cells transiently transfected with LipofectAMINE (Gibco-BRL) or stably transfected CHO cell lines and purified from conditioned media using protein A-Sepharose.

The ability of antibodies to B7-4 or PD-1 to inhibit the interaction of human B7-4Fc and human PD-1 Fc was tested using standard ELISA methods. Briefly, human PD-1Fc molecules were immobilized in 96-well plates, blocked, and washed. Biotinylated B7-4Fc molecules (100ng/ml) were added to wells at concentrations of approximately 2000, 700, 200, 70, 25, 8, and 1.18 ng/ml (Figure 25A). The wells were incubated with Streptavidin conjugated horse radish peroxidase, washed, and color was developed using standard methods. The ED50 of B7-4Fc was found to be 108 ng/ml.

The ability of murine antibodies to human B7-4 (10D9 and 11D12) or scFv portions of human immunoglobulins (B7-4-1, B7-4-6, and B7-4-12) to inhibit the binding of biotinylated human B7-4Fc to human PD-1Fc was tested at 7 concentrations of inhibitors. The IC50 was found to range from 0.5 nM to 24 nM and the data are presented in Figure 25B.

The PD-1 specific scFv were also tested for their ability to inhibit the binding of B7-4 Fc to PD-1Fc using the same ELISA methods described above. Human scFv reactive with PD-1 (PD1-17 scFv) were found to inhibit specific binding (EC50 between 10^{-7} and 10^{-8}) as shown in Figure 26. V_L and V_H domains of the PD1-17scFv were used to generate a complete IgG. In brief, the VH and VL coding regions were linked to genomic CH and CL gene sequences in expression vectors. The resulting expression vectors were transiently transfected into human 293 cells and the IgG harvested from the conditioned medium. The potency of the grafted whole IgG molecule was higher than for the scFv antibody (EC 50 between 10^{-8}M and 10^{-9}M).

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Example 22: Administration of Soluble B7-4Fc Exacerbates Disease in a Murine Model

To determine if modulation of the B7-4/PD-1 pathway has immunoregulatory activity *in vivo*, the B7-4Fc protein was evaluated in a murine model of experimental autoimmune encephalomyelitis (EAE) that shares many clinical and pathological features with the human disease multiple sclerosis. Female SJL/J mice were immunized with 100μg of proteolipid protein (PLP) in complete Freund's adjuvant. Ten days later, spleens were harvested, processed to single cell suspensions and then restimulated *in vitro* with 5μg of PLP for 96 hours. Cells were washed three times in PBS and then $15x10^6$ cells transferred to naive SJL/J mice by intraperitoneal injection. The adoptive transfer of autoreactive T cells results in acute paralysis of recipient mice which manifests as loss of tail tone with subsequent progression to full hind limb paralysis. This paralytic episode coincides with marked infiltration of activated T cells and macrophages in the CNS. Under most conditions, this is an acute model of disease with spontaneous recovery occurring after a short period of paralysis. For evaluation of B7-4Fc, mice were injected subcutaneously with 200μg of the protein in 100μl of sterile saline on days 0, 2, 4, 7 and 11 after cell transfer (n=10). Control mice (n=10) received an equal volume of saline only. All animals were monitored regularly for clinical signs of disease which were scored as follows: 1. Loss of tail tone; 2. Hind limb weakness/partial hind limb paralysis; 3. Complete hind limb paralysis; 4. Hind and forelimb paralysis; 5. Moribund.

In the experiment shown in Figure 27, the incidence and onset of clinical disease were similar in both groups. Mice treated with the B7-4Fc however, developed severe disease with the majority of animals rapidly progressing to complete hind and forelimb paralysis (9/10 and 1/10 for B7-4Fc and control mice respectively). Mortality associated with clinical signs of disease was 10% in the control group and 70% in the B7-4Fc treated mice. In addition, recovery from clinical disease was substantially delayed in the B7-4Fc treated mice that did survive despite the fact that treatment was discontinued on day 11.

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In conclusion, using an adoptive transfer model of T cell mediated autoimmunity, administration of soluble B7-4Fc exacerbates clinical signs of disease resulting in increased mortality and delayed recovery from paralysis. These findings are consistent with enhanced activation/infiltration of inflammatory cells into the CNS and clearly demonstrate the immunoregulatory potential for the B7-4Fc protein *in vivo*.

Example 23: Identification of PD-1 Signaling Molecules

This example describes a screening assay for the identification of proteins which are involved in PD-1 signaling pathways. A schematic of the screening assay is shown in Figure 31A. 1 X 10^8 (experiment 1) or 5 X 10^8 (experiment 2) Jurkat T cells were treated in the presence and absence of pervanadate. The cells were then lysed in lysis buffer (1% NP-40, 150 mM NaCl, Tris pH 7.6, protease inhibitors, and phosphatase inhibitors). Proteins were then immunoprecipitated with 100 μg biotinylated peptide for 4 hours at 4°C. The following peptides were used for immunoprecipitation: ITIM peptides Y(PO_4), F, and Y, and C' (non-ITIM). The sequences of the peptides are shown in Figure 31B. Streptavidin agarose was added for 30 minutes at 4°C, and samples were then washed 4 times in 1% lysis buffer + 0.45 M NaCl, and 2 times with PBS (phosphate buffered saline). Samples were then boiled in SDS-PAGE sample buffer and run on a 4-20% tris-glycine NOVEX gradient gel (reduced). The gel was the silver stained using the Daichi protocol (experiment 1) or the Mann protocol (experiment 2). PD-1 interacting proteins were excised from the gel and analyzed by mass spectrometry analysis.

The following PD-1 interacting proteins were identified:

Experiment 1 (ITIM peptides):

47 kD Y and pY	- pervanadate
95 kD Y, pY, and F	+ pervanadate

Experiment 2 (ITIM peptides)

75 kD Y and pY	- pervanadate
70 kD Y and pY	+ pervanadate

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C -peptide

22 kD	- pervanadate
46 kD	+ pervandaate

Example 24: Inhibition of ERK1/2 Phosphorylation Through Engagement of the PD-1:B7-4 Signaling Pathway

ERK1 and 2 are activated in response to activation of the T cell receptor. This activation is detectable via phosphorylation of the ERK1 and 2 molecules. To further elucidate the signaling pathway involved in PD-1 mediated signaling, the effect of PD-1 activation on ERK1/2 activation was investigated.

T cells (otherwise referred to as T cell blasts) were obtained from peripheral blood mononuclear cells which had been stimulated with PHA. The T cells were activated by contact with tosyl-activated beads coated with anti-CD3 (1 $\mu g/10^7$ beads) and either isotype control Ig (IgG_{2a}) or PDL1.Ig fusion protein (4 $\mu g/10^7$ beads), in the presence of soluble anti-CD28 (5 $\mu g/ml$) for either 5, 30, or 90 minutes. Following stimulation, cells and beads were washed once with ice cold PBS, lysed and fractionated by SDS-PAGE. The fractionated cells were then transferred to a nitrocellulose filter, which was then probed with a rabbit polyclonal antibody which specifically recognizes phosphorylated ERK 1 and 2 (Cell Signaling Technologies). The blot was then probed with a secondary antibody, HRP conjugated anti-rabbit IgG (Santa Cruz Biotechnologies). Bound antibody, indicating phosphorylated ERK1 and ERK2, was detected using chemiluminescence. The nitrocellulose filter was then stripped and reprobed with and antibody specific for ERK1 and ERK2, whether or not phosphorylated, to verify that equal amounts of total ERK1 and ERK2 were present in the cell extracts.

The results of probing with anti-phospho ERK specific antibody are shown in Figure 32. Unstimulated T cells had no detectable phosphorylated ERK1/2. T cells stimulated with anti-CD3 and CD28 had detectable phosphorylated ERK1/2 as little as 5 minutes after stimulation. In contrast, T cells stimulated with anti-CD3 and CD28, and

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PD-L1 (to activate PD-1), exhibited no detectable increase in ERK1/2 phosphorylation. These results indicate that PD-1 signaling suppresses ERK activation.

Example 25: Inhibition of PKC-θ Phosphorylation Through Engagement of the PD-1:B7-4 Signaling Pathway

PKC-θ is known to be activated in response to activation of the T cell receptor. To further elucidate the signaling pathway involved in PD-1 mediated signaling, the effect of activation of PD-1 on PKC-θ activation was investigated.

Human $CD28^+$ T cells, isolated as in Example 24, were activated by contact with tosyl-activated beads coated with anti-CD3 (1 μg/10^7 beads) and either isotype control Ig (IgG_{2a}) or PDL1.Ig fusion protein (4 μg/10^7 beads), in the presence of soluble anti-CD28 (5 μg/ml) for either 5 min, 30 min, or 90 min or 2 hr. Following stimulation, cells and beads were washed once with ice cold PBS, lysed and fractionated by SDS-PAGE. The fractionated cells were then transferred to a nitrocellulose filter, which was then probed with a rabbit polyclonal antibody which specifically recognizes PKC-θ which is phosphorylated at T_{538} (anti-phospho T_{538} antibody, obtained from Cell Signaling Technologies). The nitrocellulose filter was then probed with a secondary antibody, HRP-conjugated anti-murine IgG (Transduction Laboratories). The bound antibody, indicative of phosphorylated PKC-θ, was detected using chemiluminescence. The nitrocellulose filter was then stripped and reprobed with a monoclonal antibody specific for PKC-θ (anti-PKC-θ, E7, obtained from Santa Cruz Biotechnologies to verify equal amounts of PKC-θ were present in the cell extracts.

The results of probing with anti-phospho PKC-θ specific antibody are shown in Figure 33. Unstimulated T cells had no detectable phosphorylated PKC-θ. T cells stimulated with anti-phospho PKC-θ and CD28 had phosphorylated PKC-θ detectable after 30 minutes of stimulation. This phosphorylation was significantly higher than that of T cells stimulated with anti-CD3 and CD28, and also PD-L1 (to activate PD-1). The fact that similar amounts of total PKC-θ were present in all cell extracts indicates that the increase in phosphorylated PKC-θ does not result from an overall increase in the presence of the PKC-θ molecule, but rather an increase in its phosphorylation by a regulatory kinase, or a decrease in its de-phosphorylation by a regulatory phosphatase.

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This phosphorylation of the PKC-θ molecule is reported to correlate with an increased activation competent conformation of the PKC-θ enzyme (Liu et al., *Biochem. J. 361*: 255-265 (2002)). The reduced phosphorylation of the PKC-θ molecule in response to activation of PD-1 signaling indicates that PD-1 signaling suppresses PKC-θ activation.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed is:

1. A method for screening for compounds which modulate PD-1 signaling comprising:

a) contacting a T cell activated in the presence of a PD-1 ligand with a test compound; and

b) determining the ability of the test compound to modulate phosphorylation of SHP-2;

thereby identifying a compound which modulates PD-1 signaling.

2. The method of Claim 1 wherein the test compound upmodulates PD-1 signaling and thereby upmodulates SHP-2 phosphorylation.

3. The method of Claim 1 wherein the test compound downmodulates PD-1 signaling and thereby downmodulates SHP-2 phosphorylation.

4. A method for screening for compounds which modulate PD-1 signaling comprising:

a) contacting a T cell activated in the presence of a PD-1 ligand with a test compound; and

b) determining the ability of the test compound to modulate phosphorylation of ERK1 or ERK 2;

thereby identifying a compound which modulates PD-1 signaling.

5. The method of Claim 4 wherein the test compound upmodulates PD-1 signaling and thereby downmodulates ERK 1 or ERK 2 phosphorylation.

6. The method of Claim 4 wherein the test compound downmodulates PD-1 signaling and thereby upmodulates ERK 1 or ERK 2 phosphorylation.

7. A method for screening for compounds which modulate PD-1 signaling comprising:

a) contacting a T cell activated in the presence of a PD-1 ligand with a test compound; and

b) determining the ability of the test compound to modulate phosphorylation of PKC-θ;

thereby identifying a compound which modulates PD-1 signaling.

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8. The method of Claim 7 wherein the test compound upmodulates PD-1 signaling and thereby downmodulates PKC-θ phosphorylation.

9. The method of Claim 7 wherein the test compound downmodulates PD-1 signaling and thereby upmodulates PKC-θ phosphorylation.

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FIG. 1

GCTTCCCGAGGCTCCGCACCAGCCGCGCTTCTGTCCGCCTGCAGGGCATTCCA
GAAAGATGAGGATATTTGCTGTCTTTATATTCATGACCTACTGGCATTTGCTG
AACGCATTTACTGTCACGGTTCCCAAGGACCTATATGTGGTAGAGTATGGTA
GCAATATGACAATTGAATGCAAATTCCCAGTAGAAAAACAATTAGACCTGGC
TGCACTAATTGTCTATTGGGAAATGGAGGATAAGAACATTATTCAATTTGTGC
ATGGAGAGGAAGACCTGAAGGTTCAGCATAGTAGCTACAGACAGAGGGCCC
GGCTGTTGAAGGACCAGCTCTCCCTGGGAAATGCTGCACTTCAGATCACAGA
TGTGAAATTGCAGGATGCAGGGGTGTACCGCTGCATGATCAGCTATGGTGGT
GCCGACTACAAGCGAATTACTGTGAAAGTCAATGCCCCATACAACAAAATCA
ACCAAAGAATTTTGGTTGTGGATCCAGTCACCTCTGAACATGAACTGACATGT
CAGGCTGAGGGCTACCCCAAGGCCGAAGTCATCTGGACAAGCAGTGACCATC
AAGTCCTGAGTGGTAAGACCACCACCACCAATTCCAAGAGAGAGGAGAAGC
TTTTCAATGTGACCAGCACACTGAGAATCAACACAACAACTAATGAGATTTT
CTACTGCACTTTTAGGAGATTAGATCCTGAGGAAAACCATACAGCTGAATTG
GTCATCCCAGGTAATATTCTGAATGTGTCCATTAAAATATGTCTAACACTGTC
CCCTAGCACCTAGCATGATGTCTGCCTATCATAGTCATTCAGTGATTGTTGAA
TAAATGAATGAATGAATAACACTATGTTTACAAAATATATCCTAATTCCTCAC
CTCCATTCATCCAAACCATATTGTTACTTAATAAACATTCAGCAGATATTTAT
GGAATAAAAAAAAAAAAAAAAAAAA

FIG. 2

CGAGGCTCCGCACCAGCCGCGCTTCTGTCCGCCTGCAGGGCATTCCAGAAAGA
TGAGGATATTTGCTGTCTTTATATTCATGACCTACTGGCATTTGCTGAACGCATT
TACTGTCACGGTTCCCAAGGACCTATATGTGGTAGAGTATGGTAGCAATATGAC
AATTGAATGCAAATTCCCAGTAGAAAAACAATTAGACCTGGCTGCACTAATTGT
CTATTGGGAAATGGAGGATAAGAACATTATTCAATTTGTGCATGGAGAGGAAG
ACCTGAAGGTTCAGCATAGTAGCTACAGACAGAGGGCCCGGCTGTTGAAGGAC
CAGCTCTCCCTGGGAAATGCTGCACTTCAGATCACAGATGTGAAATTGCAGGAT
GCAGGGGTGTACCGCTGCATGATCAGCTATGGTGGTGCCGACTACAAGCGAAT
TACTGTGAAAGTCAATGCCCCATACAACAAAATCAACCAAAGAATTTTGGTTGT
GGATCCAGTCACCTCTGAACATGAACTGACATGTCAGGCTGAGGGCTACCCCA
AGGCCGAAGTCATCTGGACAAGCAGTGACCATCAAGTCCTGAGTGGTAAGACC
ACCACCACCAATTCCAAGAGAGAGGAGAAGCTTTTCAATGTGACCAGCACACT
GAGAATCAACACAACAACTAATGAGATTTTCTACTGCACTTTTAGGAGATTAGA
TCCTGAGGAAAACCATACAGCTGAATTGGTCATCCCAGAACTACCTCTGGCACA
TCCTCCAAATGAAAGGACTCACTTGGTAATTCTGGGAGCCATCTTATTATGCCTT
GGTGTAGCACTGACATTCATCTTCCGTTTAAGAAAAGGGAGAATGATGGATGT
GAAAAAATGTGGCATCCAAGATACAAACTCAAAGAAGCAAAGTGATACACATTT
GGAGGAGACGTAATCCAGCATTGGAACTTCTGATCTTCAAGCAGGGATTCTCA
ACCTGTGGTTTAGGGGTTCATCGGGGCTGAGCGTGACAAGAGGAAGGAATGG
GCCCGTGGGATGCAGGCAATGTGGGACTTAAAAGGCCCAAGCACTGAAAATG
GAACCTGGCGAAAGCAGAGGAGGAGAATGAAGAAAGATGGAGTCAAACAGGG
AGCCTGGAGGGAGACCTTGATACTTTCAAATGCCTGAGGGGCTCATCGACGCC
TGTGACAGGGAGAAAGGATACTTCTGAACAAGGAGCCTCCAAGCAAATCATCC
ATTGCTCATCCTAGGAAGACGGGTTGAGAATCCCTAATTTGAGGGTCAGTTCCT
GCAGAAGTGCCCTTTGCCTCCACTCAATGCCTCAATTTGTTTTCTGCATGACTGA
GAGTCTCAGTGTTGGAACGGGACAGTATTTATGTATGAGTTTTTCCTATTTATTT
TGAGTCTGTGAGGTCTTCTTGTCATGTGAGTGTGGTTGTGAATGATTTCTTTTGA
AGATATATTGTAGTAGATGTTACAATTTTGTCGCCAAACTAAACTTGCTGCTTAA
TGATTTGCTCACATCTAGTAAAACATGGAGTATTTGTAAAAAAAAAAAAAAA

FIG. 3

292 secreted (245 amino acids)

Signal/IgV/IgC/hydrophilic tail
(a) (b) (c) (d)
Ig cysteines in large bold

MRIFAVFIFMTYWHLLNA (signal)

FTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKN
IIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQD
AGVYRCMISYGGADYKRITVKVNAPY (IgV)

NKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKT
TTTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAEL
VIP (IgC)

GNILNVSIKICLTLSPST (hydrophilic tail)

FIG. 4

292 membrane (290 amino acids)

Signal/IgV/IgC/transmembrane (underlined) plus cytoplasmic

Ig cysteines in large bold

MRIFAVFIFMTYWHLLNA (signal)

FTVTVPKDLYVVEYGSNMTIE**C**KFPVEKQLDLAALIVYWEMEDKN
IIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQD
AGVYR**C**MISYGGADYKRITVKVNAPY (IgV)

NKINQRILVVDPVTSEHELT**C**QAEGYPKAEVIWTSSDHQVLSGKT
TTTNSKREEKLFNVTSTLRINTTTNEIFY**C**TFRRLDPEENHTAEL
VIP (IgC)

ELPLAHPPNER<u>THLVILGAILLCLGVALTFIF</u>RLRKGRMMDVKKC
GIQDTNSKKQSDTHLEET (transmembrane plus cytoplasmic)

FIG. 5A

AGATAGTTCCCAAAACATGAGGATATTTGCTGGCATTATATTCACAGCCTGC
TGTCACTTGCTACGGGCGTTTACTATCACGGCTCCAAAGGACTTGTACGTG
GTGGAGTATGGCAGCAACGTCACGATGGAGTGCAGATTCCCTGTAGAACG
GGAGCTGGACCTGCTTGCGTTAGTGGTGTACTGGGAAAAGGAAGATGAGC
AAGTGATTCAGTTTGTGGCAGGAGAGGAGGACCTTAAGCCTCAGCACAGCA
ACTTCAGGGGGAGAGCCTCGCTGCCAAAGGACCAGCTTTTGAAGGGAAAT
GCTGCCCTTCAGATCACAGACGTCAAGCTGCAGGACGCAGGCGTTTACTGC
TGCATAATCAGCTACGGTGGTGCGGACTACAAGCGAATCACGCTGAAAGTC
AATGCCCCATACCGCAAAATCAACCAGAGAATTTCCGTGGATCCAGCCACTT
CTGAGCATGAACTAATATGTCAGGCCGAGGGTTATCCAGAAGCTGAGGTAA
TCTGGACAAACAGTGACCACCAACCCGTGAGTGGGAAGAGAAGTGTCACCA
CTTCCCGGACAGAGGGGATGCTTCTCAATGTGACCAGCAGTCTGAGGGTCA
ACGCCACAGCGAATGATGTTTTCTACTGTACGTTTTGGAGATCACAGCCAG
GGCAAAACCACACAGCGGAGCTGATCATCCCAGAACTGCCTGCAACACATC
CTCCACAGAACAGGACTCACTGGGTGCTTCTGGGATCCATCCTGTTGTTCC
TCATTGTAGTGTCCACGGTCCTCCTCTTCTTGAGAAAACAAGTGAGAATGCT
AGATGTGGAGAAATGTGGCGTTGAAGATACAAGCTCAAAAAACCGAAATGA
TACACAATTCGAGGAGACGTAAGCAGTGTTGAACCCTCTGATCGTCGATTG
GCAGCTTGTGGTCTGTGAAAGAAAGGGCCCATGGGACATGAGTCCAAAGAC
TCAAGATGGAACCTGAGGGAGAGAACCAAGAAAGTGTTGGGAGAGGAGCC
TGGAACAACGGACATTTTTTCCAGGGAGACACTGCTAAGCAAGTTGCCCAT
CAGTCGTCTTGGGAAATGGATTGAGGGTTCCTGGCTTAGCAGCTGGTCCTT
GCACAGTGACCTTTTCCTCTGCTCAGTGCCGGGATGAGAGATGGAGTCATG
AGTGTTGAAGAATAAGTGCCTTCTATTTATTTTGAGTCTGTGTGTTCTCACTT
TGGGCATGTAATTATGACTGGTGAATTCTGACGACATGATAGATCTTAAGAT
GTAGTCACCAAACTCAACTGCTGCTTAGCATCCTCCGTAACTACTGATACAA
GCAGGGAACACAGAGGTCACCTGCTTGGTTTGACAGGCTCTTGCTGTCTGA
CTCAAATAATCTTTATTTTTCAGTCCTCAAGGCTCTTCGATAGCAGTTGTTCT
GTATCAGCCTTATAGGTGTCAGGTATAGCACTCAACATCTCATCTCATTACA
ATAGCAACCCTCATCACCATAGCAACAGCTAACCTCTGTTATCCTCACTTCA
TAGCCAGGAAGCTGAGCGACTAAGTCACTTGCCCACAGAGTATCAGCTCTC
AGATTTCTGTTCTTCAGCCACTGTCCTTTCAGGATAGAATTTGTCGTTAAGAA
ATTAATTTAAAAACTGATTATTGAGTAGCATTGTATATCAATCACAACATGCC
TTGTGCACTGTGCTGGCCTCTGAGCATAAAGATGTACGCCGGAGTACCGGT
CGGACATGTTTATGTGTGTTAAATACTCAGAGAAATGTTCATTAACAAGGAG
CTTGCATTTTAGAGACACTGGAAAGTAACTCCAGTTCATTGTCTAGCATTAC
ATTTACCTCATTTGCTATCCTTGCCATACAGTCTCTTGTTCTCCATGAAGTGT
CATGAATCTTGTTGAATAGTTCTTTTATTTTTTAAATGTTTCTATTTAAATGATA
TTGACATCTGAGGCGATAGCTCAGTTGGTAAAACCCTTTCCTCACAAGTGTG
AAACCCTGAGTCTTATCCCTAGAACCCACATAAAAAACAGTTGCGTATGTTT
GTGCATGCTTTTGATCCCAGCACTAGGGAGGCAGAGGCAGGCAGATCCTG
AGCTCTCATTGACCACCCAGCCTAGCCTACATGGTTAGCTCCAGGCCTACA
GGAGCTGGCAGAGCCTGAAAAACGATGCCTAGACACACACACACACACACA
CACACACACACACACACACACACACACCATGTACTCATAGACCTAAGTGCACC
CTCCTACACATGCACACACATACAATTCAAACACAAATCAACAGGGAATTGT

FIG. 5B

CTCAGAATGGTCCCCAAGACAAAGAAGAAGAAAAACACCAAACCAGCTCTA
TTCCCTCAGCCTATCCTCTCTACTCCTTCCTAGAAGCAACTACTATTGTTTTT
GTATATAAATTTACCCAACGACAGTTAATATGTAGAATATATATTAAAGTGTC
TGTCAATATATATTATCTCTTTCTTTCTTTCTTCCTTTCTTTCTTTCTTTCTTTC
TTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTCCTTCCTTCCTTCCTTCCTTC
CTTCCTTCCTTCCTTTCTTTCTTTCTTTCTTTTTTTCTGTCTATCTGTACCTAAA
TGGTTGCTCACTATGCATTTTCTGTGCTCTTCGCCCTTTTTATTTAATGTATG
GATATTTATGCTGCTTCCAGAATGGATCTAAAGCTCTTTGTTTCTAGGTTTTC
TCCCCCATCCTTCTAGGCATCTCTCACACTGTCTAGGCCAGACACCATGTCT
GCTGCCTGAATCTGTAGACACCATTTATAAAGCACGTACTCACCGAGTTTGT
ATTTGGCTTGTTCTGTGTCTGATTAAAGGGAGACCATGAGTCCCCAGGGTA
CACTGAGTTACCCCAGTACCAAGGGGGAGCCTTGTTTGTGTCTCCATGGCA
GAAGCAGGCCTGGAGCCATTTTGGTTTCTTCCTTGACTTCTCTCAAACACAG
ACGCCTCACTTGCTCATTACAGGTTCTCCTTTGGGAATGTCAGCATTGCTCC
TTGACTGCTGGCTGCCCTGGAAGGAGCCCATTAGCTCTGTGTGAGCCCTTG
ACAGCTACTGCCTCTCCTTACCACAGGGGCCTCTAAGATACTGTTACCTAGA
GGTCTTGAGGATCTGTGTTCTCTGGGGGGAGGAAAGGAGGAGGAACCCAG
AACTTTCTTACAGTTTTCCTTGTTCTGTCACATGTCAAGACTGAAGGAACAG
GCTGGGCTACGTAGTGAGATCCTGTCTCAAAGGAAAGACGAGCATAGCCGA
ACCCCCGGTGGAACCCCCTCTGTTACCTGTTCACACAAGCTTATTGATGAGT
CTCATGTTAATGTCTTGTTTGTATGAAGTTTAAGAAAATATCGGGTTGGGCAA
CACATTCTATTTATTCATTTTATTTGAAATCTTAATGCCATCTCATGGTGTTGG
ATTGGTGTGGCACTTTATTCTTTTGTGTTGTGTATAACCATAAATTTTATTTTG
CATCAGATTGTCAATGTATTGCATTAATTTAATAAATATTTTTATTTATTAAAAA
AAAAAAAAAAAAAAA

FIG. 6

MRIFAGIIFTACCHLLRAFTITAPKDLYVVEYGSNVTMECRFPVERELDLLALVVYWEKEDEQVIQFVAGEE
DLKPQHSNFRGRASLPKDQLLKGNAALQITDVKLQDAGVYCCIISYGGADYKRITLKVNAPYRKINQRISV
DPATSEHELICQAEGYPEAEVIWTNSDHQPVSGKRSVTTSRTEGMLLNVTSSLRVNATANDVFYCTFWR
SQPGQNHTAELIIPELPATHPPQNRTHWVLLGSILLFLIVVSTVLLFLRKQVRMLDVEKCGVEDTSSKNRN
DTQFEET.

FIG. 7

mB7-4 vs. hB7-4

69% identity

```
mB7-4    1   MRIFAGIIFTACCHLLRAFTITAPKDLYVVEYGSNVTMECRFPVERELDLLALVVYWEKE 60
             MRIFA  IF    HLL AFT+T PKDLYVVEYGSN+T+EC+FPVE++LDL AL+VYWE E
hB7-4    1   MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEME 60

mB7-4   61   DEQVIQFVAGEEDLKPQHSNFRGRASLPKDQLLKGNAALQITDVKLQDAGVYCCIISYGG 120
             D+ +IQFV GEEDLK QHS++R RA L KDQL  GNAALQITDVKLQDAGVY C+ISYGG
hB7-4   61   DKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGG 120

mB7-4  121   ADYKRITLKVNAPYRKINQRI-SVDPATSEHELICQAEGYPEAEVIWTNSDHQPVSGKRS 179
             ADYKRIT+KVNAPY KINQRI  VDP TSEHEL CQAEGYP+AEVIWT+SDHQ +SGK +
hB7-4   121   ADYKRITVKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTT 180

mB7-4   180   VTTSRTEGMLLNVTSSLRVNATANDVFYCTFWRSQPGQNHTAELIIPELPATHPPQNRTH 239
              T S+ E  L NVTS+LR+N T N++FYCTF R  P +NHTAEL+IPELP  HPP  RTH
hB7-4   181   TTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNERTH 240

mB7-4   240   WVLLGSILLFLIVVSTVLLFLRKQVRMLDVEKCGVEDTSSKNRNDTQFEET 290
              V+LG+ILL L V  T +  LRK  RM+DV+KCG++DT+SK ++DT  EET
hB7-4   241   LVILGAILLCLGVALTFIFRLRKG-RMMDVKKCGIQDTNSKKQSDTHLEET 290
```

FIG. 8

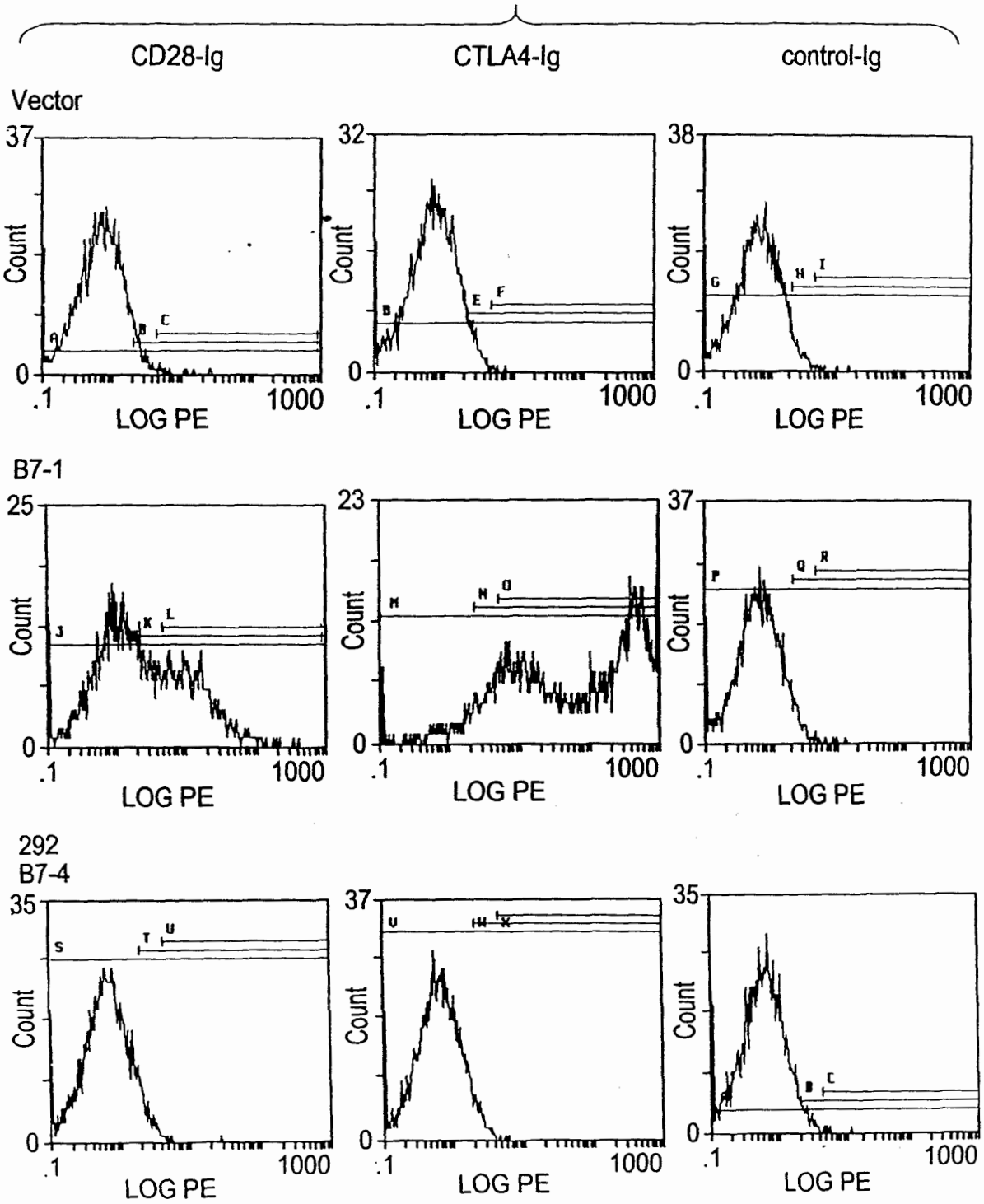

CD28-Ig
CTLA4-Ig
control-Ig
Vector
B7-1
292
B7-4
Count
LOG PE
.1
1000
0
37
32
38
25
23
37
35
37
35

FIG. 9

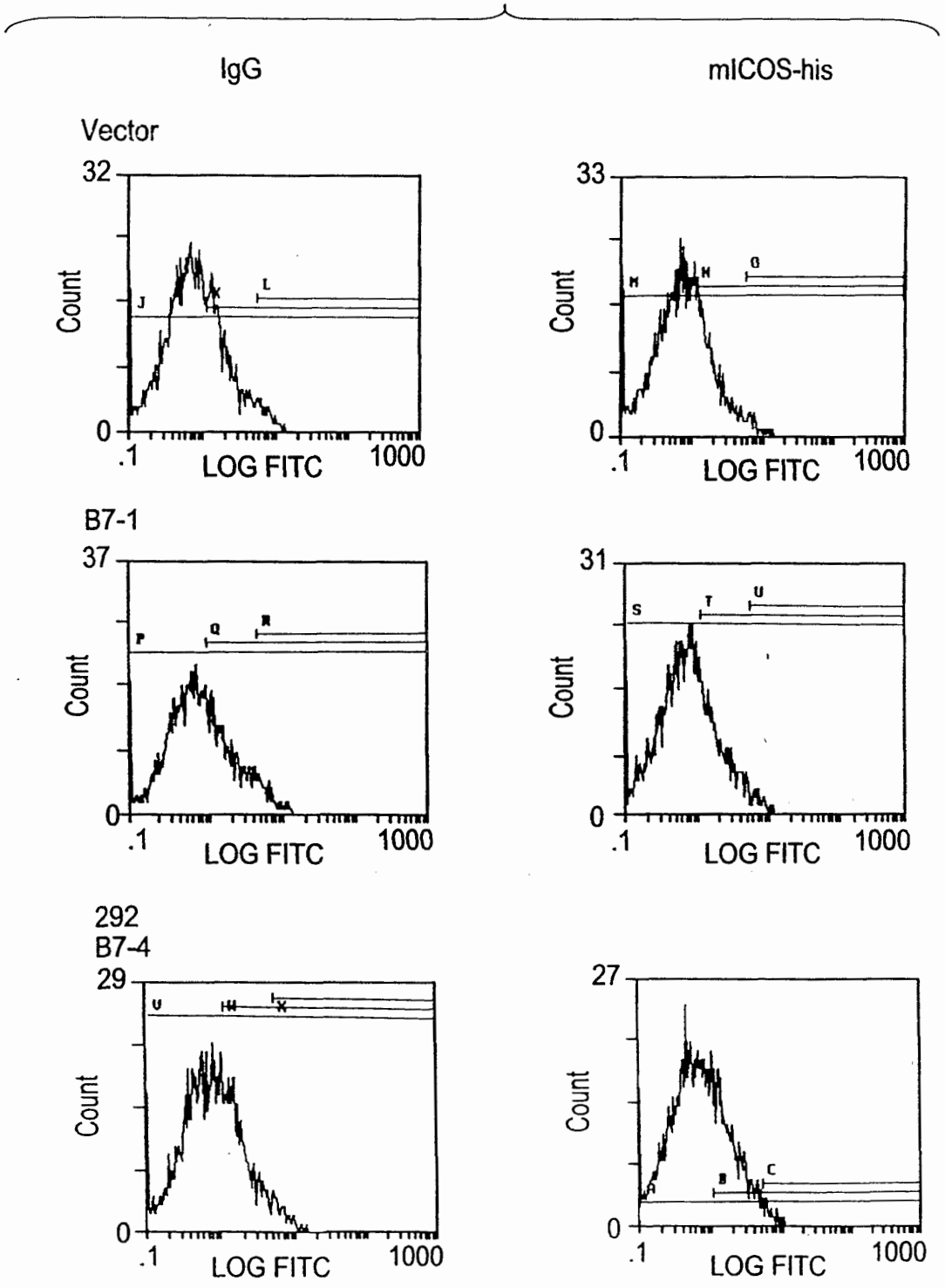

IgG
mICOS-his
Vector
32
33
Count
0
.1
LOG FITC
1000
J
L
M
N
O
B7-1
37
31
P
Q
R
S
T
U
292
B7-4
29
27
V
W
X
A
B
C

FIG. 10

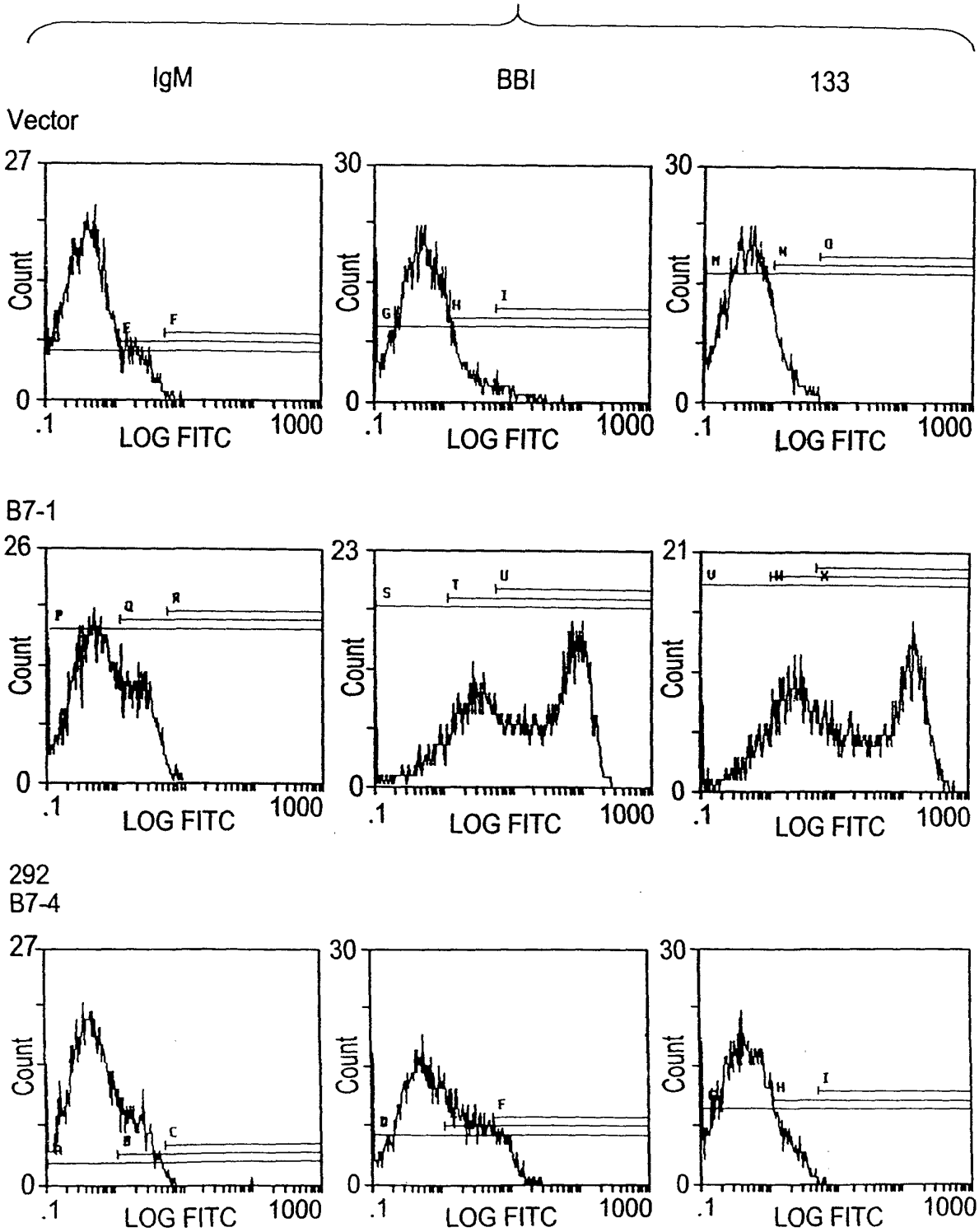

IgM
BBI
133
Vector
B7-1
292
B7-4
Count
LOG FITC
.1
1000
0
27
30
21
E
F
G
H
I
P
Q
R
S
T
U
V
A
B
C
D

FIG. 11

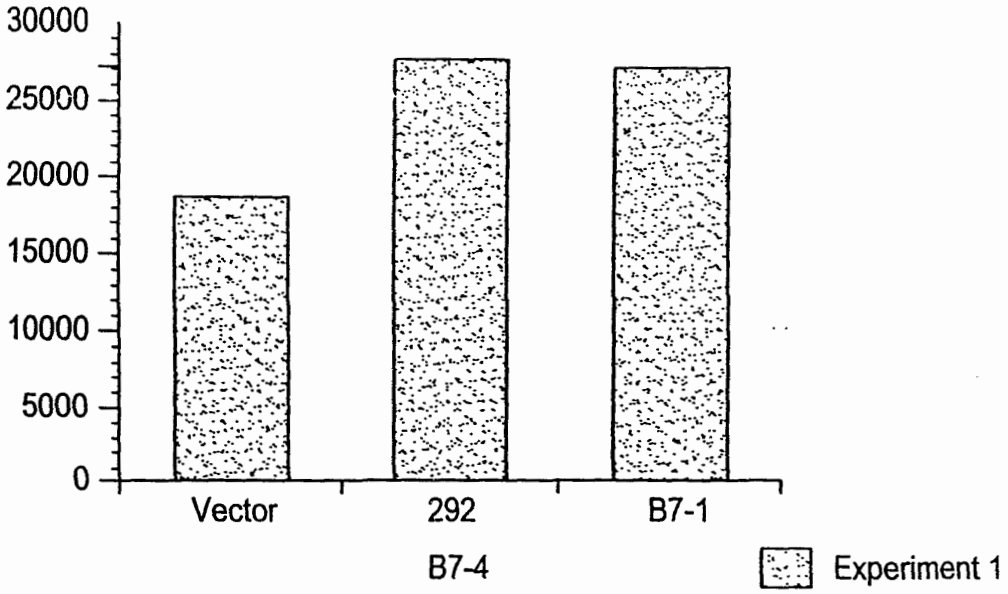

30000
25000
20000
15000
10000
5000
0
Vector
292
B7-1
B7-4
Experiment 1

FIG. 12

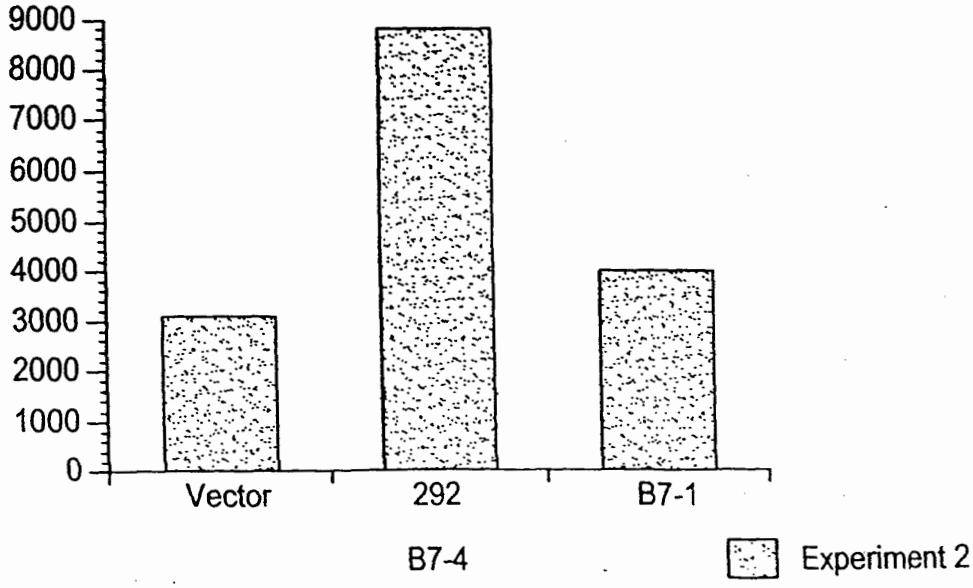

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Vector
292
B7-1
B7-4
Experiment 2

FIG. 13A

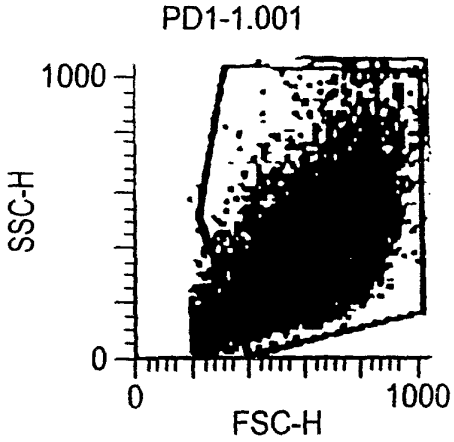


FIG. 13B

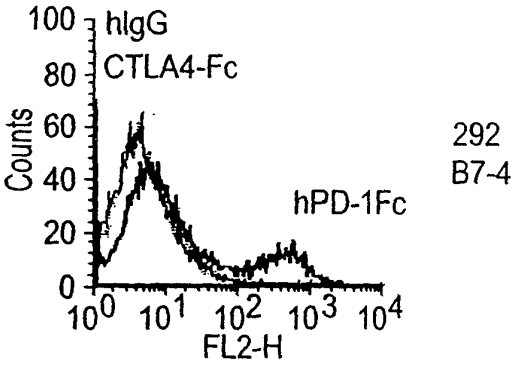


FIG. 13C

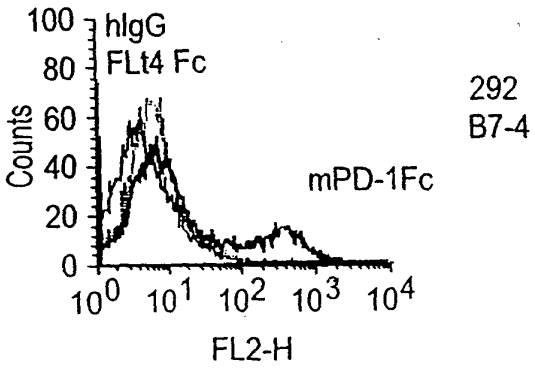
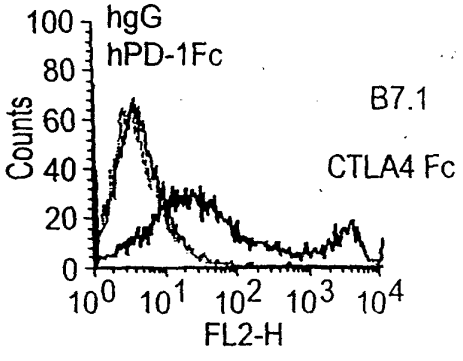


FIG. 13D



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FIG. 14A

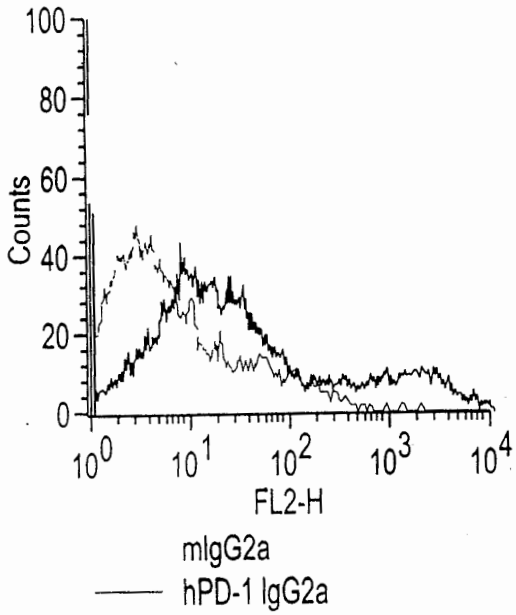


FIG. 14B

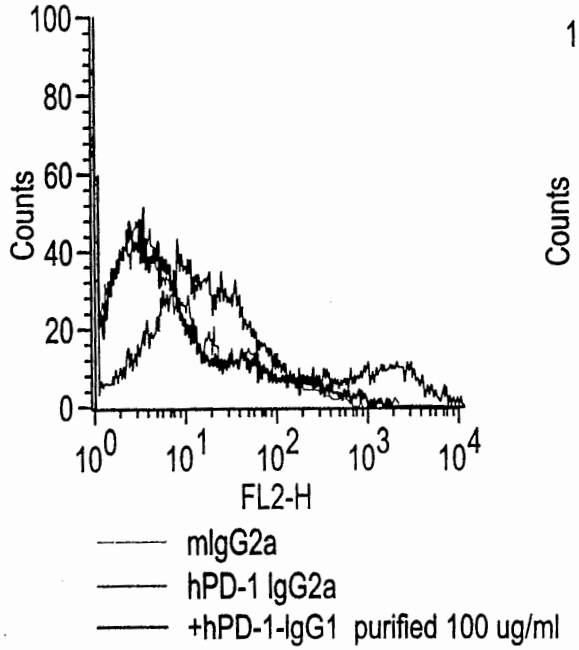
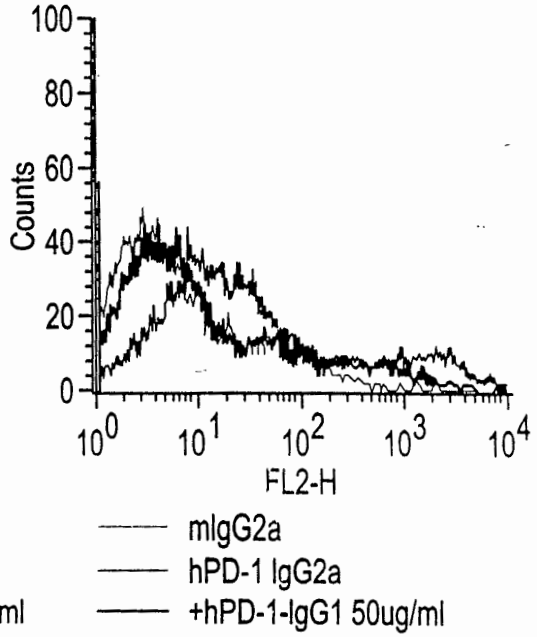


FIG. 14C



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FIG. 14D

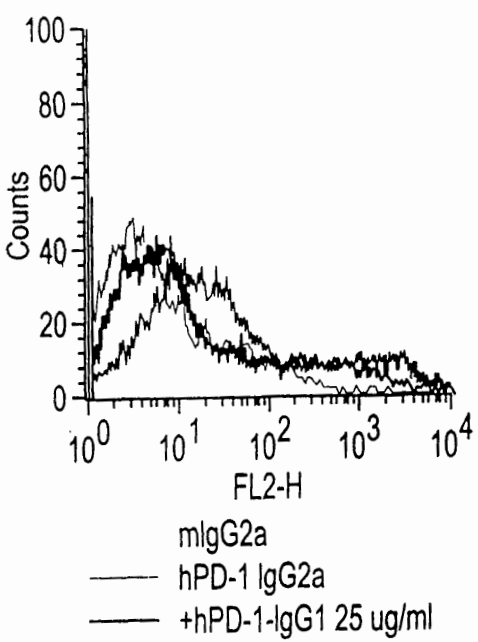


FIG. 14E

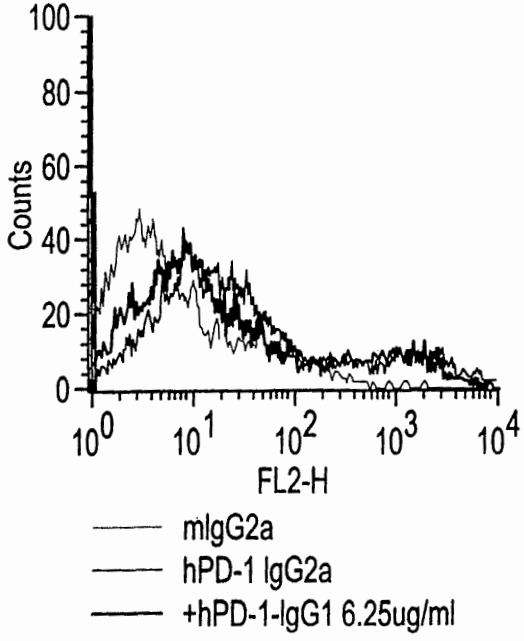
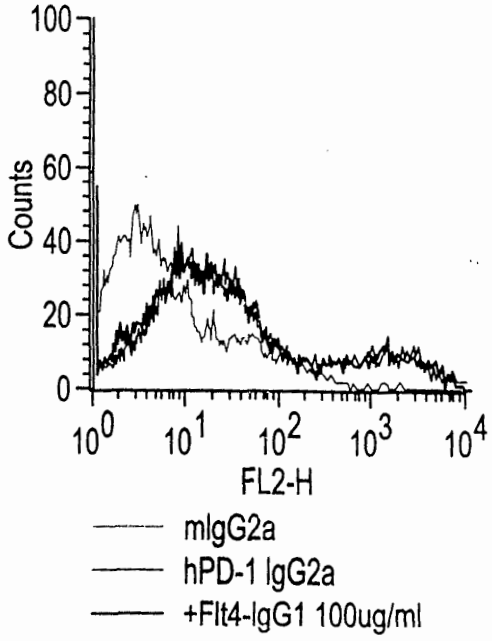


FIG. 14F



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FIG. 15A

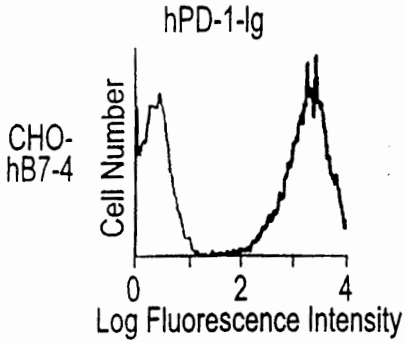


FIG. 15B

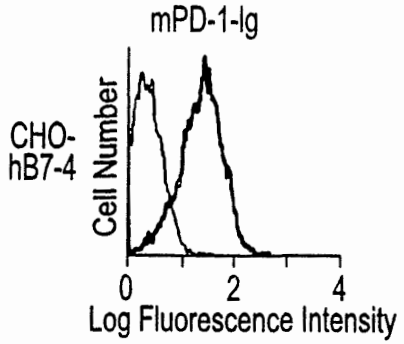


FIG. 15C

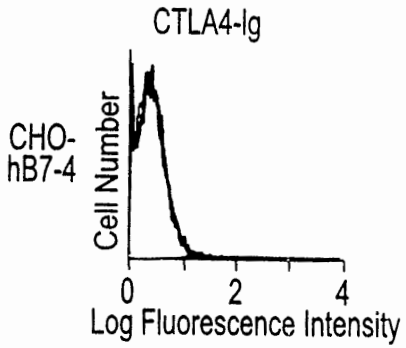


FIG. 15D

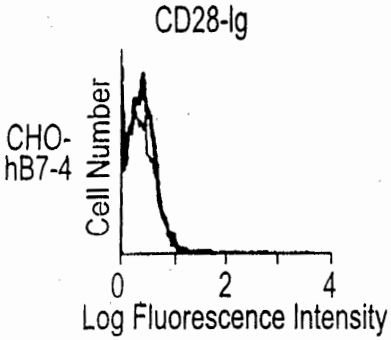


FIG. 15E

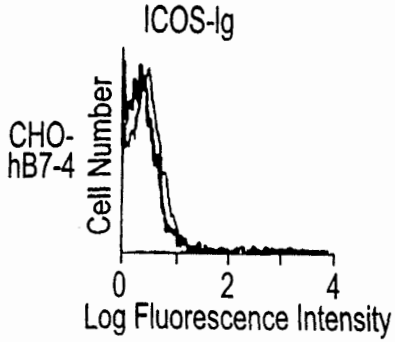


FIG. 15F

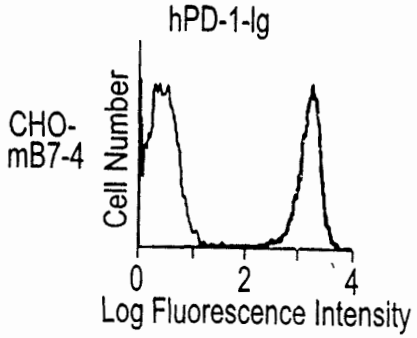


FIG. 15G

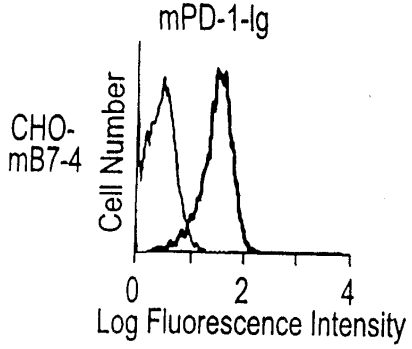


FIG. 15H

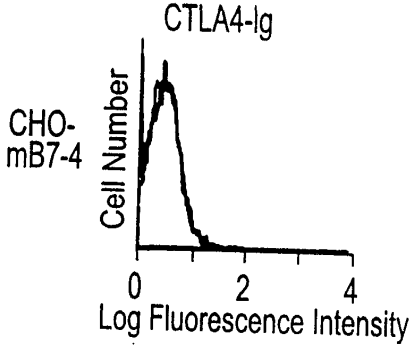


FIG. 15I

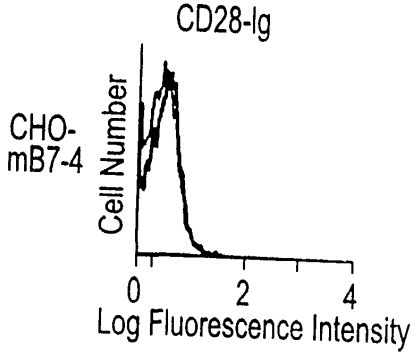


FIG. 15J

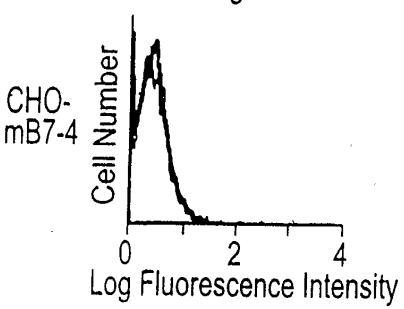


FIG. 15K

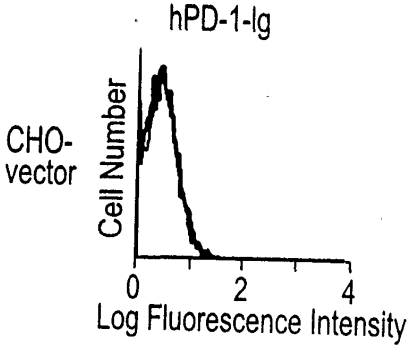


FIG. 15L

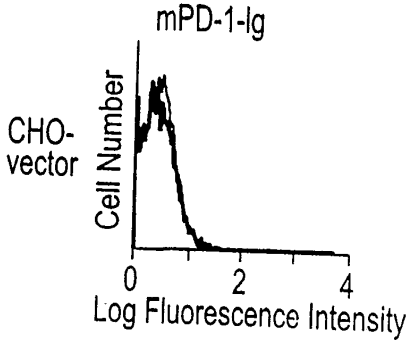


FIG. 16

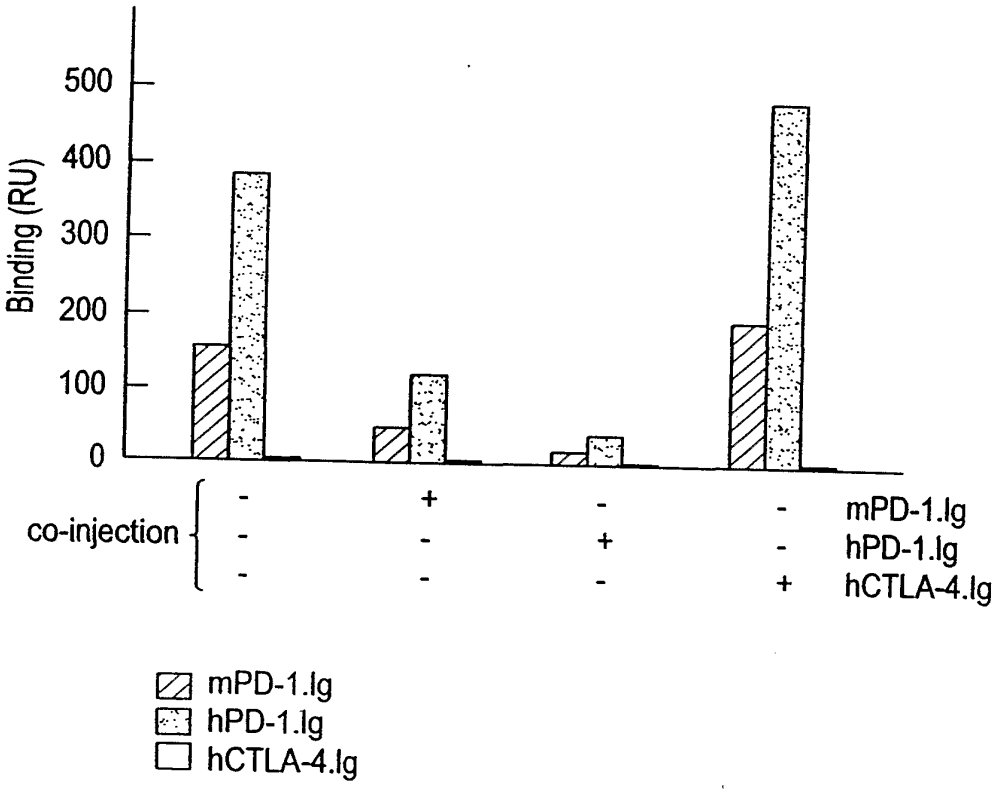

Binding (RU)
500
400
300
200
100
0
co-injection
-
+
-
-
mPD-1.lg
-
-
+
-
hPD-1.lg
-
-
-
+
hCTLA-4.lg
mPD-1.lg
hPD-1.lg
hCTLA-4.lg

FIG. 17

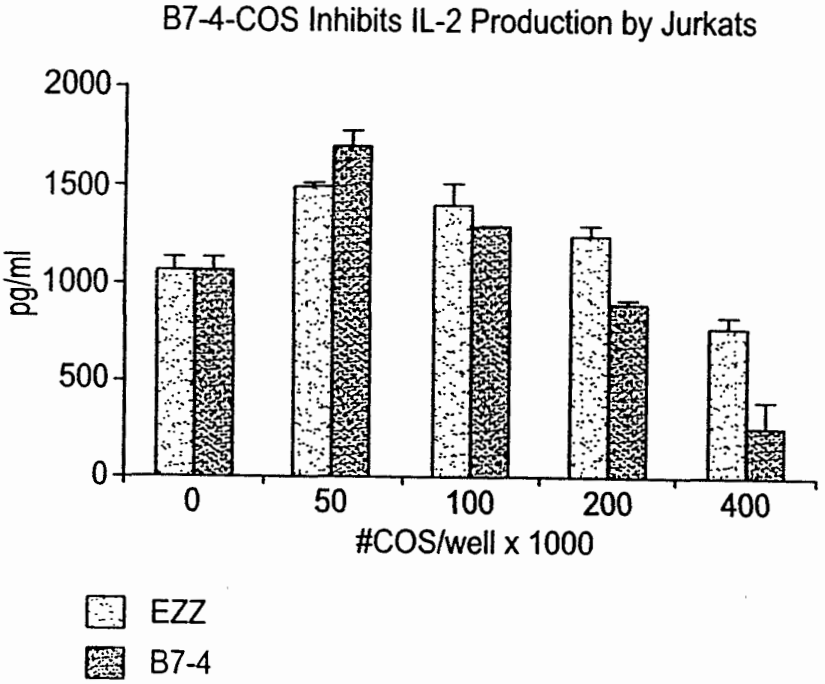

B7-4-COS Inhibits IL-2 Production by Jurkats
2000
1500
1000
500
0
pg/ml
0
50
100
200
400
#COS/well x 1000
EZZ
B7-4

FIG. 18A

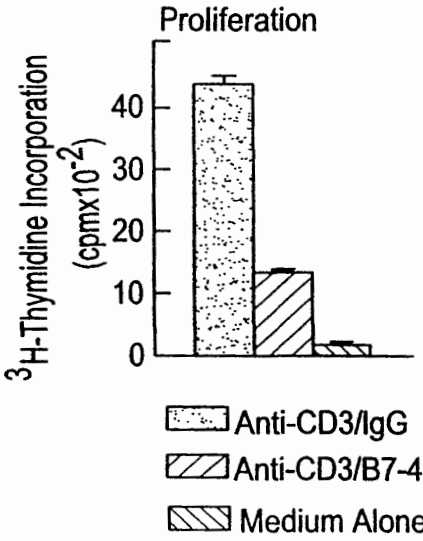


FIG. 18B

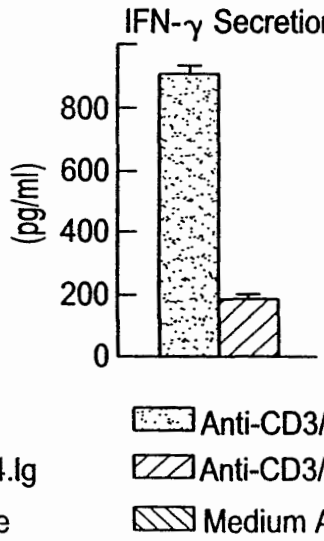


FIG. 18C

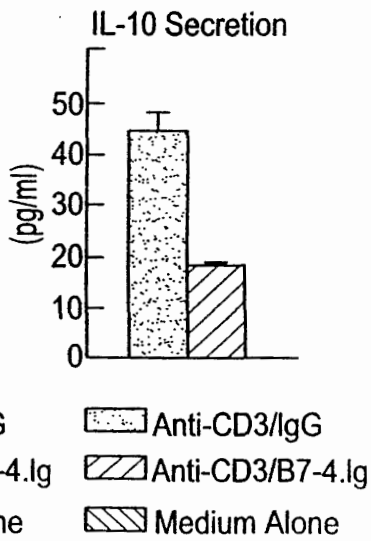


FIG. 19A

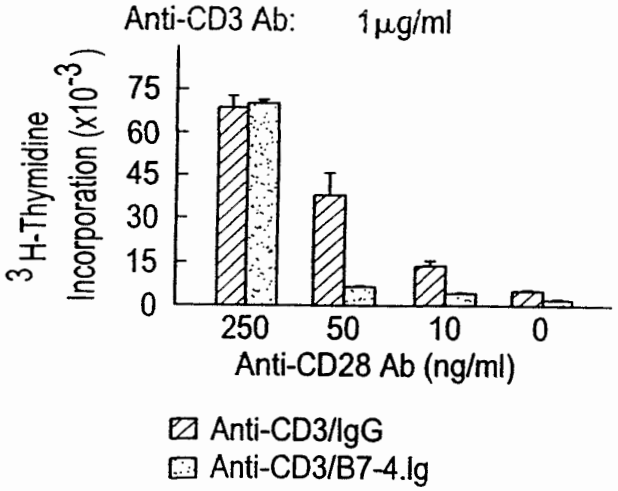
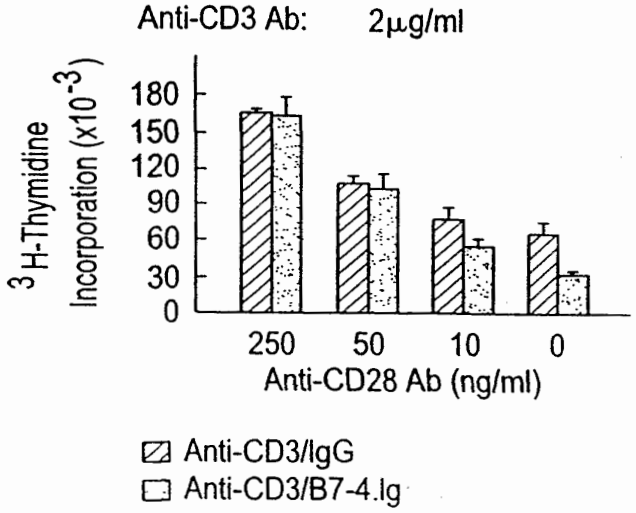


FIG. 19B



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FIG. 20A

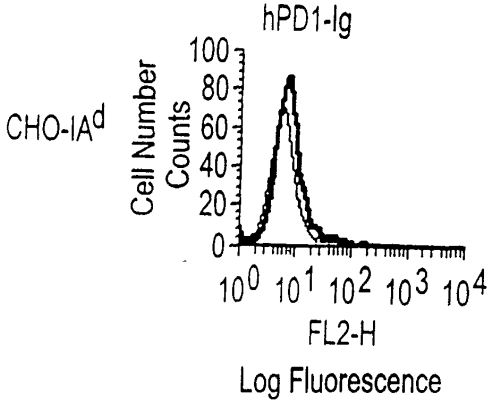


FIG. 20B

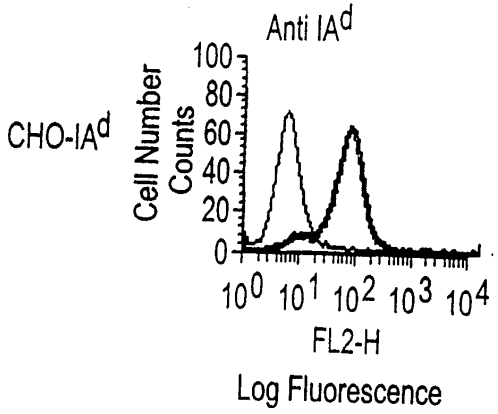


FIG. 20C

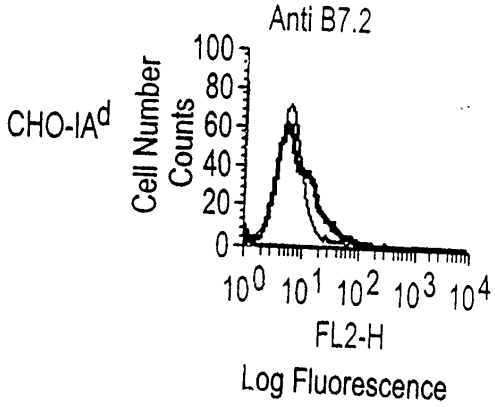


FIG. 20D

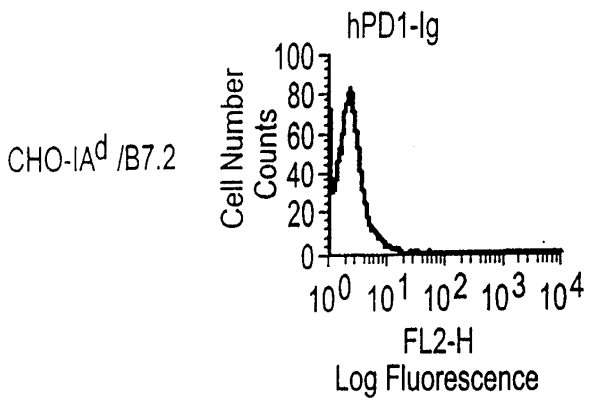


FIG. 20E

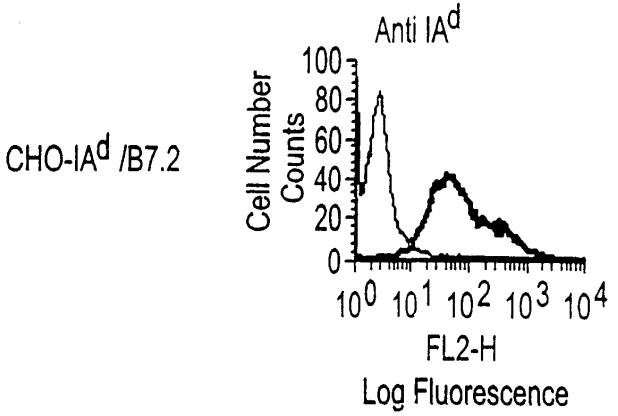


FIG. 20F

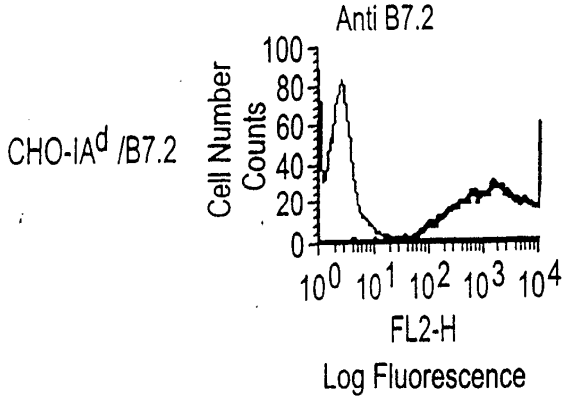


FIG. 20G

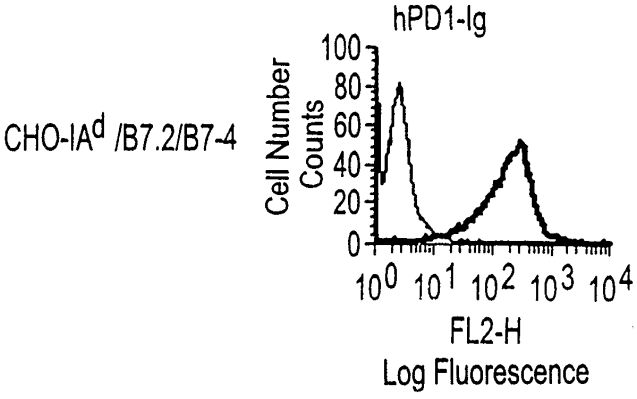


FIG. 20H

FIG. 20I

FIG. 21A

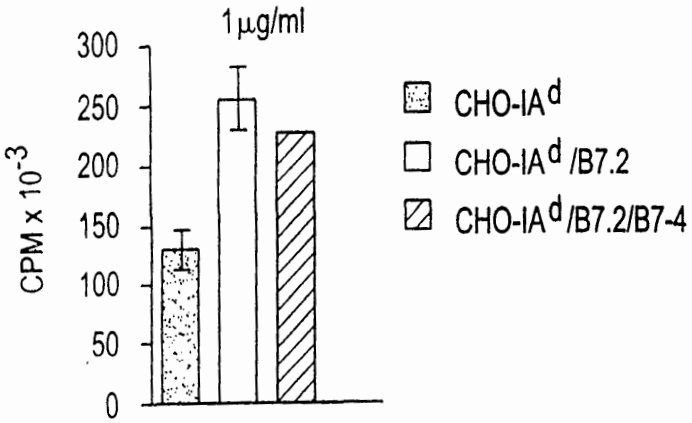


FIG. 21B

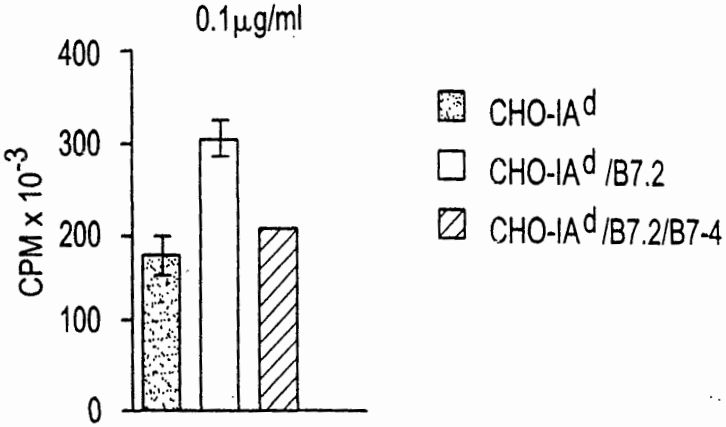


FIG. 21C

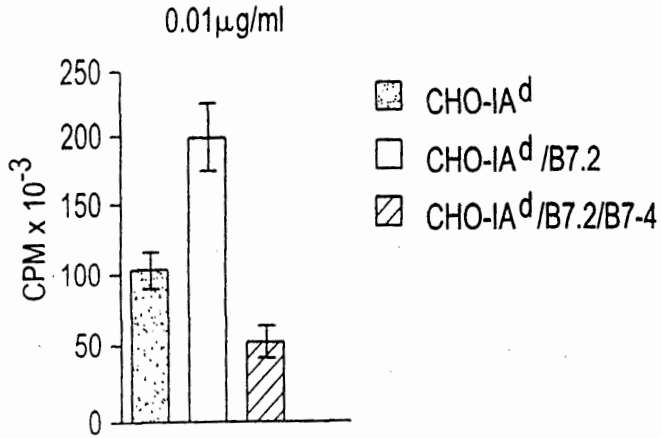
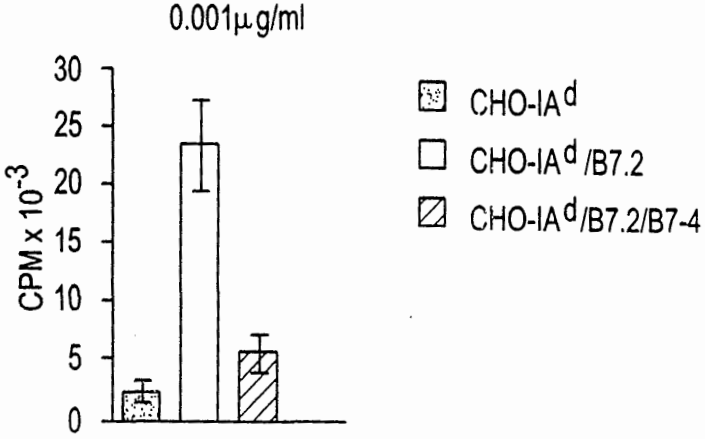


FIG.21D



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FIG. 22A

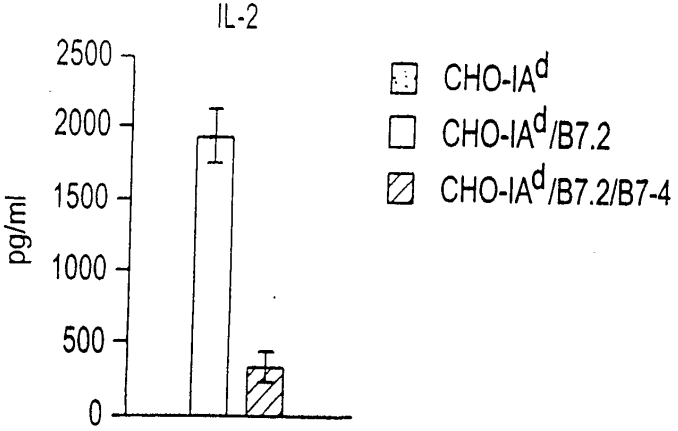


FIG. 22B

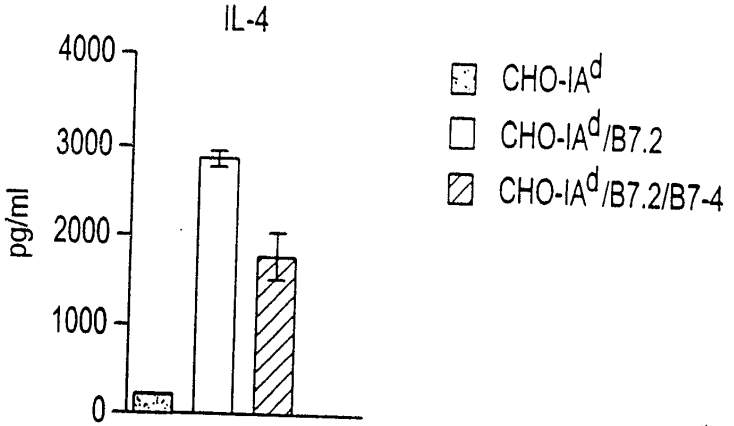


FIG. 22C

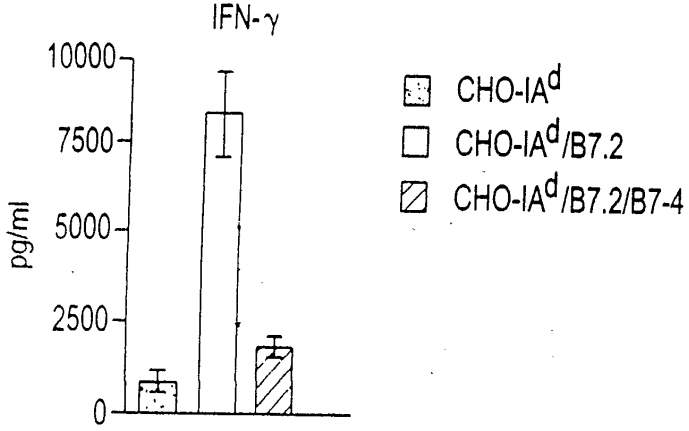


FIG. 22D

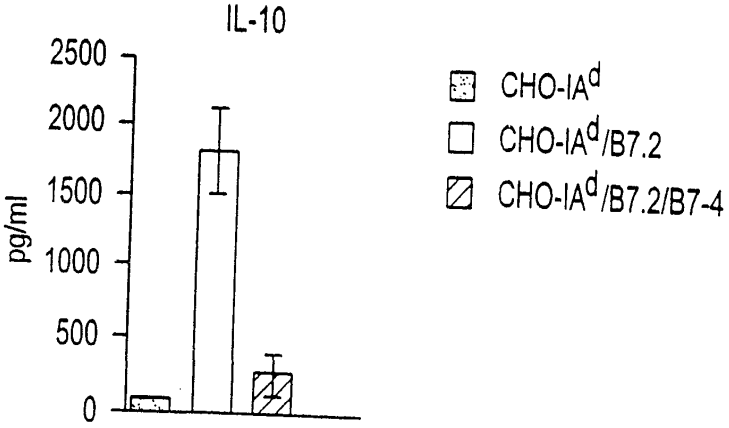


FIG. 23A

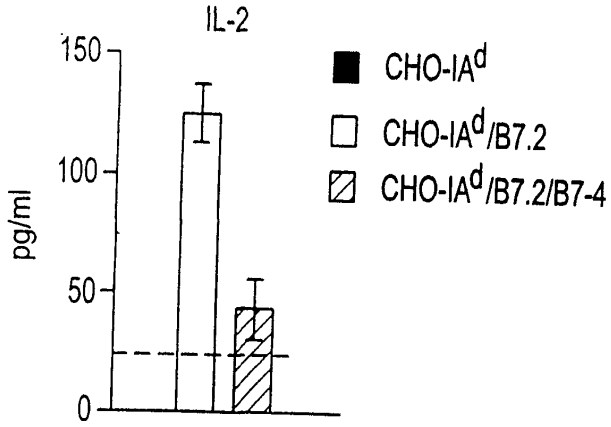


FIG. 23B

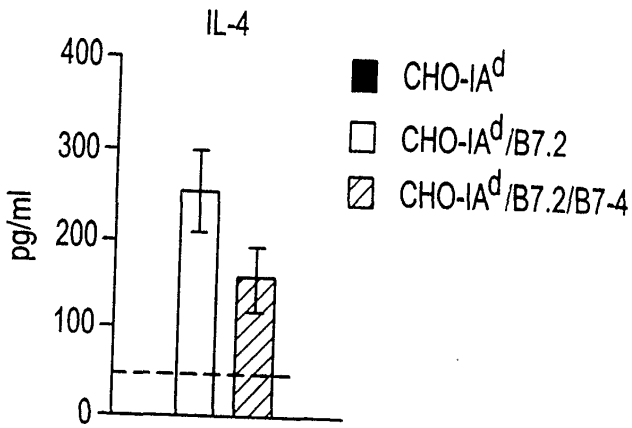
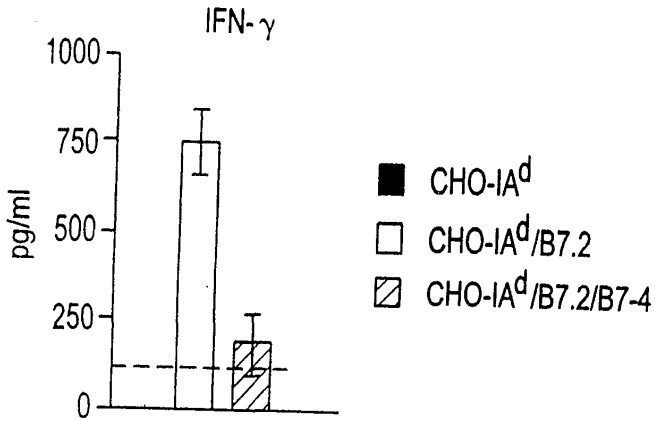


FIG. 23C



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FIG. 24A

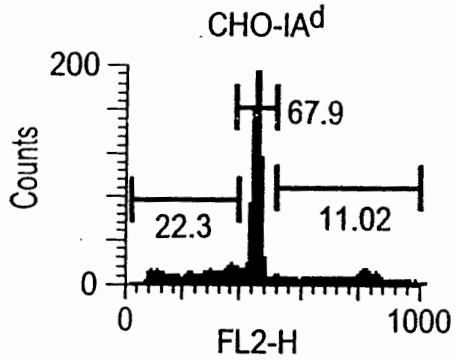


FIG. 24B

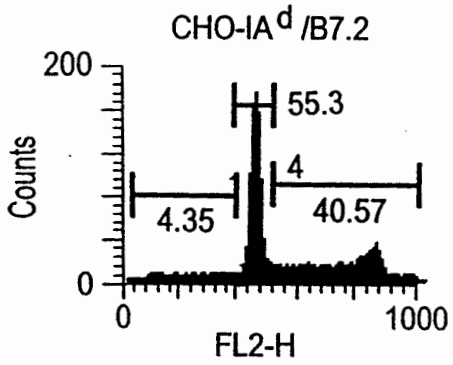


FIG. 24C

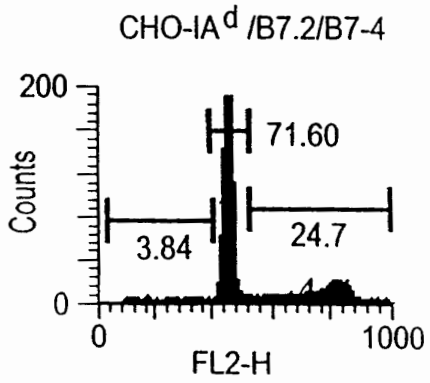


FIG. 25A

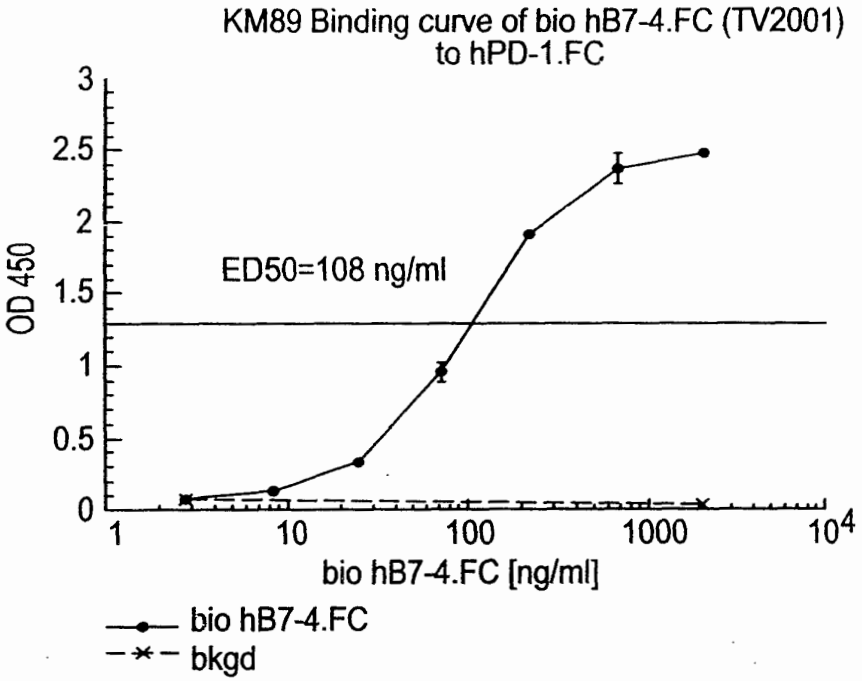
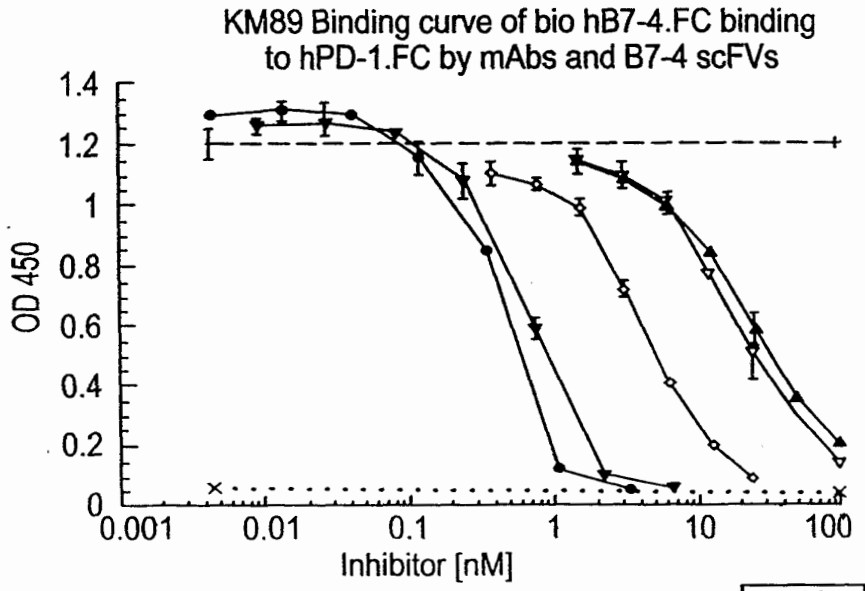


FIG. 25B



Inhibitor	IC50
10D9	0.5
11D12	0.7
B7-4-1	4
B7-4-6	19
B7-4-12	24

FIG. 26

Inhibition of B7-4 binding by PD-1 clone 17

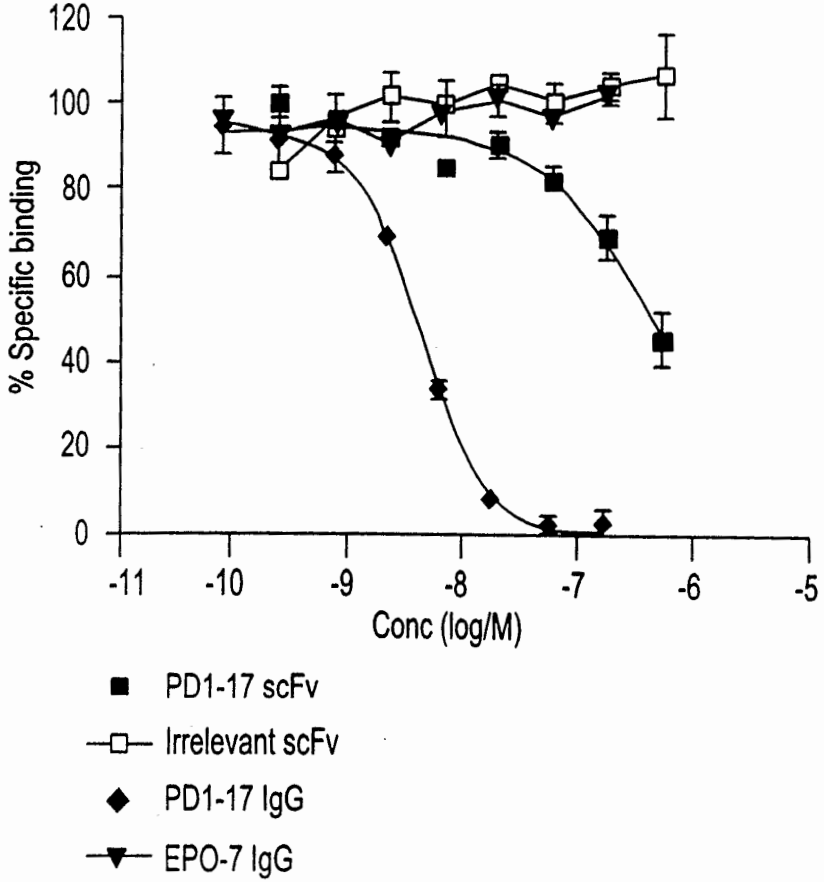


FIG. 27

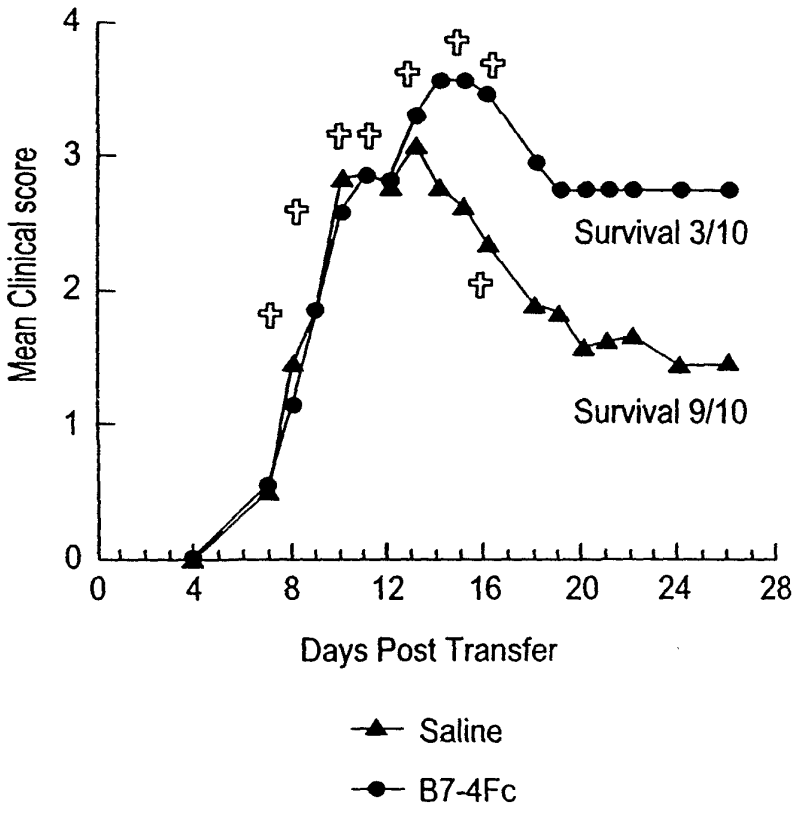

Mean Clinical score
0
1
2
3
4
0
4
8
12
16
20
24
28
Days Post Transfer
Survival 3/10
Survival 9/10
Saline
B7-4Fc

Fig. 28A

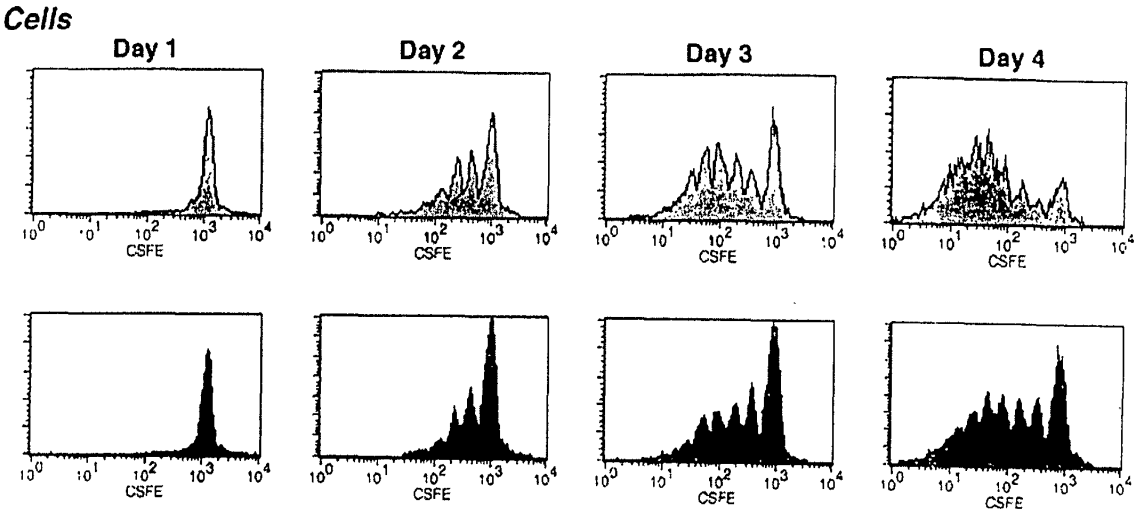
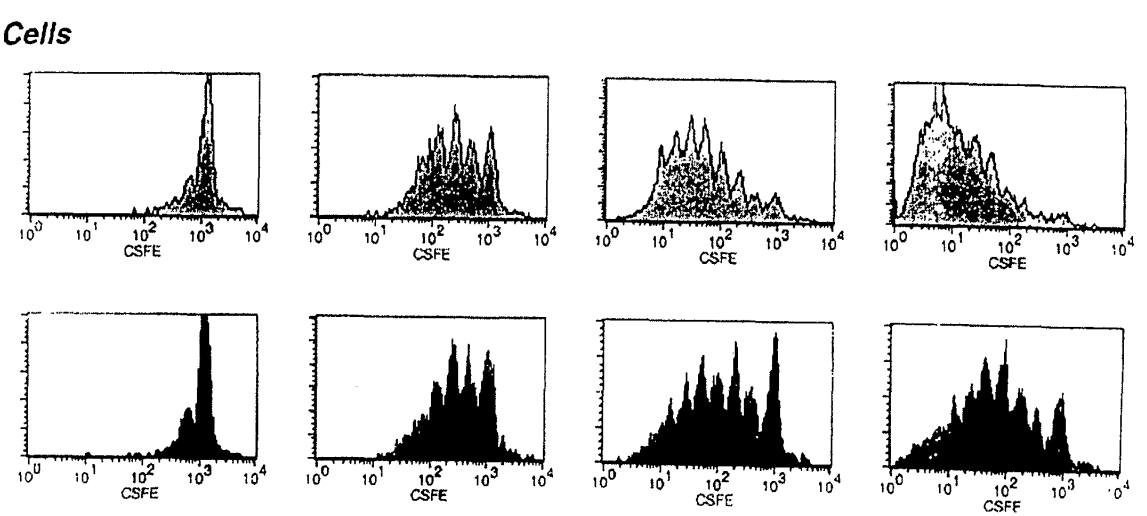


Fig. 28B



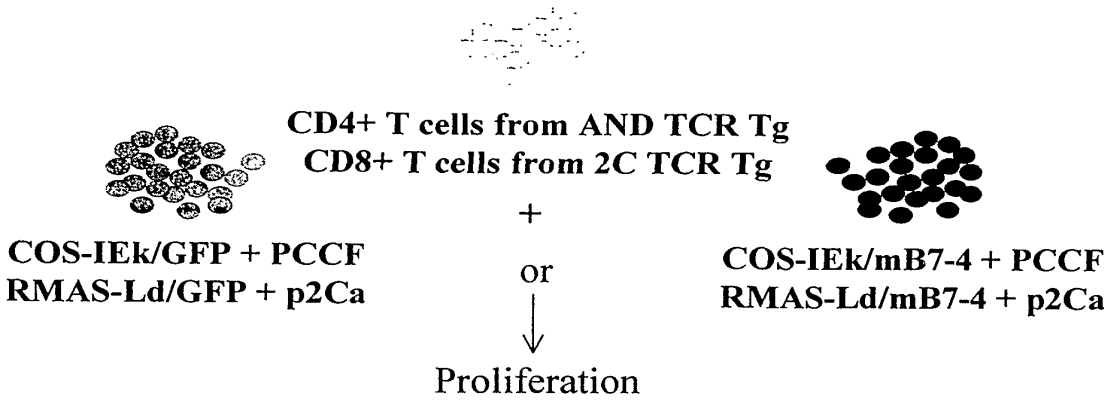


Fig. 29A

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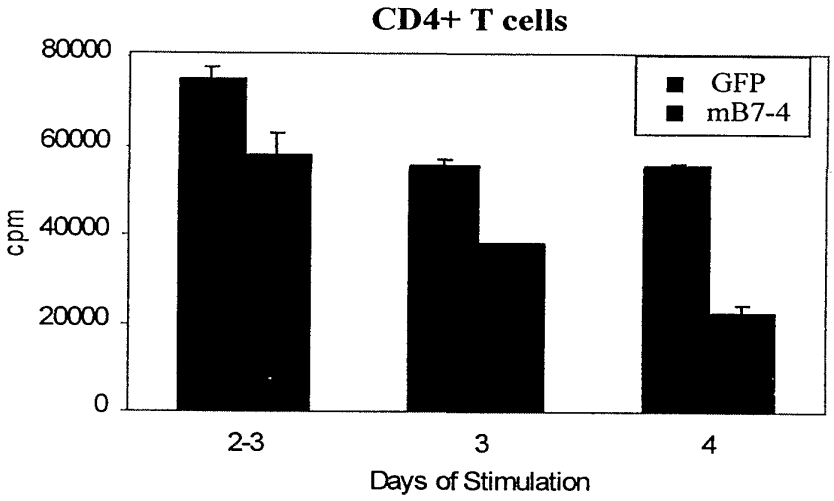

CD4+ T cells
80000
60000
40000
20000
0
cpm
GFP
mB7-4
2-3
3
4
Days of Stimulation

Fig. 29B

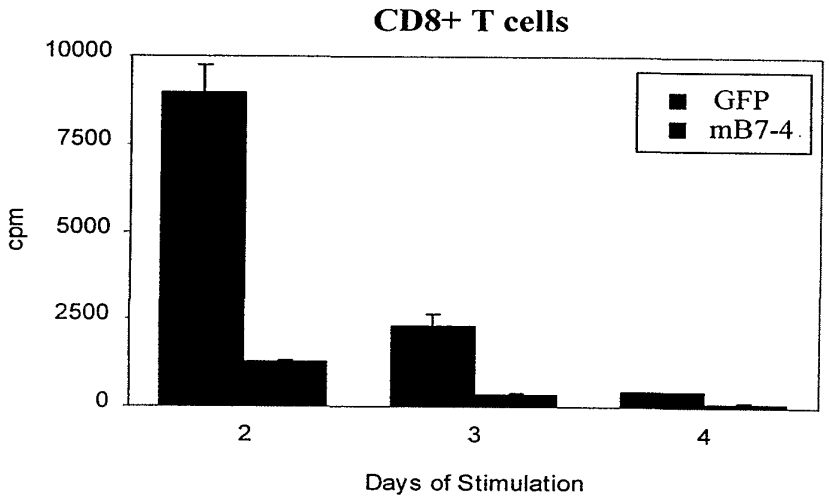

CD8+ T cells
10000
7500
5000
2500
0
cpm
GFP
mB7-4
2
3
4
Days of Stimulation

Fig. 29C

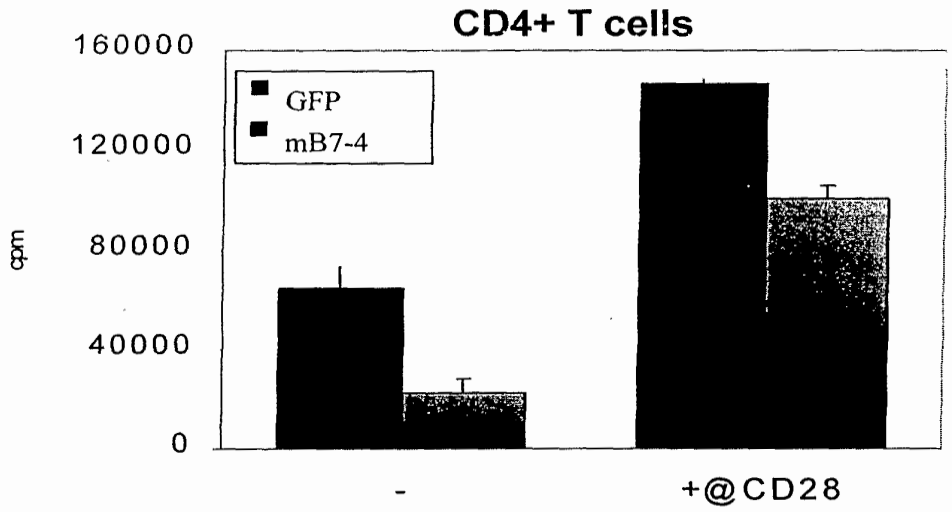


Fig. 30A

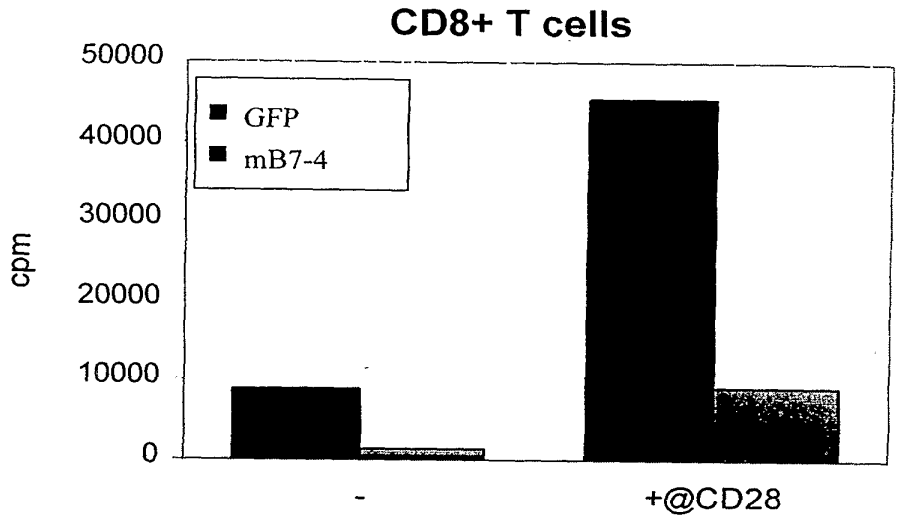


Fig. 30B

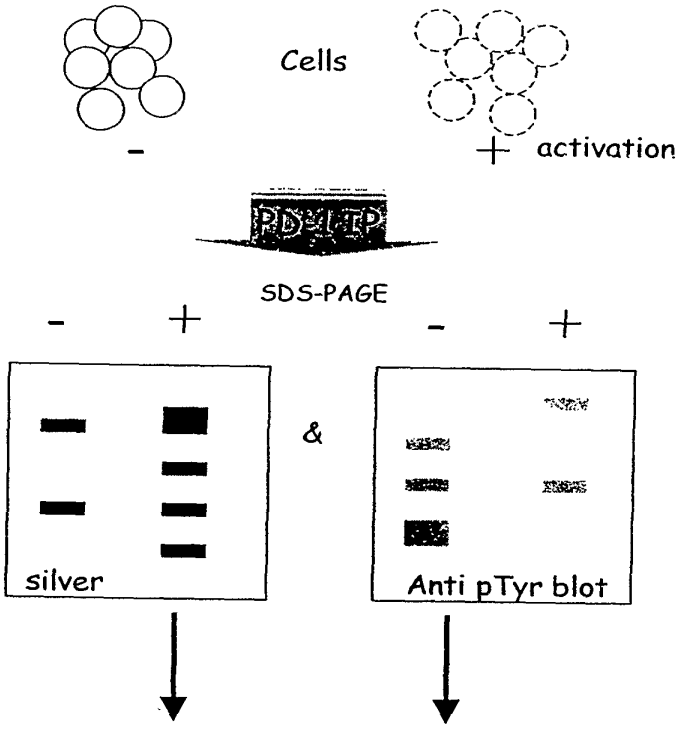
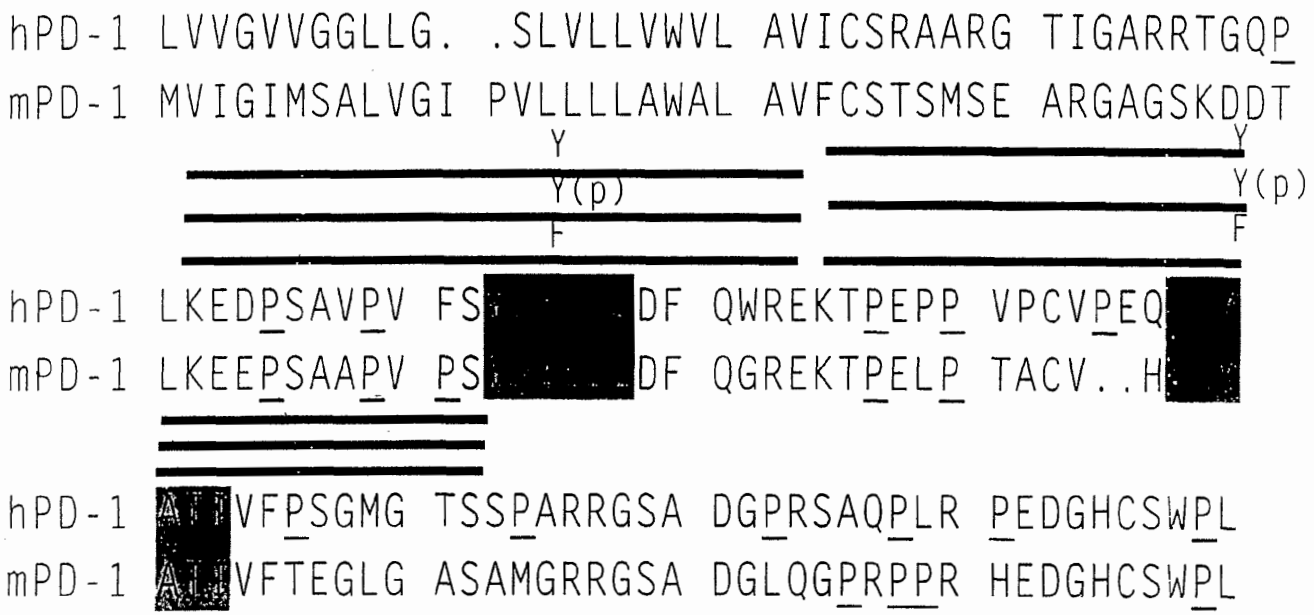


Fig 31A

Fig. 31B



ITIM Peptides	
PD-1_pY1	biotin- KEDPSAVPVFSVDY(PO4)GELDFQWRE-amide
PD-1_Y1F	biotin- KEDPSAVPVFSVDFGELDFQWRE-amide
PD-1_Y1	biotin- KEDPSAVPVFSVDYGELDFQWRE-amide
PD-1_pY2	biotin- KTPEPPVPSVPEQTEY(PO4)ATIVFPSGMGTSS-amide
PD-1_Y2F	biotin- KTPEPPVPSVPEQTEFATIVFPSGMGTSS-amide
PD-1_Y2	biotin- KTPEPPVPSVPEQTEYATIVFPSGMGTSS-amide

Other Peptides	
PD-1_K212_	biotin-RGTIGARRTGQP LKEDPSAVPVFS
PD-1_K212D	biotin-RGTIGARRTGQP LDEDPSAVPVFS
PD-1_K335	biotin-DFQWREKTPEPPVPCVPEQ
PD-1_K335D	biotin-DFQWREDTPEPPVPCVPEQ
PD-1_Ctail1	biotin-VFPSGMGTSSPARRGSADGPRSA
PD-1_Ctail2	biotin-ARRGSADGPRSAQPLRPEDGHCSWPL

PD-1 mediated inhibition of TCR induced ERK activation

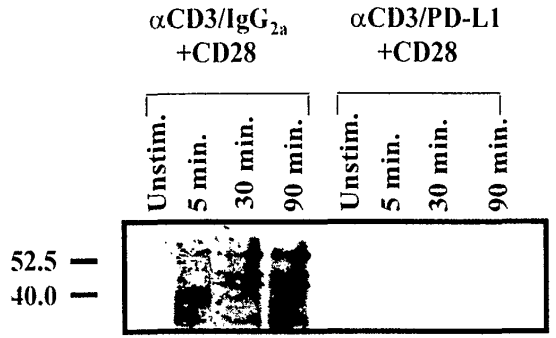


Figure 32

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PD-1 mediated inhibition of TCR induced PKCθ phosphorylation

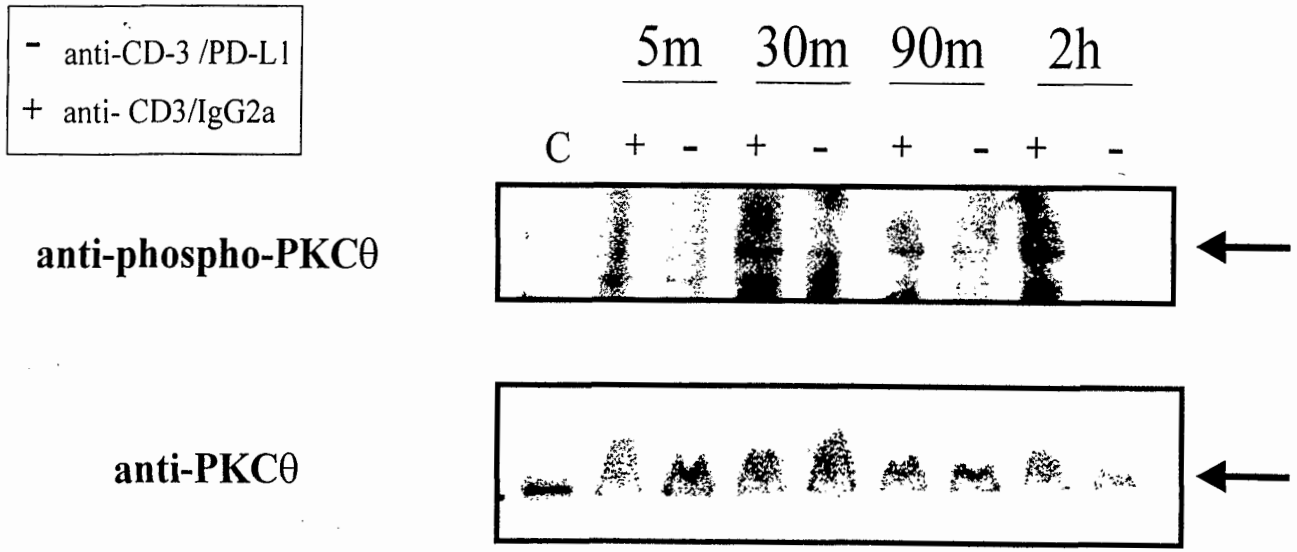


Figure 33

SEQUENCE LISTING

<110> Wyeth et al.

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<150> US 60/281064
<151> 2001-04-02

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Met Arg Ile Phe Ala Val Phe Ile Phe Met Thr Tyr Trp His Leu Leu
1 5 10 15

aac gca ttt act gtc acg gtt ccc aag gac cta tat gtg gta gag tat 154
Asn Ala Phe Thr Val Thr Val Pro Lys Asp Leu Tyr Val Val Glu Tyr
20 25 30

ggt agc aat atg aca att gaa tgc aaa ttc cca gta gaa aaa caa tta 202
Gly Ser Asn Met Thr Ile Glu Cys Lys Phe Pro Val Glu Lys Gln Leu
35 40 45

gac ctg gct gca cta att gtc tat tgg gaa atg gag gat aag aac att 250
Asp Leu Ala Ala Leu Ile Val Tyr Trp Glu Met Glu Asp Lys Asn Ile
50 55 60

att caa ttt gtg cat gga gag gaa gac ctg aag gtt cag cat agt agc 298
Ile Gln Phe Val His Gly Glu Glu Asp Leu Lys Val Gln His Ser Ser
65 70 75 80

tac aga cag agg gcc cgg ctg ttg aag gac cag ctc tcc ctg gga aat 346
Tyr Arg Gln Arg Ala Arg Leu Leu Lys Asp Gln Leu Ser Leu Gly Asn
85 90 95

gct gca ctt cag atc aca gat gtg aaa ttg cag gat gca ggg gtg tac 394
Ala Ala Leu Gln Ile Thr Asp Val Lys Leu Gln Asp Ala Gly Val Tyr
100 105 110

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Arg Cys Met Ile Ser Tyr Gly Gly Ala Asp Tyr Lys Arg Ile Thr Val
115 120 125

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aaa gtc aat gcc cca tac aac aaa atc aac caa aga att ttg gtt gtg   490
Lys Val Asn Ala Pro Tyr Asn Lys Ile Asn Gln Arg Ile Leu Val Val
    130                 135                 140

gat cca gtc acc tct gaa cat gaa ctg aca tgt cag gct gag ggc tac   538
Asp Pro Val Thr Ser Glu His Glu Leu Thr Cys Gln Ala Glu Gly Tyr
145                 150                 155                 160

ccc aag gcc gaa gtc atc tgg aca agc agt gac cat caa gtc ctg agt   586
Pro Lys Ala Glu Val Ile Trp Thr Ser Ser Asp His Gln Val Leu Ser
                165                 170                 175

ggt aag acc acc acc acc aat tcc aag aga gag gag aag ctt ttc aat   634
Gly Lys Thr Thr Thr Thr Asn Ser Lys Arg Glu Glu Lys Leu Phe Asn
            180                 185                 190

gtg acc agc aca ctg aga atc aac aca aca act aat gag att ttc tac   682
Val Thr Ser Thr Leu Arg Ile Asn Thr Thr Thr Asn Glu Ile Phe Tyr
        195                 200                 205

tgc act ttt agg aga tta gat cct gag gaa aac cat aca gct gaa ttg   730
Cys Thr Phe Arg Arg Leu Asp Pro Glu Glu Asn His Thr Ala Glu Leu
    210                 215                 220

gtc atc cca ggt aat att ctg aat gtg tcc att aaa ata tgt cta aca   778
Val Ile Pro Gly Asn Ile Leu Asn Val Ser Ile Lys Ile Cys Leu Thr
225                 230                 235                 240

ctg tcc cct agc acc tagcatgatg tctgcctatc atagtcattc agtgattgtt   833
Leu Ser Pro Ser Thr
                245

gaataaatga atgaatgaat aacactatgt ttacaaaata tatcctaatt cctcacctcc 893

attcatccaa accatattgt tacttaataa acattcagca gatatttatg gaataaaaaa 953

aaaaaaaaaa aaaaa                                                  968


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<213> Homo sapiens

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  1               5                  10                  15

Asn Ala Phe Thr Val Thr Val Pro Lys Asp Leu Tyr Val Val Glu Tyr
             20                  25                  30

Gly Ser Asn Met Thr Ile Glu Cys Lys Phe Pro Val Glu Lys Gln Leu
         35                  40                  45

Asp Leu Ala Ala Leu Ile Val Tyr Trp Glu Met Glu Asp Lys Asn Ile
     50                  55                  60

Ile Gln Phe Val His Gly Glu Glu Asp Leu Lys Val Gln His Ser Ser
 65                  70                  75                  80
```

```
Tyr Arg Gln Arg Ala Arg Leu Leu Lys Asp Gln Leu Ser Leu Gly Asn
                 85                  90                  95

Ala Ala Leu Gln Ile Thr Asp Val Lys Leu Gln Asp Ala Gly Val Tyr
            100                 105                 110

Arg Cys Met Ile Ser Tyr Gly Gly Ala Asp Tyr Lys Arg Ile Thr Val
        115                 120                 125

Lys Val Asn Ala Pro Tyr Asn Lys Ile Asn Gln Arg Ile Leu Val Val
    130                 135                 140

Asp Pro Val Thr Ser Glu His Glu Leu Thr Cys Gln Ala Glu Gly Tyr
145                 150                 155                 160

Pro Lys Ala Glu Val Ile Trp Thr Ser Ser Asp His Gln Val Leu Ser
                165                 170                 175

Gly Lys Thr Thr Thr Thr Asn Ser Lys Arg Glu Glu Lys Leu Phe Asn
            180                 185                 190

Val Thr Ser Thr Leu Arg Ile Asn Thr Thr Thr Asn Glu Ile Phe Tyr
        195                 200                 205

Cys Thr Phe Arg Arg Leu Asp Pro Glu Glu Asn His Thr Ala Glu Leu
    210                 215                 220

Val Ile Pro Gly Asn Ile Leu Asn Val Ser Ile Lys Ile Cys Leu Thr
225                 230                 235                 240

Leu Ser Pro Ser Thr
                245
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<222> (53)..(922)

<400> 3

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                                                          Met Arg
                                                            1

ata ttt gct gtc ttt ata ttc atg acc tac tgg cat ttg ctg aac gca   106
Ile Phe Ala Val Phe Ile Phe Met Thr Tyr Trp His Leu Leu Asn Ala
          5                  10                  15

ttt act gtc acg gtt ccc aag gac cta tat gtg gta gag tat ggt agc   154
Phe Thr Val Thr Val Pro Lys Asp Leu Tyr Val Val Glu Tyr Gly Ser
     20                  25                  30
```

```
aat atg aca att gaa tgc aaa ttc cca gta gaa aaa caa tta gac ctg   202
Asn Met Thr Ile Glu Cys Lys Phe Pro Val Glu Lys Gln Leu Asp Leu
 35                  40                  45                  50

gct gca cta att gtc tat tgg gaa atg gag gat aag aac att att caa   250
Ala Ala Leu Ile Val Tyr Trp Glu Met Glu Asp Lys Asn Ile Ile Gln
                 55                  60                  65

ttt gtg cat gga gag gaa gac ctg aag gtt cag cat agt agc tac aga   298
Phe Val His Gly Glu Glu Asp Leu Lys Val Gln His Ser Ser Tyr Arg
             70                  75                  80

cag agg gcc cgg ctg ttg aag gac cag ctc tcc ctg gga aat gct gca   346
Gln Arg Ala Arg Leu Leu Lys Asp Gln Leu Ser Leu Gly Asn Ala Ala
         85                  90                  95

ctt cag atc aca gat gtg aaa ttg cag gat gca ggg gtg tac cgc tgc   394
Leu Gln Ile Thr Asp Val Lys Leu Gln Asp Ala Gly Val Tyr Arg Cys
    100                 105                 110

atg atc agc tat ggt ggt gcc gac tac aag cga att act gtg aaa gtc   442
Met Ile Ser Tyr Gly Gly Ala Asp Tyr Lys Arg Ile Thr Val Lys Val
115                 120                 125                 130

aat gcc cca tac aac aaa atc aac caa aga att ttg gtt gtg gat cca   490
Asn Ala Pro Tyr Asn Lys Ile Asn Gln Arg Ile Leu Val Val Asp Pro
                135                 140                 145

gtc acc tct gaa cat gaa ctg aca tgt cag gct gag ggc tac ccc aag   538
Val Thr Ser Glu His Glu Leu Thr Cys Gln Ala Glu Gly Tyr Pro Lys
            150                 155                 160

gcc gaa gtc atc tgg aca agc agt gac cat caa gtc ctg agt ggt aag   586
Ala Glu Val Ile Trp Thr Ser Ser Asp His Gln Val Leu Ser Gly Lys
        165                 170                 175

acc acc acc acc aat tcc aag aga gag gag aag ctt ttc aat gtg acc   634
Thr Thr Thr Thr Asn Ser Lys Arg Glu Glu Lys Leu Phe Asn Val Thr
    180                 185                 190

agc aca ctg aga atc aac aca aca act aat gag att ttc tac tgc act   682
Ser Thr Leu Arg Ile Asn Thr Thr Thr Asn Glu Ile Phe Tyr Cys Thr
195                 200                 205                 210

ttt agg aga tta gat cct gag gaa aac cat aca gct gaa ttg gtc atc   730
Phe Arg Arg Leu Asp Pro Glu Glu Asn His Thr Ala Glu Leu Val Ile
                215                 220                 225

cca gaa cta cct ctg gca cat cct cca aat gaa agg act cac ttg gta   778
Pro Glu Leu Pro Leu Ala His Pro Pro Asn Glu Arg Thr His Leu Val
            230                 235                 240

att ctg gga gcc atc tta tta tgc ctt ggt gta gca ctg aca ttc atc   826
Ile Leu Gly Ala Ile Leu Leu Cys Leu Gly Val Ala Leu Thr Phe Ile
        245                 250                 255

ttc cgt tta aga aaa ggg aga atg atg gat gtg aaa aaa tgt ggc atc   874
Phe Arg Leu Arg Lys Gly Arg Met Met Asp Val Lys Lys Cys Gly Ile
    260                 265                 270
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Gln Asp Thr Asn Ser Lys Lys Gln Ser Asp Thr His Leu Glu Glu Thr
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Asn Ala Phe Thr Val Thr Val Pro Lys Asp Leu Tyr Val Val Glu Tyr
20 25 30

Gly Ser Asn Met Thr Ile Glu Cys Lys Phe Pro Val Glu Lys Gln Leu
35 40 45

Asp Leu Ala Ala Leu Ile Val Tyr Trp Glu Met Glu Asp Lys Asn Ile
50 55 60

Ile Gln Phe Val His Gly Glu Glu Asp Leu Lys Val Gln His Ser Ser
65 70 75 80

Tyr Arg Gln Arg Ala Arg Leu Leu Lys Asp Gln Leu Ser Leu Gly Asn
85 90 95

Ala Ala Leu Gln Ile Thr Asp Val Lys Leu Gln Asp Ala Gly Val Tyr
100 105 110

Arg Cys Met Ile Ser Tyr Gly Gly Ala Asp Tyr Lys Arg Ile Thr Val
115 120 125

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Lys Val Asn Ala Pro Tyr Asn Lys Ile Asn Gln Arg Ile Leu Val Val
    130                 135                 140

Asp Pro Val Thr Ser Glu His Glu Leu Thr Cys Gln Ala Glu Gly Tyr
145                 150                 155                 160

Pro Lys Ala Glu Val Ile Trp Thr Ser Ser Asp His Gln Val Leu Ser
                165                 170                 175

Gly Lys Thr Thr Thr Thr Asn Ser Lys Arg Glu Glu Lys Leu Phe Asn
            180                 185                 190

Val Thr Ser Thr Leu Arg Ile Asn Thr Thr Thr Asn Glu Ile Phe Tyr
        195                 200                 205

Cys Thr Phe Arg Arg Leu Asp Pro Glu Glu Asn His Thr Ala Glu Leu
    210                 215                 220

Val Ile Pro Glu Leu Pro Leu Ala His Pro Pro Asn Glu Arg Thr His
225                 230                 235                 240

Leu Val Ile Leu Gly Ala Ile Leu Leu Cys Leu Gly Val Ala Leu Thr
                245                 250                 255

Phe Ile Phe Arg Leu Arg Lys Gly Arg Met Met Asp Val Lys Lys Cys
            260                 265                 270

Gly Ile Gln Asp Thr Asn Ser Lys Lys Gln Ser Asp Thr His Leu Glu
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Glu Thr
    290
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<211> 25
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<212> DNA
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<212> DNA
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ctgctcgtgg tgaccgaagg ggacaacgcc accttcacct gcagcttctc caacacatcg 180

gagagcttcg tgctaaactg gtaccgcatg agccccagca accagacgga caagctggcc 240

gccttccccg aggaccgcag ccagcccggc caggactgcc gcttccgtgt cacacaactg 300

cccaacgggc gtgacttcca catgagcgtg gtcagggccc ggcgcaatga cagcggcacc 360

tacctctgtg gggccatctc cctggccccc aaggcgcaga tcaaagagag cctgcgggca 420

gagctcaggg tgacagagag aagggcagaa gtgcccacag cccaccccag cccctcaccc 480

aggtcagccg gccagttcca aaccctggtg gttggtgtcg tgggcggcct gctgggcagc 540

ctggtgctgc tagtctgggt cctggccgtc atctgctccc gggccgcacg agggacaata 600

ggagccaggc gcaccggcca gcccctgaag gaggaccccc cagccgtgcc tgtgttctct 660

gtggactatg gggagctgga tttccagtgg cgagagaaga ccccggagcc ccccgtgccc 720

tgtgtccctg agcagacgga gtatgccacc attgtctttc ctagcggaat gggcacctca 780

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gatggacact gctcttggcc cctc 864

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<222> (25)..(888)

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1 5

gtc gtc tgg gcg gtg cta caa ctg ggc tgg cgg cca gga tgg ttc tta 99
Val Val Trp Ala Val Leu Gln Leu Gly Trp Arg Pro Gly Trp Phe Leu
10 15 20 25

gac tcc cca gac agg ccc tgg aac ccc ccc acc ttc tcc cca gcc ctg 147
Asp Ser Pro Asp Arg Pro Trp Asn Pro Pro Thr Phe Ser Pro Ala Leu
30 35 40

ctc gtg gtg acc gaa ggg gac aac gcc acc ttc acc tgc agc ttc tcc 195
Leu Val Val Thr Glu Gly Asp Asn Ala Thr Phe Thr Cys Ser Phe Ser
45 50 55

aac aca tcg gag agc ttc gtg cta aac tgg tac cgc atg agc ccc agc 243
Asn Thr Ser Glu Ser Phe Val Leu Asn Trp Tyr Arg Met Ser Pro Ser
60 65 70

aac cag acg gac aag ctg gcc gcc ttc ccc gag gac cgc agc cag ccc 291
Asn Gln Thr Asp Lys Leu Ala Ala Phe Pro Glu Asp Arg Ser Gln Pro
75 80 85

ggc cag gac tgc cgc ttc cgt gtc aca caa ctg ccc aac ggg cgt gac 339
Gly Gln Asp Cys Arg Phe Arg Val Thr Gln Leu Pro Asn Gly Arg Asp
90 95 100 105

ttc cac atg agc gtg gtc agg gcc cgg cgc aat gac agc ggc acc tac 387
Phe His Met Ser Val Val Arg Ala Arg Arg Asn Asp Ser Gly Thr Tyr
110 115 120

ctc tgt ggg gcc atc tcc ctg gcc ccc aag gcg cag atc aaa gag agc 435
Leu Cys Gly Ala Ile Ser Leu Ala Pro Lys Ala Gln Ile Lys Glu Ser
125 130 135

ctg cgg gca gag ctc agg gtg aca gag aga agg gca gaa gtg ccc aca 483
Leu Arg Ala Glu Leu Arg Val Thr Glu Arg Arg Ala Glu Val Pro Thr
140 145 150

gcc cac ccc agc ccc tca ccc agg tca gcc ggc cag ttc caa acc ctg 531
Ala His Pro Ser Pro Ser Pro Arg Ser Ala Gly Gln Phe Gln Thr Leu
155 160 165

gtg gtt ggt gtc gtg ggc ggc ctg ctg ggc agc ctg gtg ctg cta gtc 579
Val Val Gly Val Val Gly Gly Leu Leu Gly Ser Leu Val Leu Leu Val
170 175 180 185

tgg gtc ctg gcc gtc atc tgc tcc cgg gcc gca cga ggg aca ata gga 627
Trp Val Leu Ala Val Ile Cys Ser Arg Ala Ala Arg Gly Thr Ile Gly
190 195 200

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gcc agg cgc acc ggc cag ccc ctg aag gag gac ccc tca gcc gtg cct   675
Ala Arg Arg Thr Gly Gln Pro Leu Lys Glu Asp Pro Ser Ala Val Pro
            205                 210                 215

gtg ttc tct gtg gac tat ggg gag ctg gat ttc cag tgg cga gag aag   723
Val Phe Ser Val Asp Tyr Gly Glu Leu Asp Phe Gln Trp Arg Glu Lys
        220                 225                 230

acc ccg gag ccc ccc gtg ccc tgt gtc cct gag cag acg gag tat gcc   771
Thr Pro Glu Pro Pro Val Pro Cys Val Pro Glu Gln Thr Glu Tyr Ala
    235                 240                 245

acc att gtc ttt cct agc gga atg ggc acc tca tcc ccc gcc cgc agg   819
Thr Ile Val Phe Pro Ser Gly Met Gly Thr Ser Ser Pro Ala Arg Arg
250                 255                 260                 265

ggc tca gct gac ggc cct cgg agt gcc cag cca ctg agg cct gag gat   867
Gly Ser Ala Asp Gly Pro Arg Ser Ala Gln Pro Leu Arg Pro Glu Asp
                270                 275                 280

gga cac tgc tct tgg ccc ctc tgaccggctt ccttggccac cagtgttctg cag  921
Gly His Cys Ser Trp Pro Leu
            285

<210> 12
<211> 288
<212> PRT
<213> Homo sapiens

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Met Gln Ile Pro Gln Ala Pro Trp Pro Val Val Trp Ala Val Leu Gln
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Leu Gly Trp Arg Pro Gly Trp Phe Leu Asp Ser Pro Asp Arg Pro Trp
             20                  25                  30

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

Asn Ala Thr Phe Thr Cys Ser Phe Ser Asn Thr Ser Glu Ser Phe Val
     50                  55                  60

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

Ala Phe Pro Glu Asp Arg Ser Gln Pro Gly Gln Asp Cys Arg Phe Arg
                 85                  90                  95

Val Thr Gln Leu Pro Asn Gly Arg Asp Phe His Met Ser Val Val Arg
            100                 105                 110

Ala Arg Arg Asn Asp Ser Gly Thr Tyr Leu Cys Gly Ala Ile Ser Leu
        115                 120                 125

Ala Pro Lys Ala Gln Ile Lys Glu Ser Leu Arg Ala Glu Leu Arg Val
    130                 135                 140
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Thr Glu Arg Arg Ala Glu Val Pro Thr Ala His Pro Ser Pro Ser Pro
145                 150                 155                 160

Arg Ser Ala Gly Gln Phe Gln Thr Leu Val Val Gly Val Val Gly Gly
                165                 170                 175

Leu Leu Gly Ser Leu Val Leu Leu Val Trp Val Leu Ala Val Ile Cys
            180                 185                 190

Ser Arg Ala Ala Arg Gly Thr Ile Gly Ala Arg Arg Thr Gly Gln Pro
        195                 200                 205

Leu Lys Glu Asp Pro Ser Ala Val Pro Val Phe Ser Val Asp Tyr Gly
    210                 215                 220

Glu Leu Asp Phe Gln Trp Arg Glu Lys Thr Pro Glu Pro Pro Val Pro
225                 230                 235                 240

Cys Val Pro Glu Gln Thr Glu Tyr Ala Thr Ile Val Phe Pro Ser Gly
                245                 250                 255

Met Gly Thr Ser Ser Pro Ala Arg Arg Gly Ser Ala Asp Gly Pro Arg
            260                 265                 270

Ser Ala Gln Pro Leu Arg Pro Glu Asp Gly His Cys Ser Trp Pro Leu
        275                 280                 285
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caccaccacc aattccaaga 20

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acgtgaccaa ggaagtgaaa gaa 23

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tgccagctct tcaacagaaa cat 23

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tggcaacgct gtcctgtggt cac 23

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gcccttgtcc ttgatctgaa ga 22

<210> 21
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1 5 10

tgt cac ttg cta cgg gcg ttt act atc acg gct cca aag gac ttg tac 100
Cys His Leu Leu Arg Ala Phe Thr Ile Thr Ala Pro Lys Asp Leu Tyr
15 20 25

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gtg gtg gag tat ggc agc aac gtc acg atg gag tgc aga ttc cct gta   148
Val Val Glu Tyr Gly Ser Asn Val Thr Met Glu Cys Arg Phe Pro Val
     30                  35                  40

gaa cgg gag ctg gac ctg ctt gcg tta gtg gtg tac tgg gaa aag gaa   196
Glu Arg Glu Leu Asp Leu Leu Ala Leu Val Val Tyr Trp Glu Lys Glu
 45                  50                  55                  60

gat gag caa gtg att cag ttt gtg gca gga gag gag gac ctt aag cct   244
Asp Glu Gln Val Ile Gln Phe Val Ala Gly Glu Glu Asp Leu Lys Pro
                 65                  70                  75

cag cac agc aac ttc agg ggg aga gcc tcg ctg cca aag gac cag ctt   292
Gln His Ser Asn Phe Arg Gly Arg Ala Ser Leu Pro Lys Asp Gln Leu
             80                  85                  90

ttg aag gga aat gct gcc ctt cag atc aca gac gtc aag ctg cag gac   340
Leu Lys Gly Asn Ala Ala Leu Gln Ile Thr Asp Val Lys Leu Gln Asp
         95                 100                 105

gca ggc gtt tac tgc tgc ata atc agc tac ggt ggt gcg gac tac aag   388
Ala Gly Val Tyr Cys Cys Ile Ile Ser Tyr Gly Gly Ala Asp Tyr Lys
    110                 115                 120

cga atc acg ctg aaa gtc aat gcc cca tac cgc aaa atc aac cag aga   436
Arg Ile Thr Leu Lys Val Asn Ala Pro Tyr Arg Lys Ile Asn Gln Arg
125                 130                 135                 140

att tcc gtg gat cca gcc act tct gag cat gaa cta ata tgt cag gcc   484
Ile Ser Val Asp Pro Ala Thr Ser Glu His Glu Leu Ile Cys Gln Ala
                145                 150                 155

gag ggt tat cca gaa gct gag gta atc tgg aca aac agt gac cac caa   532
Glu Gly Tyr Pro Glu Ala Glu Val Ile Trp Thr Asn Ser Asp His Gln
            160                 165                 170

ccc gtg agt ggg aag aga agt gtc acc act tcc cgg aca gag ggg atg   580
Pro Val Ser Gly Lys Arg Ser Val Thr Thr Ser Arg Thr Glu Gly Met
        175                 180                 185

ctt ctc aat gtg acc agc agt ctg agg gtc aac gcc aca gcg aat gat   628
Leu Leu Asn Val Thr Ser Ser Leu Arg Val Asn Ala Thr Ala Asn Asp
    190                 195                 200

gtt ttc tac tgt acg ttt tgg aga tca cag cca ggg caa aac cac aca   676
Val Phe Tyr Cys Thr Phe Trp Arg Ser Gln Pro Gly Gln Asn His Thr
205                 210                 215                 220

gcg gag ctg atc atc cca gaa ctg cct gca aca cat cct cca cag aac   724
Ala Glu Leu Ile Ile Pro Glu Leu Pro Ala Thr His Pro Pro Gln Asn
                225                 230                 235

agg act cac tgg gtg ctt ctg gga tcc atc ctg ttg ttc ctc att gta   772
Arg Thr His Trp Val Leu Leu Gly Ser Ile Leu Leu Phe Leu Ile Val
            240                 245                 250
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gtg tcc acg gtc ctc ctc ttc ttg aga aaa caa gtg aga atg cta gat   820
Val Ser Thr Val Leu Leu Phe Leu Arg Lys Gln Val Arg Met Leu Asp
        255                 260                 265

gtg gag aaa tgt ggc gtt gaa gat aca agc tca aaa aac cga aat gat   868
Val Glu Lys Cys Gly Val Glu Asp Thr Ser Ser Lys Asn Arg Asn Asp
    270                 275                 280

aca caa ttc gag gag acg taa gcagtgttga accctctgat cgtcgattgg      919
Thr Gln Phe Glu Glu Thr
285                 290

cagcttgtgg tctgtgaaag aaagggccca tgggacatga gtccaaagac tcaagatgga 979

acctgaggga gagaaccaag aaagtgttgg gagaggagcc tggaacaacg gacatttttt 1039

ccagggagac actgctaagc aagttgccca tcagtcgtct tgggaaatgg attgagggtt 1099

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aatcaacagg gaattgtctc agaatggtcc ccaagacaaa gaagaagaaa aacaccaaac 2359
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gaacccagaa ctttcttaca gttttccttg ttctgtcaca tgtcaagact gaaggaacag 3259

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ggaacccccct ctgttacctg ttcacacaag cttattgatg agtctcatgt taatgtcttg 3379

tttgtatgaa gtttaagaaa atatcgggtt gggcaacaca ttctatttat tcattttatt 3439

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tgtataacca taaattttat tttgcatcag attgtcaatg tattgcatta atttaataaa 3559

tatttttatt tattaaaaaa aaaaaaaaaa aaaa 3593

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<213> Mus musculus

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Arg Ala Phe Thr Ile Thr Ala Pro Lys Asp Leu Tyr Val Val Glu Tyr
20 25 30

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

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Asp Leu Leu Ala Leu Val Val Tyr Trp Glu Lys Glu Asp Glu Gln Val
     50                  55                  60

Ile Gln Phe Val Ala Gly Glu Glu Asp Leu Lys Pro Gln His Ser Asn
 65                  70                  75                  80

Phe Arg Gly Arg Ala Ser Leu Pro Lys Asp Gln Leu Leu Lys Gly Asn
                85                  90                  95

Ala Ala Leu Gln Ile Thr Asp Val Lys Leu Gln Asp Ala Gly Val Tyr
            100                 105                 110

Cys Cys Ile Ile Ser Tyr Gly Gly Ala Asp Tyr Lys Arg Ile Thr Leu
        115                 120                 125

Lys Val Asn Ala Pro Tyr Arg Lys Ile Asn Gln Arg Ile Ser Val Asp
    130                 135                 140

Pro Ala Thr Ser Glu His Glu Leu Ile Cys Gln Ala Glu Gly Tyr Pro
145                 150                 155                 160

Glu Ala Glu Val Ile Trp Thr Asn Ser Asp His Gln Pro Val Ser Gly
                165                 170                 175

Lys Arg Ser Val Thr Thr Ser Arg Thr Glu Gly Met Leu Leu Asn Val
            180                 185                 190

Thr Ser Ser Leu Arg Val Asn Ala Thr Ala Asn Asp Val Phe Tyr Cys
        195                 200                 205

Thr Phe Trp Arg Ser Gln Pro Gly Gln Asn His Thr Ala Glu Leu Ile
    210                 215                 220

Ile Pro Glu Leu Pro Ala Thr His Pro Pro Gln Asn Arg Thr His Trp
225                 230                 235                 240

Val Leu Leu Gly Ser Ile Leu Leu Phe Leu Ile Val Val Ser Thr Val
                245                 250                 255

Leu Leu Phe Leu Arg Lys Gln Val Arg Met Leu Asp Val Glu Lys Cys
            260                 265                 270

Gly Val Glu Asp Thr Ser Ser Lys Asn Arg Asn Asp Thr Gln Phe Glu
        275                 280                 285

Glu Thr
    290
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<210> 24
<211> 120
<212> PRT
<213> Homo sapiens

<400> 24

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Leu Val Val Gly Val Val Gly Gly Leu Leu Gly Ser Leu Val Leu Leu
 1               5                  10                  15
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Val Trp Val Leu Ala Val Ile Cys Ser Arg Ala Ala Arg Gly Thr Ile
            20                  25                  30
Gly Ala Arg Arg Thr Gly Gln Pro Leu Lys Glu Asp Pro Ser Ala Val
        35                  40                  45
Pro Val Phe Ser Val Asp Tyr Gly Glu Leu Asp Phe Gln Trp Arg Glu
    50                  55                  60
Lys Thr Pro Glu Pro Pro Val Pro Cys Val Pro Glu Gln Thr Glu Tyr
65                  70                  75                  80
Ala Thr Ile Val Phe Pro Ser Gly Met Gly Thr Ser Ser Pro Ala Arg
                85                  90                  95
Arg Gly Ser Ala Asp Gly Pro Arg Ser Ala Gln Pro Leu Arg Pro Glu
            100                 105                 110
Asp Gly His Cys Ser Trp Pro Leu
        115                 120
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<210> 25
<211> 120
<212> PRT
<213> Mus musculus

<400> 25

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Met Val Ile Gly Ile Met Ser Ala Leu Val Gly Ile Pro Val Leu Leu
 1               5                  10                  15
Leu Leu Ala Trp Ala Leu Ala Val Phe Cys Ser Thr Ser Met Ser Glu
            20                  25                  30
Ala Arg Gly Ala Gly Ser Lys Asp Asp Thr Leu Lys Glu Glu Pro Ser
        35                  40                  45
Ala Ala Pro Val Pro Ser Val Ala Tyr Glu Glu Leu Asp Phe Gln Gly
    50                  55                  60
Arg Glu Lys Thr Pro Glu Leu Pro Thr Ala Cys Val His Thr Glu Tyr
65                  70                  75                  80
Ala Thr Ile Val Phe Thr Glu Gly Leu Gly Ala Ser Ala Met Gly Arg
                85                  90                  95
Arg Gly Ser Ala Asp Gly Leu Gln Gly Pro Arg Pro Pro Arg His Glu
            100                 105                 110
Asp Gly His Cys Ser Trp Pro Leu
        115                 120
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<400> 26

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Lys Glu Asp Pro Ser Ala Val Pro Val Phe Ser Val Asp Tyr Gly Glu
 1               5                  10                  15
Leu Asp Phe Gln Trp Arg Glu
            20
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Lys Glu Asp Pro Ser Ala Val Pro Val Phe Ser Val Asp Phe Gly Glu
1 5 10 15

Leu Asp Phe Gln Trp Arg Glu
20

<210> 28
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<220>
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Lys Glu Asp Pro Ser Ala Val Pro Val Phe Ser Val Asp Tyr Gly Glu
1 5 10 15
Leu Asp Phe Gln Trp Arg Glu
20

<210> 29
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<213> Artificial sequence

<220>
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<400> 29
Lys Thr Pro Glu Pro Pro Val Pro Ser Val Pro Glu Gln Thr Glu Tyr
1 5 10 15
Ala Thr Ile Val Phe Pro Ser Gly Met Gly Thr Ser Ser
20 25

<210> 30
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<212> PRT
<213> Artificial sequence

<220>
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Lys Thr Pro Glu Pro Pro Val Pro Ser Val Pro Glu Gln Thr Glu Phe
1 5 10 15
Ala Thr Ile Val Phe Pro Ser Gly Met Gly Thr Ser Ser
20 25

<210> 31
<211> 29
<212> PRT
<213> Artificial sequence

<220>
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Lys Thr Pro Glu Pro Pro Val Pro Ser Val Pro Glu Gln Thr Glu Tyr
1 5 10 15
Ala Thr Ile Val Phe Pro Ser Gly Met Gly Thr Ser Ser
20 25

<210> 32
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<213> Artificial sequence

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1 5 10 15
Pro Ser Ala Val Pro Val Phe Ser
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<210> 33
<211> 24
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<213> Artificial sequence

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Arg Gly Thr Ile Gly Ala Arg Arg Thr Gly Gln Pro Leu Asp Glu Asp
1 5 10 15
Pro Ser Ala Val Pro Val Phe Ser
20

<210> 34
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/10371

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/02
US CL : 435/4
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)
U.S. : 435/4

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FREEMAN et al. Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. J. Exp. Med. 02 October 2000, Vol 192. No. 7, pages 1027-1034, see entire document.	1-9
Y	LATCHMAN et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nature Immunol. March 2001, Vol 2. No. 3, pages 261-268, see entire document.	1-9
Y	ADACHI et al. CD72 Negatively Regulates Signaling Through the Antigen Receptor of B Cells. J. Immunol. 01 February 2000, Vol. 164., pages 1223-1229, see entire document.	1-9

☐ Further documents are listed in the continuation of Box C. ☐ 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

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"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

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Continuation of B. FIELDS SEARCHED Item 3:
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(54) Title: AGENTS THAT MODULATE IMMUNE CELL ACTIVATION AND METHODS OF USE THEREOF

(57) Abstract: Disclosed are methods for identifying an agent that modulates an immune response. One such method comprises screening for agents which inhibit the interaction between a PD-1 ligand and a PD-1 polypeptide, and determining whether the agents inhibit the interaction between a PD-1 ligand and a B7 polypeptide, to identify an agent that inhibits PD-1 ligand and PD-1 polypeptide interaction, while not inhibiting the interaction between a PD-1 ligand and a B7 polypeptide, as an agent that modulates an immune response. Another such method comprises screening for agents which inhibit the interaction between a PD-1 ligand and a B7 polypeptide, and determining whether the agents inhibit the interaction between a PD-1 ligand and a PD-1 polypeptide, to identify an agent that inhibits the PD-1 ligand and B7 polypeptide interaction, which does not inhibit the interaction between a PD-1 ligand and a PD-1 polypeptide, as an agent that modulates the immune response. Further disclosed is a method for inhibiting the interaction between a B7 polypeptide and a PD-1 ligand, the method comprising contacting an immune cell bearing a PD-1 ligand, or an immune cell bearing a B7 polypeptide, with an agent that inhibits the interaction between the PD-1 ligand and the B7 polypeptide. Such agents may be an anti- PD-1 ligand antibody or a small molecule. Also disclosed is a method for modulating an immune response. The method comprises contacting an immune cell bearing the PD-1 ligand, or an immune cell bearing the PD-1 polypeptide, with an agent that inhibits interactions between the PD-1 ligand and the PD-1 polypeptide, without inhibiting interactions between the PD-1 ligand and a B7 polypeptide, to thereby modulate an immune response. The agent may be an anti-PD-1 ligand antibody or a small molecule.

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AGENTS THAT MODULATE IMMUNE CELL ACTIVATION AND METHODS OF USE THEREOF

Government Funding

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Related Applications

This application claims priority to U.S. Provisional Application 60/337,817, filed November 13, 2001, the contents of which are incorporated herein by reference.

Background of the Invention

In order for T cells to respond to foreign proteins, two signals must be provided by antigen-presenting cells (APCs) to resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165:302-319; Mueller, D. L. *et al.* (1990) *J. Immunol.* 144:3701-3709). The first signal, which confers specificity to the immune response, is transduced via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Lenschow *et al.* (1996) *Annu. Rev. Immunol.* 14:233). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface polypeptides expressed by APCs (Jenkins, M. K. *et al.* (1988) *J. Immunol.* 140:3324-3330; Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 173:721-730; Gimmi, C. D., *et al.* 1991 *Proc. Natl. Acad. Sci. USA* 88:6575-6579; Young, J. W. *et al.* (1992) *J. Clin. Invest.* 90:229-237; Koulova, L. *et al.* (1991) *J. Exp. Med.* 173:759-762; Reiser, H. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:271-275; van-Seventer, G. A. *et al.* (1990) *J. Immunol.* 144:4579-4586; LaSalle, J. M. *et al.* (1991) *J. Immunol.* 147:774-80; Dustin, M. I. *et al.* (1989) *J. Exp. Med.* 169:503; Armitage, R. J. *et al.* (1992) *Nature* 357:80-82; Liu, Y. *et al.* (1992) *J. Exp. Med.* 175:437-445).

The CD80 (B7-1) and CD86 (B7-2) proteins, expressed on APCs, are critical costimulatory polypeptides (Freeman *et al.* (1991) *J. Exp. Med.* 174:625; Freeman *et al.* (1989) *J. Immunol.* 143:2714; Azuma *et al.* (1993) *Nature* 366:76; Freeman *et al.* (1993) *Science* 262:909). B7-2 appears to play a predominant role during primary immune responses, while B7-1, which is upregulated later in the course of an immune response, may be important in prolonging primary T cell responses or costimulating secondary T cell responses (Bluestone (1995) *Immunity* 2:555).

One receptor to which B7-1 and B7-2 bind, CD28, is constitutively expressed on resting T cells and increases in expression after activation. After signaling through the T cell receptor, ligation of CD28 and transduction of a costimulatory signal induces T cells to proliferate and secrete IL-2 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 173:721-730; Gimmi, C. D. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:6575-6579; June, C. H. *et al.* (1990) *Immunol. Today.* 11:211-6; Harding, F. A. *et al.* (1992) *Nature* 356:607-609). A second receptor, termed CTLA4 (CD152) is homologous to CD28 but is not expressed on resting T cells and appears following T cell activation (Brunet, J. F. *et al.* (1987) *Nature* 328:267-270). CTLA4 appears to be critical in negative regulation of T cell responses (Waterhouse *et al.* (1995) *Science* 270:985). Blockade of CTLA4 has been found to remove inhibitory signals, while aggregation of CTLA4 has been found to provide inhibitory signals that downregulate T cell responses (Allison and Krummel (1995) *Science* 270:932). The B7 polypeptides have a higher affinity for CTLA4 than for CD28 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 174:561-569) and B7-1 and B7-2 have been found to bind to distinct regions of the CTLA4 polypeptide and have different kinetics of binding to CTLA4 (Linsley *et al.* (1994) *Immunity* 1:793). A new polypeptide related to CD28 and CTLA4, ICOS, has been identified and seems to be important in IL-10 production (Hutloff *et al.* (1999) *Nature* 397:263; WO 98/38216), as has its ligand, which is a new B7 family member (Aicher A. *et al.* (2000) *J. Immunol.* 164:4689-96; Mages H.W. *et al.* (2000) *Eur. J. Immunol.* 30:1040-7; Brodie D. *et al.* (2000) *Curr. Biol.* 10:333-6; Ling V. *et al.* (2000) *J. Immunol.* 164:1653-7; Yoshinaga S.K. *et al.* (1999) *Nature* 402:827-32). If T cells are only stimulated through the T cell receptor, without receiving an additional costimulatory signal, they become nonresponsive, anergic, or die, resulting in downmodulation of the immune response.

The importance of the B7:CD28/CTLA4 costimulatory pathway has been demonstrated *in vitro* and in several *in vivo* model systems. Blockade of this costimulatory pathway results in the development of antigen specific tolerance in murine and human systems (Harding, F. A. *et al.* (1992) *Nature* 356:607-609; Lenschow, D. J. *et al.* (1992) *Science* 257:789-792; Turka, L. A. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:11102-11105; Gimmi, C. D. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6586-6590; Boussiotis, V. *et al.* (1993) *J. Exp. Med.* 178:1753-1763). Conversely, expression of B7 by B7 negative murine tumor cells induces T-cell mediated specific immunity accompanied by tumor rejection and long lasting protection to tumor challenge (Chen, L. *et al.* (1992) *Cell* 71:1093-1102; Townsend, S. E. and Allison, J. P. (1993) *Science* 259:368-370; Baskar, S. *et al.* (1993) *Proc. Natl. Acad. Sci.* 90:5687-5690.).

Inhibitory receptors that bind to costimulatory polypeptides have also been identified on immune cells. Activation of CTLA4, for example, transmits a negative signal to a T cell. Engagement of CTLA4 inhibits IL-2 production and can induce cell cycle arrest (Krummel and Allison (1996) *J. Exp. Med.* 183:2533). In addition, mice that lack CTLA4 develop lymphoproliferative disease (Tivol *et al.* (1995) *Immunity* 3:541; Waterhouse *et al.* (1995) *Science* 270:985). The blockade of CTLA4 with antibodies may remove an inhibitory signal, whereas aggregation of CTLA4 with antibody transmits an inhibitory signal. Therefore, depending upon the receptor to which a costimulatory polypeptide binds (*i.e.*, a costimulatory receptor such as CD28 or an inhibitory receptor such as CTLA4), certain B7 polypeptides can promote T cell costimulation or inhibition.

PD-1 has been identified as a receptor which binds to PD-L1 and PD-L2. PD-1 is a member of the immunoglobulin gene superfamily. PD-1 (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; U.S. Patent 5,698,520) has an extracellular region containing immunoglobulin superfamily domain, a transmembrane domain, and an intracellular region including an immunoreceptor tyrosine-based inhibitory motif (ITIM). These features also define a larger family of polypeptides, called the immunoinhibitory receptors, which also includes gp49B, PIR-B, and the killer inhibitory receptors (KIRs) (Vivier and Daeron (1997) *Immunol. Today* 18:286). It is often assumed that the tyrosyl phosphorylated ITIM motif of these

receptors interacts with SH2-domain containing phosphatases, which leads to inhibitory signals. A subset of these immunoinhibitory receptors bind to MHC polypeptides, for example the KIRs, and CTLA4 bind to B7-1 and B7-2. It has been proposed that there is a phylogenetic relationship between the MHC and B7 genes (Henry *et al.* (1999) *Immunol. Today* 20(6):285-8).

The nucleotide and amino acid sequence of PD-1 is published in Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; and U.S. Patent 5,698,520. PD-1 was previously identified using a subtraction cloning based approach to select for proteins involved in apoptotic cell death. PD-1 is identified herein as a member of the CD28/CTLA-4 family of polypeptides. Like CTLA4, PD-1 is rapidly induced on the surface of T-cells in response to anti-CD3 (Agata *et al.* (1996) *Int. Immunol.* 8:765). In contrast to CTLA4, however, PD-1 is also induced on the surface of B-cells (in response to anti-IgM). PD-1 is also expressed on a subset of thymocytes and myeloid cells (Agata *et al.* (1996) *supra*; Nishimura *et al.* (1996) *Int. Immunol.* 8:773).

Two types of human PD-1 ligand polypeptides have been identified. PD-1 ligand proteins comprise a signal sequence, and an IgV domain, an IgC domain, a transmembrane domain, and a short cytoplasmic tail. Both PD-L1 (See Freeman et al. (2000) J. Exp. Med. 192:1027 for sequence data) and PD-L2 (See Latchman et al. (2001) Nat. Immunol. 2:261 for sequence data) are members of the B7 family of polypeptides. Both PD-L1 and PD-L2 are expressed in placenta, spleen, lymph nodes, thymus, and heart. Only PD-L2 is expressed in pancreas, lung and liver while only PD-L1 is expressed in fetal liver. Both PD-1 ligands are upregulated on activated monocytes and dendritic cells.

The fact that PD-1 binds to PD-L1 and PD-L2 places PD-1 in a family of inhibitory receptors with CTLA4. While engagement of a costimulatory receptor results in a costimulatory signal in an immune cell, engagement of an inhibitory receptor, *e.g.*, CTLA4 or PD-1 (for example by crosslinking or by aggregation), leads to the transmission of an inhibitory signal in an immune cell, resulting in downmodulation of immune cell responses and/or in immune cell anergy. While transmission of an inhibitory signal leads to downmodulation in immune cell responses (and a resulting

downmodulation in the overall immune response), the prevention of an inhibitory signal (*e.g.*, by using a non-activating antibody against PD-1) in immune cells leads to upmodulation of immune cell responses (and a resulting upmodulation of an immune response).

The identification of additional agents useful in modulation of an immune response would be of tremendous benefit.

Summary of the Invention

The present invention is based, at least in part, on the discovery that PD-1 ligand, in addition to binding PD-1, binds to B7-1. PD-1 transmits a negative signal to immune cells, similar to CTLA4. PD-1 ligand polypeptides are expressed on the surface of antigen presenting cells and can provide a costimulatory signal to immune cells or can transmit downmodulatory signals to immune cells, depending upon the polypeptide to which they bind. For example, PD-1 ligand binding to PD-1 transmits a negative signal, whereas PD-1 ligand binding to a B7 polypeptide does not. Thus, modulation of the interaction between PD-1 and PD-1 ligand or between PD-1 ligand and a B7 polypeptide results in modulation of the immune response.

Thus, one aspect of the invention relates to a method for inhibiting the interaction between a B7 polypeptide and a PD-1 ligand. The method comprises contacting an immune cell bearing a PD-1 ligand, or an immune cell bearing a B7 polypeptide, with an agent that inhibits the interaction between the PD-1 ligand and the B7 polypeptide. In one embodiment, the interaction between a B7 polypeptide and a PD-1 ligand polypeptide prevents PD-1 ligand from binding to PD-1 and, thus, inhibits delivery of an inhibitory immune signal. In one embodiment, agents which block the interaction between PD-1 and PD-1 ligand can prevent inhibitory signaling. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand allow the PD-1 ligand to bind PD-1, and provide an inhibitory signal to an immune cell, thus enhancing signaling inhibition.

PD-L1, by binding to a B7 polypeptide, also reduces the B7 polypeptide binding to the inhibitory receptor CTLA4. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand polypeptide allow the B7 polypeptide to bind CTLA4

and provide an inhibitory signal to an immune cell, and agents that promote the binding of the B7 polypeptide to the PD-1 ligand inhibit binding of the B7 polypeptide to CTLA4, and thus inhibit a negative signal.

In another embodiment, a PD-1 ligand, by binding to a B7 polypeptide, also reduces the B7 polypeptide binding to the costimulatory receptor CD28. Thus, in one embodiment, agents that block the binding of a the B7 polypeptide to the PD-1 ligand polypeptide allow the B7 polypeptide to bind CD28 and provide a costimulatory signal to an immune cell and agents that promote the binding of the B7 polypeptide to the PD-1 ligand inhibit the binding of the B7 polypeptide to CD28, and thereby inhibit a costimulatory signal.

Accordingly, one aspect of the invention relates to a method for modulating (e.g., inhibiting or stimulating) an immune response, comprising contacting an immune cell bearing a PD-1 ligand or an immune cell bearing a B7 polypeptide with an agent that modulates (e.g., inhibits or stimulates) the interaction between the PD-1 ligand and the B7 polypeptide, to thereby modulate(e.g., inhibit or stimulate) the immune response.

In one embodiment, the agent is an anti-PD-1 ligand antibody.

In one embodiment, the agent is an anti-B7-1 antibody.

In one embodiment, the agent is a small molecule.

In one embodiment, the agent is a peptide.

In one embodiment, the agent is a fusion protein.

Another aspect of the invention relates to a method for modulating an immune response comprising, contacting an immune cell bearing a PD-1 ligand, or an immune cell bearing PD-1, with an agent that inhibits the interaction between the PD-1 ligand and PD-1, without inhibiting an interaction between a PD-1 ligand and a B7 polypeptide, to thereby modulate the immune response.

In one embodiment, the agent is an anti-PD-1 ligand antibody, an anti-PD-1 antibody, a peptide, or a small molecule, wherein the agent inhibits the interaction between PD-1 and a PD-1 ligand, and does not inhibit the interaction between the PD-1 ligand and a B7 polypeptide.

In one embodiment, the PD-1 ligand is PD-L1.

In one embodiment, the PD-1 ligand is PD-L2.

In one embodiment, the B7 polypeptide is B7-1

Another aspect of the invention relates to a method for modulating an immune response by inhibiting the interaction between a B7 polypeptide on a first immune cell and CTLA4 on a second immune cell, comprising contacting said first or second immune cell with a PD-1 ligand, to thereby modulate the immune response.

Another aspect of the invention relates to a method for modulating an immune response comprising contacting an immune cell bearing a B7 polypeptide, or an immune cell bearing a CTLA4 polypeptide with an agent that inhibits the interaction between a B7 polypeptide and a CTLA4 polypeptide, and does not inhibit the interaction between a B7 polypeptide and the PD-1 ligand, to thereby modulate an immune response.

Yet another aspect of the invention relates to a method of modulating an immune response by inhibiting the interaction between a B7 polypeptide on a first immune cell and CD28 on a second immune cell, comprising contacting said first or second immune cell with a PD-1 ligand, to thereby modulate an immune response.

Another aspect of the invention relates to a method for modulating an immune response comprising contacting an immune cell bearing a B7 polypeptide, or an immune cell bearing CD28, with an agent that inhibits the interaction between the B7 polypeptide and a CD28, and does not inhibit the interaction between the B7 polypeptide and the PD-1 ligand, to thereby modulate the immune response.

Another aspect of the invention relates to a method for identifying an agent that modulates an immune response, comprising, screening for agents which inhibit the interaction between a PD-1 ligand and a PD-1 polypeptide, and determining whether the agents identified in the screen inhibit the interaction between a PD-1 ligand and a B7 polypeptide, wherein an agent identified in the screen which is determined not to affect (e.g., inhibit) the interaction between a PD-1 ligand and a B7 polypeptide, is identified as an agent that modulates the immune response.

Another aspect of the invention relates to a method for identifying an agent that modulates an immune response, comprising, screening for agents which inhibit the interaction between a B7 polypeptide and CTL4, and determining whether the agents

identified in the screen inhibit the interaction between a PD-1 ligand and the B7 polypeptide, wherein an agent identified in the screen which is determined not to affect (e.g., inhibit) the interaction between the B7 polypeptide and CTLA4, but does not inhibit the interaction between the B7 polypeptide and the PD-1 ligand, is identified as an agent that modulates the immune response.

Yet another aspect of the invention relates to a method for identifying an agent that modulates an immune response comprising, screening for agents which inhibit the interaction between a PD-1 ligand and a B7 polypeptide, and determining whether the agents identified in the screen inhibit the interaction between the PD-1 ligand and the B7 polypeptide, wherein an agent identified in the screen which is determined not to affect (e.g., inhibit) the interaction between a PD-1 ligand and a PD-1 polypeptide, is identified as an agent that modulates the immune response.

Brief Description of the Drawings

Figure 1 is a bar graph of data representing the binding of cells expressing PD-L1 to B7-1 Ig, in the presence of absence of 1G10 (anti-B7-1) antibody, 10F.9G2 (anti-PD-1) antibody, 10F.2H11 (anti-PD-1) antibody, and PD-1 Ig polypeptide.

Figure 2 is a bar graph of data representing the binding of cells expressing PD-L1 to PD-1 Ig, in the presence and absence of the 10F.9G2 antibody. The data indicates that the 10F.9G2 antibody inhibits this interaction.

Figure 3 is a bar graph of data representing the binding of cells expressing PD-L1 to B7-1 and B7-2. illustrates the compares the ability of B7-1 and B7-2 to bind to cells expressing PD-L1.

Figure 4 is a graphical representation of data generated by FACS analysis, which indicates murine PD-1 binding to murine PD-L1 and PD-L2.

Detailed Description of the Invention

I. Definitions

As used herein, the term "modulate" includes up-regulation and down-regulation, e.g., enhancing or inhibiting a response.

As used herein, the term "inhibit" includes the decrease, limitation, or blockage, of, for example a particular action, function, or interaction.

As used herein, the term "immune cell" refers to cells that play a role in the immune response. Immune cells are of hematopoietic origin, and include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

As used herein, the term "T cell" includes CD4+ T cells and CD8+ T cells. The term T cell also includes both T helper 1 type T cells and T helper 2 type T cells. The term "antigen presenting cell" includes professional antigen presenting cells (*e.g.*, B lymphocytes, monocytes, dendritic cells, Langerhans cells) as well as other antigen presenting cells (*e.g.*, keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes).

As used herein, the term "immune response" includes T cell mediated and/or B cell mediated immune responses that are influenced by modulation of T cell costimulation. Exemplary immune responses include T cell responses, *e.g.*, cytokine production, and cellular cytotoxicity. In addition, the term immune response includes immune responses that are indirectly effected by T cell activation, *e.g.*, antibody production (humoral responses) and activation of cytokine responsive cells, *e.g.*, macrophages.

As used herein, the term "costimulatory receptor" includes receptors which transmit a costimulatory signal to a immune cell, *e.g.*, CD28. As used herein, the term "inhibitory receptors" includes receptors which transmit a negative signal to an immune cell (*e.g.*, CTLA4 or PD-1). An inhibitory signal as transduced by an inhibitory receptor can occur even if a costimulatory receptor (such as CD28) is not present on the immune cell and, thus, is not simply a function of competition between inhibitory receptors and costimulatory receptors for binding of costimulatory polypeptides (Fallarino *et al.* (1998) *J. Exp. Med.* 188:205). Transmission of an inhibitory signal to an immune cell can result in unresponsiveness or anergy or programmed cell death in the immune cell. Preferably transmission of an inhibitory signal operates through a mechanism that does not involve apoptosis. As used herein the term "apoptosis" includes programmed cell death which can be characterized using techniques which are known in the art.

Apoptotic cell death can be characterized, *e.g.*, by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation. Cells undergoing apoptosis also display a characteristic pattern of internucleosomal DNA cleavage.

Depending upon the form of the PD-1 ligand polypeptide that binds to a receptor, a signal can either be transmitted (*e.g.*, by a multivalent form of a PD-1 ligand polypeptide or a form of a PD-1 ligand polypeptide that binds to Fc receptors that results in crosslinking of receptor) or a signal can be inhibited (*e.g.*, by a soluble, monovalent form of a PD-1 ligand polypeptide or a form of PD-1 ligand polypeptide lacking Fc receptors), for instance by competing with activating forms of PD-1 ligand polypeptides for binding to the receptor. However, there are instances in which a soluble polypeptide can be stimulatory. The effects of a modulatory agent can be easily demonstrated using routine screening assays as described herein.

As used herein, the term "costimulate," as used with reference to activated immune cells, includes the ability of a costimulatory polypeptide to provide a second, non-activating receptor mediated signal (a "costimulatory signal") that induces proliferation or effector function. For example, a costimulatory signal can result in cytokine secretion, *e.g.*, in a T cell that has received a T cell-receptor-mediated signal. Immune cells that have received a cell-receptor mediated signal, *e.g.*, via an activating receptor are referred to herein as "activated immune cells."

As used herein, the term "activating receptor" includes immune cell receptors that bind antigen, complexed antigen (*e.g.*, in the context of MHC polypeptides), or bind to antibodies. Such activating receptors include T cell receptors (TCR), B cell receptors (BCR), cytokine receptors, LPS receptors, complement receptors, and Fc receptors.

T cell receptors are present on T cells and are associated with CD3 polypeptides. T cell receptors are stimulated by antigen in the context of MHC polypeptides (as well as by polyclonal T cell activating reagents). T cell activation via the TCR results in numerous changes, *e.g.*, protein phosphorylation, membrane lipid changes, ion fluxes, cyclic nucleotide alterations, RNA transcription changes, protein synthesis changes, and cell volume changes.

B cell receptors are present on B cells. B cell antigen receptors are a complex between membrane Ig (mIg) and other transmembrane polypeptides (*e.g.*, Igα and Igβ). The signal transduction function of mIg is triggered by crosslinking of receptor polypeptides by oligomeric or multimeric antigens. B cells can also be activated by anti-immunoglobulin antibodies. Upon BCR activation, numerous changes occur in B cells, including tyrosine phosphorylation.

Fc receptors are found on many cells which participate in immune responses. Fc receptors (FcRs) are cell surface receptors for the Fc portion of immunoglobulin polypeptides (Igs). Among the human FcRs that have been identified so far are those which recognize IgG (designated Fcγ R), IgE (Fcε R1), IgA (Fcα), and polymerized IgM/A (Fcμα R). FcRs are found in the following cell types: Fcε R I (mast cells), Fcε R.II (many leukocytes), Fcα R (neutrophils), and Fcμα R (glandular epithelium, hepatocytes) (Hogg, N. (1988) *Immunol. Today* 9:185-86). The widely studied FcγRs are central in cellular immune defenses, and are responsible for stimulating the release of mediators of inflammation and hydrolytic enzymes involved in the pathogenesis of autoimmune disease (Unkeless, J. C. *et al.* (1988) *Annu. Rev. Immunol.* 6:251-81). The FcγRs provide a crucial link between effector cells and the lymphocytes that secrete Ig, since the macrophage/monocyte, polymorphonuclear leukocyte, and natural killer (NK) cell FcγRs confer an element of specific recognition mediated by IgG. Human leukocytes have at least three different receptors for IgG: h Fcγ RI (found on monocytes/macrophages), hFcγ RII (on monocytes, neutrophils, eosinophils, platelets, possibly B cells, and the K562 cell line), and Fcγ III (on NK cells, neutrophils, eosinophils, and macrophages).

With respect to T cells, transmission of a costimulatory signal to a T cell involves a signaling pathway that is not inhibited by cyclosporine A. In addition, a costimulatory signal can induce cytokine secretion (*e.g.*, IL-2 and/or IL-10) in a T cell and/or can prevent the induction of unresponsiveness to antigen, the induction of anergy, or the induction of cell death in the T cell.

As used herein, the term "inhibitory signal" refers to a signal transmitted via an inhibitory receptor (*e.g.*, CTLA4 or PD-1) for a polypeptide on a immune cell. Such a signal antagonizes a signal via an activating receptor (*e.g.*, via a TCR, CD3, BCR, or Fc polypeptide) and can result in, *e.g.*, inhibition of second messenger generation; an inhibition of proliferation; an inhibition of effector function in the immune cell, *e.g.*, reduced phagocytosis, reduced antibody production, reduced cellular cytotoxicity, the failure of the immune cell to produce mediators, (such as cytokines (*e.g.*, IL-2) and/or mediators of allergic responses); or the development of anergy.

As used herein, the term "unresponsiveness" includes refractivity of immune cells to stimulation, *e.g.*, stimulation via an activating receptor or a cytokine. Unresponsiveness can occur, *e.g.*, because of exposure to immunosuppressants or exposure to high doses of antigen. As used herein, the term "anergy" or "tolerance" includes refractivity to activating receptor-mediated stimulation. Such refractivity is generally antigen-specific and persists after exposure to the tolerizing antigen has ceased. For example, anergy in T cells (as opposed to unresponsiveness) is characterized by lack of cytokine production, *e.g.*, IL-2. T cell anergy occurs when T cells are exposed to antigen and receive a first signal (a T cell receptor or CD-3 mediated signal) in the absence of a second signal (a costimulatory signal). Under these conditions, reexposure of the cells to the same antigen (even if reexposure occurs in the presence of a costimulatory polypeptide) results in failure to produce cytokines and, thus, failure to proliferate. Anergic T cells can, however, proliferate if cultured with cytokines (*e.g.*, IL-2). For example, T cell anergy can also be observed by the lack of IL-2 production by T lymphocytes as measured by ELISA or by a proliferation assay using an indicator cell line. Alternatively, a reporter gene construct can be used. For example, anergic T cells fail to initiate IL-2 gene transcription induced by a heterologous promoter under the control of the 5' IL-2 gene enhancer or by a multimer of the AP1 sequence that can be found within the enhancer (Kang *et al.* (1992) *Science* 257:1134).

The PD-1 ligands and B7 polypeptides comprise a family of polypeptides having certain conserved structural and functional features. Similarly, the PD-1 proteins are members of a family of polypeptides having conserved structural and functional

features. The term "family" when used to refer to proteins or nucleic acid molecules, is intended to mean two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide sequence homology, as defined herein. Such family members can be naturally or non-naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or alternatively, can contain homologues of non-human origin. Members of a family may also have common functional characteristics. The PD-1 ligand polypeptides described herein are members of the B7 family of polypeptides. The term "B7 family" or "B7 polypeptides" as used herein includes costimulatory polypeptides that share sequence homology with B7 polypeptides, *e.g.*, with B7-1, B7-2, B7h (Swallow *et al.* (1999) *Immunity* 11:423), and/or PD-1 ligands (e.g., PD-L1 or PD-L2). For example, human B7-1 and B7-2 share approximately 26% amino acid sequence identity when compared using the BLAST program at NCBI with the default parameters (Blosum62 matrix with gap penalties set at existence 11 and extension 1 (See the NCBI website). The term B7 family also includes variants of these polypeptides which are capable of modulating immune cell function.

Preferred B7 polypeptides are capable of providing costimulatory or inhibitory signals to immune cells to thereby promote or inhibit immune cell responses. For example, B7 family members that bind to costimulatory receptors increase T cell activation and proliferation, while B7 family members that bind to inhibitory receptors reduce costimulation. Moreover, the same B7 family member may increase or decrease T cell costimulation. For example, when bound to a costimulatory receptor, PD-1 ligand can induce costimulation of immune cells or can inhibit immune cell costimulation, e.g., when present in soluble form. When bound to an inhibitory receptor, PD-1 ligand polypeptides can transmit an inhibitory signal to an immune cell. Preferred B7 family members include B7-1, B7-2, B7h, PD-L1 or PD-L2 and soluble fragments or derivatives thereof. In one embodiment, B7 family members bind to one or more receptors on an immune cell, *e.g.*, CTLA4, CD28, ICOS, PD-1 and/or other receptors, and, depending on the receptor, have the ability to transmit an inhibitory signal or a costimulatory signal to an immune cell, preferably a T cell.

PD-1 polypeptides are inhibitory receptors capable of transmitting an inhibitory signal to an immune cell to thereby inhibit immune cell effector function, or are capable of promoting costimulation (*e.g.*, by competitive inhibition) of immune cells, *e.g.*, when present in soluble, monomeric form. Preferred PD-1 family members share sequence identity with PD-1 and bind to one or more B7 family members, *e.g.*, B7-1, B7-2, PD-1 ligand, and/or other polypeptides on antigen presenting cells.

As used herein, the term "activity," when used with respect to a polypeptide, e.g., a PD-1 ligand, PD-1, CTLA4, CD28, or a B7 polypeptide includes activities which are inherent in the structure of the protein. For example, with regard to PD-1 ligand, the term "activity" includes the ability to modulate immune cell costimulation (*e.g.* by modulating a costimulatory signal in an activated immune cell) or to modulate inhibition by modulating an inhibitory signal in an immune cell (*e.g.*, by engaging a natural receptor on an immune cell) Those of skill in the art will recognize that when an activating form of the PD-1 ligand polypeptide binds to a costimulatory receptor, a costimulatory signal is generated in the immune cell. When an activating form of the PD-1 ligand polypeptide binds to an inhibitory receptor, an inhibitory signal is generated in the immune cell. When a PD-1 ligand binds to a B7 polypeptide, a costimulatory signal may be generated because the inhibitory signal of the PD-1 ligand binding to PD-1 is inhibited.

As used herein, the term "PD-1 ligand" includes both PD-L1 (Freeman *et al.* (2000*) J. Exp. Med.* 192:1027) and PD-L2 (Latchman *et al.* (2001) *Nat. Immunol.* 2:261).

Modulation of a costimulatory signal results in modulation of effector function of an immune cell. Thus, the term "PD-1 ligand activity" includes the ability of a PD-1 ligand polypeptide to bind its natural receptor(s) (*e.g.* PD-1 or B7-1), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

With respect to PD-1, the term "activity" includes the ability of a PD-1 polypeptide to modulate an inhibitory signal in an activated immune cell, *e.g.*, by engaging a natural PD-1 ligand on an antigen presenting cell. PD-1 transmits an inhibitory signal to an immune cell in a manner similar to CTLA4. Modulation of an

inhibitory signal in an immune cell results in modulation of proliferation of, and/or cytokine secretion by, an immune cell. Thus, the term "PD-1 activity" includes the ability of a PD-1 polypeptide to bind its natural ligand(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

As used herein, the term "interaction", when referring to an interaction between two molecules, refers to the physical contact (e.g., binding) of the molecules with one another. Generally, such an interaction results in an activity (which produces a biological effect) of one or both of said molecules. The activity may be a direct activity of one or both of the molecules, (e.g., signal transduction). Alternatively, one or both molecules in the interaction may be prevented from binding their ligand, and thus be held inactive with respect to ligand binding activity (e.g., binding its ligand and triggering or inhibiting costimulation). To inhibit such an interaction results in the disruption of the activity of one or more molecules involved in the interaction. To enhance such an interaction is to prolong or increase the likelihood of said physical contact, and prolong or increase the likelihood of said activity.

As used herein, a "naturally-occurring" nucleic acid polypeptide refers to an RNA or DNA polypeptide having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

As used herein, an "antisense" nucleic acid polypeptide comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA polypeptide, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid polypeptide can hydrogen bond to a sense nucleic acid polypeptide.

As used herein, the term "coding region" refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the term "noncoding region" refers to regions of a nucleotide sequence that are not translated into amino acids (*e.g.*, 5' and 3' untranslated regions).

As used herein, the term "vector" refers to a nucleic acid capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, an "isolated protein" refers to a protein that is substantially free of other proteins, cellular material, separation medium, and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other

contaminating proteins from the cell or tissue source from which the antibody, polypeptide, peptide or fusion protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of PD-1 ligand, PD-1 or B7 polypeptide, in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of PD-1 ligand, PD-1 or B7 fusion protein, having less than about 30% (by dry weight) of non- PD-1 ligand, PD-1 or B7 fusion protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non- PD-1 ligand, PD-1 or B7 fusion protein, still more preferably less than about 10% of PD-1 ligand, PD-1 or B7 fusion protein, and most preferably less than about 5% non- PD-1 ligand, PD-1 or B7 fusion protein. When antibody, polypeptide, peptide or fusion protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of antibody, polypeptide, peptide or fusion protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of antibody, polypeptide, peptide or fusion protein having less than about 30% (by dry weight) of chemical precursors or non-antibody, polypeptide, peptide or fusion protein chemicals, more preferably less than about 20% chemical precursors or non-antibody, polypeptide, peptide or fusion protein chemicals, still more preferably less than about 10% chemical precursors or non-antibody, polypeptide, peptide or fusion protein chemicals, and most preferably less than about 5% chemical precursors or non- antibody, polypeptide, peptide or fusion protein chemicals.

The term “antibody” as used herein also includes an "antigen-binding portion" of an antibody (or simply "antibody portion"). The term “antigen-binding portion”, as used herein, refers to one or more fragments of an antibody that retain the ability to

specifically bind to an antigen (*e.g.*, PD-1 ligand or a B7 polypeptide). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent polypeptides (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Osbourn et al. 1998, Nature Biotechnology 16: 778). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Any VH and VL sequences of specific scFv can be linked to human immunoglobulin constant region cDNA or genomic sequences, in order to generate expression vectors encoding complete IgG polypeptides or other isotypes. VH and Vl can also be used in the generation of Fab , Fv or other fragments of immunoglobulins using either protein chemistry or recombinant DNA technology. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R. J., *et al.* (1994) *Structure* 2:1121-1123).

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Still further, an antibody or antigen-binding portion thereof may be part of larger immunoadhesion polypeptides, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion polypeptides include use of the streptavidin core region to make a tetrameric scFv polypeptide (Kipriyanov, S.M., *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv polypeptides (Kipriyanov, S.M., *et al.* (1994) *Mol. Immunol.* 31:1047-1058). Antibody portions, such as Fab and $F(ab')_2$ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion polypeptides can be obtained using standard recombinant DNA techniques, as described herein.

Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof (e.g. humanized, chimeric, etc.). Antibodies may also be fully human. Preferably, antibodies of the invention bind specifically or substantially specifically to PD-1 ligand, PD-1, or B7-1 polypeptides. The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody polypeptides that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal antibodies" and "polyclonal antibody composition" refer to a population of antibody polypeptides that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody composition typically displays a single binding affinity for a particular antigen with which it immunoreacts.

The term "humanized antibody", as used herein, is intended to include antibodies made by a non-human cell having variable and constant regions which have been altered to more closely resemble antibodies that would be made by a human cell. For example, by altering the non-human antibody amino acid sequence to incorporate amino acids found in human germline immunoglobulin sequences. The humanized antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific

mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs. The term "humanized antibody", as used herein, also includes antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds PD-1 ligand is substantially free of antibodies that specifically bind antigens other than PD-1 ligand). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

There is a known and definite correspondence between the amino acid sequence of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite correspondence between the nucleotide sequence of a particular nucleic acid and the amino acid sequence encoded by that nucleic acid, as defined by the genetic code.

GENETIC CODE	
Alanine (Ala, A)	GCA, GCC, GCG, GCT
Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
Asparagine (Asn, N)	AAC, AAT
Aspartic acid (Asp, D)	GAC, GAT
Cysteine (Cys, C)	TGC, TGT
Glutamic acid (Glu, E)	GAA, GAG
Glutamine (Gln, Q)	CAA, CAG
Glycine (Gly, G)	GGA, GGC, GGG, GGT
Histidine (His, H)	CAC, CAT
Isoleucine (Ile, I)	ATA, ATC, ATT
Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
Lysine (Lys, K)	AAA, AAG
Methionine (Met, M)	ATG
Phenylalanine (Phe, F)	TTC, TTT
Proline (Pro, P)	CCA, CCC, CCG, CCT
Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
Threonine (Thr, T)	ACA, ACC, ACG, ACT
Tryptophan (Trp, W)	TGG
Tyrosine (Tyr, Y)	TAC, TAT
Valine (Val, V)	GTA, GTC, GTG, GTT
Termination signal (end)	TAA, TAG, TGA

An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed (illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA coding for a fusion protein or polypeptide of the invention (or any portion thereof) can be used to derive the fusion protein or polypeptide amino acid sequence, using the genetic code to translate the DNA or RNA into an amino acid sequence. Likewise, for fusion protein or polypeptide amino acid sequence, corresponding nucleotide sequences that can encode the fusion protein or polypeptide can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure herein of a nucleotide sequence which encodes a fusion protein or polypeptide should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a fusion protein or polypeptide amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

II. Agents that Modulate Immune Cell Activation

The agents of this invention can up or down regulate the immune system and, thereby, up or downregulate an immune response. For example, modulation of the interaction between PD-1 and PD-1 ligand, or between PD-1 ligand and a B7 polypeptide, results in modulation of the immune response. The interaction between a B7 polypeptide and a PD-1 ligand polypeptide prevents PD-1 ligand from binding to

PD-1 and, thus, inhibits delivery of an inhibitory immune signal. Thus, in one embodiment, agents which block the interaction between PD-1 and PD-1 ligand can prevent inhibitory signaling. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand polypeptide allow PD-1 ligand to bind PD-1 and provide an inhibitory signal to an immune cell. PD-1 ligand, by binding to a B7 polypeptide, also reduces the B7 polypeptide binding to the inhibitory receptor CTLA4. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand polypeptide allow the B7 polypeptide to bind CTLA4 and provide an inhibitory signal to an immune cell. In another embodiment, PD-L1, by binding to a B7 polypeptide, also reduces the B7 polypeptide binding to the costimulatory receptor CD28. Thus, in one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand polypeptide allow the B7 polypeptide to bind CD28, and provide a costimulatory signal to an immune cell.

For example, in one embodiment, agents that increase the interaction between a PD-1 ligand and a B7 polypeptide can enhance an immune response while agents that decrease the interaction between a PD-1 ligand and a B7 polypeptide can reduce an immune response by enhancing the interaction between the PD-1 ligand and PD-1 and/ or the interaction between the B7 polypeptide and CTLA4. In one embodiment, agents that modulate the interaction between a PD-1 ligand and a B7 polypeptide do not produce inhibition of the interaction between a PD-1 ligand and PD-1 and/ or between the B7 polypeptide and CTLA4. In another embodiment, agents that increase a PD-1 ligand interaction with a B7 polypeptide, also decrease the interaction between the PD-1 ligand and PD-1, and/or between the B7 polypeptide and CTLA4. In yet another embodiment, agents that decrease the interaction of a PD-1 ligand and a B7 polypeptide, enhance or increase the interaction between the PD-1 ligand and PD-1, and/or between the B7 polypeptide and CTLA4. Exemplary agents for modulating (e.g., reducing, an immune response) include antibodies against a PD-1 ligand or a B7 polypeptide which inhibit the interaction of the PD-1 ligand with the B7 polypeptide; small molecules or peptides which inhibit the interaction of the PD-1 ligand with the B7 polypeptide; and fusion proteins (*e.g.* the extracellular portion of the PD-1 ligand or B7 polypeptide,

fused to the Fc portion of an antibody) which bind to the B7 polypeptide or PD-1 ligand, respectively, and prevent the interaction between the PD-1 ligand and B7 polypeptide.

In another embodiment, agents that increase the interaction between a PD-1 ligand and a B7 polypeptide, decrease an immune response by decreasing the ability of the B7 polypeptide to bind to CD28. In yet another embodiment, agents that decrease the interaction between a PD-1 ligand and a B7 polypeptide can increase the immune response by increasing the interaction between the B7 polypeptide and CD28.

Agents that modulate the interaction between a PD-1 ligand and a PD-1 polypeptide can also be used to up or down regulate the immune response. For example, agents that increase the interaction between the PD-1 ligand and PD-1 polypeptide can decrease an immune response while agents that decrease the interaction between the PD-1 ligand and PD-1 polypeptide can increase an immune response. Preferably, agents that modulate the interaction between the PD-1 ligand and PD-1, do not modulate (have no direct affect on) the interaction between the PD-1 ligand and a B7 polypeptide. In another embodiment, agents that increase the interaction between the PD-1 ligand and PD-1, decrease the interaction between the PD-1 ligand and the B7 polypeptide. In yet another embodiment, agents that decrease the interaction between the PD-L1 ligand and PD-1 increase the interaction between the PD-1 ligand and the B7 polypeptide. Exemplary agents for modulating (e.g., enhancing, an immune response) include antibodies against PD-1 or a PD-1 ligand which block the interaction between PD-1 and the PD-1 ligand; small molecules or peptides which block the interaction between PD-1 and the PD-1 ligand; and fusion proteins (*e.g.* the extracellular portion of a PD-1 ligand or PD-1 polypeptide fused to the Fc portion of an antibody) which bind to PD-1 or a PD-1 ligand and prevent the interaction between the PD-1 ligand and PD-1.

In another embodiment, at least a portion of a PD-1 ligand which binds to a B7 polypeptide, or a mimetic of such a portion, can be used to enhance an immune response by binding to the B7 polypeptide and inhibiting the interaction between the B7 polypeptide on a first immune cell and CTLA4 on a second immune cell.

In another embodiment, at least a portion of a PD-1 ligand which binds to a B7 polypeptide, or a mimetic of such a portion, can be used to inhibit an immune response by binding to the B7 polypeptide and inhibiting the interaction between the B7 polypeptide on a first immune cell and CD28 on a second immune cell.

An isolated PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28, or a portion or fragment thereof (or a nucleic acid encoding such a polypeptide), can be used as an immunogen to generate antibodies that bind to the respective PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28, using standard techniques for polyclonal and monoclonal antibody preparation. A full-length PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 can be used, or alternatively, the invention relates to antigenic peptide fragments of PD-1, PD-1 ligand, B7, CTLA4, or a CD28 polypeptide for use as immunogens. An antigenic peptide of PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 comprises at least 8 amino acid residues and encompasses an epitope present in the respective full length molecule such that an antibody raised against the peptide forms a specific immune complex with the respective full length molecule. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptides are regions of PD-1, PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 that are located on the surface of the protein, *e.g.*, hydrophilic regions. A standard hydrophobicity analysis of the polypeptide molecule can be performed to identify hydrophilic regions. Highly preferred epitopes encompassed by the antigenic peptides are the regions of the polypeptide molecule which are in the extracellular domain, and therefore are involved in binding. In one embodiment such epitopes can be specific for a given polypeptide molecule from one species, such as mouse or human (*i.e.*, an antigenic peptide that spans a region of the polypeptide molecule that is not conserved across species is used as immunogen; such non conserved residues can be determined using an alignment such as that provided herein).

In one embodiment, an antibody binds substantially specifically to PD-1 without binding to a B7 polypeptide or a PD-1 ligand. In another embodiment, an antibody binds substantially specifically to a PD-1 ligand. In another embodiment, an antibody

binds substantially specifically to a B7 polypeptide. In a preferred embodiment, an antibody binds to a PD-1 ligand and blocks the interaction between the PD-1 ligand and a B7 polypeptide. In another preferred embodiment, an antibody binds to a B7 polypeptide and blocks the interaction between a PD-1 ligand and the B7 polypeptide. In another preferred embodiment, an antibody binds to a PD-1 ligand and blocks the interaction between PD-1 and the PD-1 ligand, without blocking the interaction between the PD-1 ligand and a B7 polypeptide.

A PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, a recombinantly expressed or chemically synthesized molecule or fragment thereof to which the immune response is to be generated. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic preparation induces a polyclonal antibody response to the antigenic peptide contained therein.

Polyclonal antibodies can be prepared as described above by immunizing a suitable subject with a polypeptide immunogen. The polypeptide antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired, the antibody directed against the antigen can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci.* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp.

77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally Kenneth, R. H. in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.* 54:387-402; Gefter, M. L. *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to the polypeptide antigen, preferably specifically.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-PD-1, anti-PD-1 ligand or anti-B7 polypeptide monoclonal antibody (see, *e.g.*, Galfre, G. *et al.* (1977) *Nature* 266:55052; Gefter *et al.* (1977) *supra*; Lerner (1981) *supra*; Kenneth (1980) *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind a given polypeptide, *e.g.*, using a standard ELISA assay.

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal specific for one of the above described polypeptides antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with the appropriate polypeptide to thereby isolate immunoglobulin library members that bind the polypeptide. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP*^{TM} *Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening an antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Biotechnology (NY)* 9:1369-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Biotechnology (NY)* 9:1373-1377; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al.* (1990) *Nature* 348:552-554.

Additionally, recombinant anti-PD-1, anti-PD-1 ligand or anti-B7 polypeptide antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira *et al.* European Patent Application 184,187; Taniguchi, M. European Patent Application 171,496; Morrison *et al.* European Patent

Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *Biotechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

In addition, humanized antibodies can be made according to standard protocols such as those disclosed in US patent 5,565,332. In another embodiment, antibody chains or specific binding pair members can be produced by recombination between vectors comprising nucleic acid molecules encoding a fusion of a polypeptide chain of a specific binding pair member and a component of a replicable generic display package and vectors containing nucleic acid molecules encoding a second polypeptide chain of a single binding pair member using techniques known in the art, *e.g.*, as described in US patents 5,565,332, 5,871,907, or 5,733,743. The use of intracellular antibodies to inhibit protein function in a cell is also known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T. M. *et al.* (1990) *FEBS Lett.* 274:193-198; Carlson, J. R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W. A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Biotechnology (NY)* 12:396-399; Chen, S-Y. *et al.* (1994) *Hum. Gene Ther.* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R. R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R. R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J. H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

Additionally, fully human antibodies could be made against a PD-1 ligand, PD-1, or a B7 polypeptide. Fully human antibodies can be made in mice that are transgenic for human immunoglobulin genes, *e.g.* according to Hogan, *et al.*, "Manipulating the Mouse Embryo: A Laboratory Manuel," Cold Spring Harbor Laboratory. Briefly, transgenic mice are immunized with purified PD-1 ligand, PD-1, or a B7 polypeptide. Spleen cells are harvested and fused to myeloma cells to produce hybridomas. Hybridomas are selected based on their ability to produce antibodies which bind to PD-1 ligand, PD-1 or a B7 polypeptide. Fully human antibodies would reduce the immunogenicity of such antibodies in a human.

In one embodiment, an antibody for use in the instant invention is a bispecific antibody. A bispecific antibody has binding sites for two different antigens within a single antibody polypeptide. Antigen binding may be simultaneous or sequential. Triomas and hybrid hybridomas are two examples of cell lines that can secrete bispecific antibodies. Examples of bispecific antibodies produced by a hybrid hybridoma or a trioma are disclosed in U.S. Pat. 4,474,893. Bispecific antibodies have been constructed by chemical means (Staerz *et al.* (1985) *Nature* 314:628, and Perez *et al.* (1985) *Nature* 316:354) and hybridoma technology (Staerz and Bevan (1986) *Proc. Natl. Acad. Sci. USA*, 83:1453, and Staerz and Bevan (1986) *Immunol. Today* 7:241). Bispecific antibodies are also described in U.S. patent 5,959,084. Fragments of bispecific antibodies are described in US patent 5,798,229. Bispecific agents can also be generated by making heterohybridomas by fusing hybridomas or other cells making different antibodies, followed by identification of clones producing and co-assembling both antibodies. They can also be generated by chemical or genetic conjugation of complete immunoglobulin chains or portions thereof such as Fab and Fv sequences. The antibody component can bind to PD-1, PD-1 ligand, B7, CTLA4, or a CD28 polypeptide. In one embodiment, the bispecific antibody could specifically bind to both PD-1 ligand and a B7 polypeptide.

Yet another aspect of the invention pertains to anti-PD-1, anti-PD-1 ligand or anti-B7 polypeptide antibodies that are obtainable by a process comprising, immunizing an animal with an immunogenic PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28, or an immunogenic portion thereof unique to PD-1, the PD-1 ligand, the B7 polypeptide,

CTLA4, or CD28; and then isolating from the animal antibodies that specifically bind to the polypeptide.

In another aspect of this invention, peptides or peptide mimetics can be used to antagonize or promote the interaction between a PD-1 ligand and a B7 polypeptide or the interaction between PD-1 and a PD-1 ligand (e.g., without interfering with the interaction between the PD-1 ligand and the B7 polypeptide). In one embodiment, variants of PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 which function as a modulating agent for the respective full length protein, can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, for antagonist activity. In one embodiment, a variegated library of PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 variants can be produced, for instance, by enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential polypeptide sequences is expressible as individual polypeptides containing the set of polypeptide sequences therein. There are a variety of methods which can be used to produce libraries of polypeptide variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential polypeptide sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S. A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of a polypeptide coding sequence can be used to generate a variegated population of polypeptide fragments for screening and subsequent selection of variants of a given polypeptide. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a polypeptide coding sequence with a nuclease under conditions wherein nicking occurs only about once per polypeptide, denaturing the double stranded DNA,

renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the polypeptide.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of polypeptides. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 (Arkin and Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delagrave *et al.* (1993) *Protein Eng.* 6(3):327-331). In one embodiment, cell based assays can be exploited to analyze a variegated polypeptide library. For example, a library of expression vectors can be transfected into a cell line which ordinarily synthesizes PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28. The transfected cells are then cultured such that the full length polypeptide and a particular mutant polypeptide are produced and the effect of expression of the mutant on the full length polypeptide activity in cell supernatants can be detected, *e.g.*, by any of a number of functional assays. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of full length polypeptide activity, and the individual clones further characterized.

Systematic substitution of one or more amino acids of a polypeptide amino acid sequence with a D-amino acid of the same type (*e.g.*, D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides comprising a polypeptide amino acid sequence of interest or a substantially identical sequence variation can be generated by methods known in the art (Rizo and Gierasch (1992) *Annu. Rev. Biochem.* 61:387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences of disclosed herein will enable those of skill in the art to produce polypeptides corresponding peptide sequences and sequence variants thereof. Such polypeptides can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding the peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis *et al. Molecular Cloning: A Laboratory Manual* (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) *J. Am. Chem. Soc.* 91:501; Chaiken I. M. (1981) *CRC Crit. Rev. Biochem.* 11: 255; Kaiser *et al.* (1989) *Science* 243:187; Merrifield, B. (1986) *Science* 232:342; Kent, S. B. H. (1988) *Annu. Rev. Biochem.* 57:957; and Offord, R. E. (1980) *Semisynthetic Proteins*, Wiley Publishing, which are incorporated herein by reference).

In a one embodiment, the peptide has an amino acid sequence identical or similar to the B7 binding site of a PD-1 ligand polypeptide. In another embodiment, the peptide has an amino acid sequence identical or similar to the PD-L1 binding site of a B7 polypeptide. In one embodiment, the peptide competes with PD-1 ligand for binding to a B7 polypeptide or the peptide competes with a B7 polypeptide for binding to PD-1 ligand. In a preferred embodiment, the peptide competes for the binding of PD-1 ligand to a B7 polypeptide, but not for the binding between PD-1 and PD-1

ligand. In another embodiment, the peptide competes for the binding between PD-1 and PD-1 ligand but not between PD-1 ligand and a B7 polypeptide.

Peptides can be produced, typically by direct chemical synthesis, and used *e.g.*, as antagonists of the interaction between PD-1 and PD-1 ligand or between PD-1 ligand and a B7 polypeptide. Peptides can be produced as modified peptides, with nonpeptide moieties attached by covalent linkage to the N-terminus and/or C-terminus. In certain preferred embodiments, either the carboxy-terminus or the amino-terminus, or both, are chemically modified. The most common modifications of the terminal amino and carboxyl groups are acetylation and amidation, respectively. Amino-terminal modifications such as acylation (*e.g.*, acetylation) or alkylation (*e.g.*, methylation) and carboxy-terminal-modifications such as amidation, as well as other terminal modifications, including cyclization, can be incorporated into various embodiments of the invention. Certain amino-terminal and/or carboxy-terminal modifications and/or peptide extensions to the core sequence can provide advantageous physical, chemical, biochemical, and pharmacological properties, such as: enhanced stability, increased potency and/or efficacy, resistance to serum proteases, desirable pharmacokinetic properties, and others. Peptides disclosed herein can be used therapeutically to treat disease, *e.g.*, by altering costimulation in a patient.

Peptidomimetics (Fauchere, J. (1986) *Adv. Drug Res.* 15:29; Veber and Freidinger (1985) TINS p.392; and Evans *et al.* (1987) *J. Med. Chem.* 30:1229, which are incorporated herein by reference) are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), such as human PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH2NH-, -CH2S-, -CH2-CH2-, -CH=CH- (cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-, by methods known in the art and further described in the following references: Spatola, A. F. in "*Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*" Weinstein, B., ed., Marcel Dekker, New York, p. 267 (1983);

Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J. S. (1980) *Trends Pharm. Sci.* pp. 463-468 (general review); Hudson, D. *et al.* (1979) *Int. J. Pept. Prot. Res.* 14:177-185 (-CH2NH-, CH2CH2-); Spatola, A. F. *et al.* (1986) *Life Sci.* 38:1243-1249 (-CH2-S); Hann, M. M. (1982) *J. Chem. Soc. Perkin Trans. I.* 307-314 (-CH-CH-, cis and trans); Almquist, R. G. *et al.* (190) *J. Med. Chem.* 23:1392-1398 (-COCH2-); Jennings-White, C. *et al.* (1982) *Tetrahedron Lett.* 23:2533 (-COCH2-); Szelke, M. *et al.* European Appln. EP 45665 (1982) CA: 97:39405 (1982)(-CH(OH)CH2-); Holladay, M. W. *et al.* (1983) *Tetrahedron Lett.* (1983) 24:4401-4404 (-C(OH)CH2-); and Hruby, V. J. (1982) *Life Sci.* (1982) 31:189-199 (-CH2-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH2NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macropolypeptides(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

Also encompassed by the present invention are small molecules which can modulate (either enhance or inhibit) interactions, e.g., the interaction between PD-1 ligand and a B7 polypeptide or between PD-1 and PD-1 ligand. The small molecules of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. (Lam, K. S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*). Compounds can be screened in cell based or non-cell based assays. Compounds can be screened in pools (e.g. multiple compounds in each testing sample) or as individual compounds.

In one embodiment, the small molecule binds to the binding site involved in the PD-1 ligand / B7 polypeptide interaction, or to the binding site involved in the PD-1 / PD-1 ligand interaction. In one embodiment, the small molecule antagonizes the interaction between PD-1 ligand and a B7 polypeptide. In a preferred embodiment, the small molecule antagonizes the interaction between PD-1 ligand and a B7 polypeptide, but not the interaction between PD-1 and PD-1 ligand or between B7 and CTLA4. In another embodiment, the small molecule antagonizes the interaction between PD-1 and PD-1 ligand or between B7 and CTLA4 without antagonizing the interaction between PD-1 ligand and a B7 polypeptide.

The invention also relates to PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 chimeric or fusion proteins. As used herein, a PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 "chimeric protein" or "fusion protein" comprises a PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 molecule operatively linked to a non-PD-1, non-PD-1 ligand, non-CTLA4, non-CD28, or non-B7 polypeptide molecule. A "PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 molecule" refers to a polypeptide having an amino acid sequence corresponding to PD-1, PD-1 ligand, B7

polypeptide, CTLA4, or CD28, whereas a " a non PD-1, non-PD-1 ligand, non-B7 polypeptide, non-CTLA4, or non-CD28, molecule" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the respective PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 molecule, *e.g.*, a protein which is different from the PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 molecule, and which is derived from the same or a different organism. Within a PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 fusion protein, the PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 portion can correspond to all or a portion of a full length PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 molecule. In a preferred embodiment, the fusion protein comprises at least one biologically active portion of a PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 molecule, *e.g.*, an extracellular domain. Within the fusion protein, the term "operatively linked" is intended to indicate that the PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 amino acid sequences and the non PD-1, non-PD-1 ligand or non-B7 polypeptide sequences are fused in-frame to each other in such a way as to preserve functions exhibited when expressed independently of the fusion. The non PD-1, non-PD-1 ligand, non-B7 polypeptide, non-CTLA4, or non-CD28 molecules can be fused to the N-terminus or C-terminus of the PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 sequences, respectively.

Such a fusion protein can be produced by recombinant expression of a nucleotide sequence encoding the first peptide and a nucleotide sequence encoding the second peptide. The second peptide may optionally correspond to a moiety that alters the solubility, affinity, stability or valency of the first peptide, for example, an immunoglobulin constant region. Preferably, the first peptide consists of a portion of the PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 that is sufficient to modulate costimulation or inhibition of activated immune cells. In another preferred embodiment, the first peptide consists of a portion of a biologically active molecule (*e.g* the extracellular portion of the polypeptide or the ligand binding portion). The second peptide can include an immunoglobulin constant region, for example, a human Cγ1 domain or Cγ4 domain (*e.g.*, the hinge, CH2 and CH3 regions of human IgCγ1, or human IgCγ4, see *e.g.*, Capon *et al.* US patent 5,116,964; 5,580,756; 5,844,095 and the

like, incorporated herein by reference). Such constant regions may retain regions which mediate effector function (e.g. Fc receptor binding) or may be altered to reduce effector function. A resulting fusion protein may have altered solubility, binding affinity, stability and/or valency (*i.e.*, the number of binding sites available per polypeptide) as compared to the independently expressed first peptide, and may increase the efficiency of protein purification. Fusion proteins and peptides produced by recombinant techniques can be secreted and isolated from a mixture of cells and medium containing the protein or peptide. Alternatively, the protein or peptide can be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. Protein and peptides can be isolated from cell culture media, host cells, or both using techniques known in the art for purifying proteins and peptides. Techniques for transfecting host cells and purifying proteins and peptides are known in the art.

Particularly preferred Ig fusion proteins include the extracellular domain portion or variable region-like domain of human PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28, coupled to an immunoglobulin constant region (*e.g.*, the Fc region). The immunoglobulin constant region may contain genetic modifications which reduce or eliminate effector activity inherent in the immunoglobulin structure. For example, DNA encoding the extracellular portion of PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 can be joined to DNA encoding the hinge, CH2 and CH3 regions of human IgGγ1 and/or IgGγ4 modified by site directed mutagenesis, *e.g.*, as taught in WO 97/28267.

Preferably, a fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to

complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). A polypeptide encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the PD-1, PD-1 ligand, B7 polypeptide ,CTLA4, or CD28 encoding sequences.

In another embodiment, the fusion protein contains a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of a polypeptide can be increased through use of a heterologous signal sequence.

In a preferred embodiment, the fusion protein binds to PD-1 or PD-1 ligand and blocks the interaction of PD-1 with PD-1 ligand, without blocking the interaction between PD-1 ligand and a B7 polypeptide. In another preferred embodiment, the PD-1 ligand or B7 polypeptide fusion proteins bind to PD-1 ligand or a B7 polypeptide and block the interaction between PD-1 ligand and the B7 polypeptide.

Use of PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 fusion proteins can be useful therapeutically for the treatment of immunological disorders, *e.g.*, autoimmune diseases, or in the case of inhibiting rejection of transplants.

The fusion proteins of the invention can be used as immunogens to produce antibodies in a subject. Such antibodies may be used to purify the respective natural polypeptides from which the fusion proteins were generated, or in screening assays to identify polypeptides which inhibit the interaction of a PD-1 ligand with a B7 polypeptide or the interaction of PD-1 with a PD-1 ligand.

The modulatory agents described herein (e.g. antibodies, small molecules, peptides, or fusion proteins) can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. The compositions may contain a single such molecule or agent or any combination of modulatory agents described herein.

III. Methods of Selecting Agents that Modulate Immune Cell Activation

Another aspect of the invention relates to methods of selecting agents (e.g., antibodies, fusion proteins, peptides, or small molecules) which modulate an immune response by modulating costimulation (such as agents that inhibit the interaction of PD-1 ligand with a B7 polypeptide or the interaction of PD-1 with a PD-1 ligand). Such methods utilize screening assays, including cell based and non-cell based assays. In one embodiment, the assays provide a method for identifying agents which inhibit the interaction of a PD-1 ligand and PD-1 (e.g., with or without inhibiting the interaction of PD-1 ligand and a B7 polypeptide). In another embodiment, the assays provide a method for identifying agents which modulate the interaction between a PD-1 ligand and a B7 polypeptide (e.g., with or without inhibiting the interaction of the PD-1 ligand and PD-1; the interaction of the B7 polypeptide and CTLA4; and/or the interaction of the B7 polypeptide and CD28).

In one embodiment, the invention relates to assays for screening candidate or test compounds which bind to, or modulate the activity of, PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28, *e.g.*, modulate the ability of a PD-1 ligand or PD-1 to interact with (*e.g.* bind to) its cognate binding partner. In one embodiment, a method for identifying an agent to modulate an immune response entails determining the ability of the agent to modulate, *e.g.* enhance or inhibit, the interaction between PD-1 and a PD-1 ligand, and further determining the ability of the agent to modulate the interaction between a PD-1 ligand and a B7 polypeptide. In one embodiment, an agent that modulates the interaction between the PD-1 ligand and PD-1 (e.g., without modulating the interaction between the PD-1 ligand and the B7 polypeptide is selected). In another embodiment, an agent that modulates the interaction between a PD-1 ligand and a B7 polypeptide (e.g., without modulating the interaction between the PD-1 ligand and PD-1) is selected. Such agents include, without limitation, antibodies, proteins, fusion proteins and small molecules.

In one embodiment, a method for identifying an agent which enhances an immune response entails determining the ability of the candidate agent to enhance the interaction between a PD-1 ligand and a B7 polypeptide (e.g., without modulating or while inhibiting the interaction between the PD-1 ligand and PD-1). In another

embodiment, the method entails determining the ability of the candidate agent to inhibit the interaction between a PD-1 ligand and PD-1 (e.g., without modulating or while enhancing the interaction between the PD-1 ligand and a B7 polypeptide).

In another embodiment, a method for identifying an agent to decrease an immune response entails determining the ability of a candidate agent to inhibit the interaction between a PD-1 ligand and a B7 polypeptide (e.g., without modulating or while enhancing the interaction between the PD-1 ligand and PD-1) and selecting an agent that inhibits the interaction between the PD-1 ligand and the B7 polypeptide. In another embodiment, a method for identifying an agent to decrease an immune response entails determining the ability of the candidate agent to enhance the interaction between a PD-1 ligand and PD-1 (e.g., that does not modulate, or inhibits, the interaction between the PD-1 ligand and the B7 polypeptide) and selecting an agent that enhances the interaction between the PD-1 ligand and PD-1. In a preferred embodiment, the agent selected for decreasing an immune response inhibits the interaction between the PD-1 ligand and a B7 polypeptide, but does not inhibit the interaction between the PD-1 ligand and PD-1.

In one embodiment, an assay is a cell-based assay, comprising contacting a cell expressing PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28, with a test compound and determining the ability of the test compound to modulate (*e.g.* stimulate or inhibit) the binding of PD-1, the PD-1 ligand, the B7 polypeptide, CTLA4, or CD28 target to its binding partner. Determining the ability of the PD-1, PD-1 ligand or B7 polypeptide to bind to, or interact with, its binding partner can be accomplished, *e.g.*, by measuring direct binding or by measuring a parameter of immune cell activation.

For example, in a direct binding assay, the PD-1, PD-1 ligand or B7 polypeptide protein (or their respective target polypeptides) can be coupled with a radioisotope or enzymatic label such that binding of PD-1 ligand to PD-1 or to the B7 polypeptide can be determined by detecting the labeled protein in a complex. For example, PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 can be enzymatically labeled with, for example,

horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a compound to modulate the interaction between PD-1 and a PD-1 ligand or between a PD-1 ligand and a B7 polypeptide, without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of PD-1 and a PD-1 ligand, or between a PD-1 ligand and a B7 polypeptide, with its target polypeptide, without the labeling of either PD-1, PD-1 ligand, B7 polypeptide, or the target polypeptide (McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912). As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between compound and receptor.

In a preferred embodiment, determining the ability of the blocking agents (*e.g.* antibodies, fusion proteins, peptides, or small molecules) to antagonize the interaction between a given set of polypeptides can be accomplished by determining the activity of one or more members of the set of polypeptides. For example, the activity of PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 can be determined by detecting induction of a cellular second messenger (*e.g.*, tyrosine kinase activity), detecting catalytic/enzymatic activity of an appropriate substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, chloramphenicol acetyl transferase), or detecting a cellular response regulated by PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28. Determining the ability of the blocking agent to bind to or interact with said polypeptide can be accomplished, for example, by measuring the ability of a compound to modulate immune cell costimulation or inhibition in a proliferation assay, or by interfering with the ability of said polypeptide to bind to antibodies that recognize a portion thereof.

Agents that block or inhibit interaction of a PD-1 ligand with a costimulatory receptor (e.g., soluble forms of PD-1 ligand or blocking antibodies to PD-1 ligand) as well as agents that promote a PD-1 ligand-mediated inhibitory signal (e.g., agents which

block the interaction of the PD-1 ligand with a B7 polypeptide) can be identified by their ability to inhibit immune cell proliferation, and/or effector function, or to induce anergy when added to an *in vitro* assay. For example, cells can be cultured in the presence of an agent that stimulates signal transduction via an activating receptor. A number of recognized readouts of cell activation can be employed to measure, cell proliferation or effector function (e.g., antibody production, cytokine production, phagocytosis) in the presence of the activating agent. The ability of a test agent to block this activation can be readily determined by measuring the ability of the agent to effect a decrease in proliferation or effector function being measured, using techniques known in the art.

For example, agents of this invention can be tested for the ability to inhibit or enhance costimulation in a T cell assay, as described in Freeman *et al.* (2000) *J. Exp. Med.* 192:1027 and Latchman *et al.* (2001) *Nat. Immunol.* 2:261. CD4+ T cells can be isolated from human PBMCs and stimulated with activating anti-CD3 antibody. Proliferation of T cells can be measured by ^{3}H thymidine incorporation. An assay can be performed with or without CD28 costimulation in the assay. Similar assays can be performed with Jurkat T cells and PHA-blasts from PBMCs.

In yet another embodiment, an assay of the present invention is a cell-free assay in which PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28, or a biologically active portion thereof, is contacted with a test compound, and the ability of the test compound to bind to the polypeptide, or biologically active portion thereof, is determined. Binding of the test compound to the PD-1, PD-1 ligand, B7 polypeptide, CTLA4, or CD28 can be determined either directly or indirectly as described above. In a preferred embodiment, the assay includes contacting the polypeptide, or biologically active portion thereof, with its binding partner to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the polypeptide in the assay mixture, wherein determining the ability of the test compound to interact with the polypeptide comprises determining the ability of the test compound to preferentially bind to the polypeptide or biologically active portion thereof, as compared to the binding partner.

For example, a PD-1 ligand and a B7 polypeptide can be used to form an assay mixture and the ability of a polypeptide to block this interaction can be tested by determining the ability of PD-1 to bind the PD-1 ligand and determining the ability of the PD-1 ligand to bind the B7 polypeptide, by one of the methods described above for determining direct binding. Determining the ability of the PD-1 to bind the PD-1 ligand and determining the ability of the PD-1 ligand to bind the B7 polypeptide can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al*. (1995) *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological polypeptides. PD-1, PD-1 ligand, and B7 polypeptide can be immobilized on a BIAcore chip and multiple agents (blocking antibodies, fusion proteins, peptides, or small molecules) can be tested for binding to PD-1, PD-1 ligand, and B7 polypeptide. An example of using the BIA technology is described by Fitz *et al.* (1997) *Oncogene* 15:613.

The cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of proteins (*e.g.*, a PD-1 ligand or PD-1 proteins or biologically active portions thereof, or binding partners to which a PD-1 ligand or PD-1 binds). In the case of cell-free assays in which a membrane-bound form protein is used (*e.g.*, a cell surface PD-1 ligand or PD-1 receptor) it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In one or more embodiments of the above described assay methods, it may be desirable to immobilize either PD-1, a PD-1 ligand, and a B7 polypeptide, or an appropriate target polypeptide, to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to PD-1, a PD-1 ligand, or a B7 polypeptide, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/ PD-1, PD-1 ligand, or B7 polypeptide fusion proteins, or glutathione-S-transferase/target fusion proteins, can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of PD-1, PD-1 ligand, or B7 polypeptide binding or activity determined using standard techniques.

In an alternative embodiment, determining the ability of the test compound to modulate the activity of PD-1, a PD-1 ligand, or a B7 polypeptide can be accomplished by determining the ability of the test compound to modulate the activity of a polypeptide that functions downstream of PD-1, the PD-1 ligand, or the B7 polypeptide, *e.g.*, a polypeptide that interacts with the PD-1 ligand, or a polypeptide that functions downstream of PD-1, *e.g.*, by interacting with the cytoplasmic domain of PD-1. For example, levels of second messengers can be determined, the activity of the interactor polypeptide on an appropriate target can be determined, or the binding of the interactor to an appropriate target can be determined as previously described.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an

agent identified as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

IV. Pharmaceutical Compositions

PD-1, PD-1 ligand, B7, CTLA4, or CD28 modulating agents (*e.g.*, agents that inhibit or promote the interaction of PD-1 and PD-1 ligand without blocking the interaction of PD-1 ligand and a B7 polypeptide or agents that block the interaction of PD-1 ligand with a B7 polypeptide, including, e.g., blocking antibodies, peptides, fusion proteins, or small molecules) can be incorporated into pharmaceutical compositions suitable for administration to a subject. Such compositions typically comprise the antibody, peptide, fusion protein or small molecule and a pharmaceutically acceptable carrier. As used herein the language "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. 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 the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or

sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition should be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, antibodies against PD-1, a PD-1 ligand, a B7 polypeptide, CTLA4, or CD28 fragments of said molecules; or small molecules that block the interactions of said polypeptides) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound

into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, modulatory agents are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations should be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by, and directly dependent on, the unique characteristics of the active compound, the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a

delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

The above described modulating agents may be administered it he form of expressible nucleic acids which encode said agents. Such nucleic acids and compositions in which they are contained, are also encompassed by the present invention. For instance, the nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

V. Uses and Methods of the Invention

The modulatory agents described herein can be used for methods of treatment (*e.g.*, by up- or down-modulating the immune response). For example, PD-1 ligand binding to PD-1 transmits a negative signal, whereas PD-1 ligand binding to a B7 polypeptide such as B7-1 does not. Thus, modulation of the interaction between PD-1 and a PD-1 ligand, or between a PD-1 ligand and a B7 polypeptide, results in modulation of the immune response. The interaction between a B7 polypeptide and a PD-1 ligand also prevents the PD-1 ligand from binding to PD-1, and thus, inhibits delivering an inhibitory immune signal. Thus, in one embodiment, agents which block the interaction between PD-1 and a PD-1 ligand can prevent inhibitory signaling. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand allow the PD-1 ligand to bind PD-1 and provide an inhibitory signal to an immune cell. The PD-1 ligand, by binding to a B7 polypeptide, also reduces B7 binding to the inhibitory receptor CTLA4. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand allow the B7 polypeptide to bind CTLA4 and provide an inhibitory signal to an immune cell. In another embodiment, a PD-1 ligand, by binding to a B7 polypeptide, also reduces the B7 polypeptide binding to the costimulatory receptor CD28. Thus, in one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand polypeptide allow the B7 polypeptide to bind CD28 and provide a costimulatory signal to an immune cell.

1. Prophylactic Methods

In one aspect, the invention relates to a method for preventing in a subject, a disease or condition associated with an unwanted or less than desirable immune response. Subjects at risk for a disease that would benefit from treatment with the claimed agents or methods can be identified, for example, by any or a combination of diagnostic or prognostic assays known in the art. Administration of a prophylactic agent can occur prior to the manifestation of symptoms associated with an unwanted or less than desirable immune response. The appropriate agent used for treatment (*e.g.* antibodies, peptides, fusion proteins or small molecules) can be determined based on clinical indications and can be identified, *e.g.*, using screening assays described herein.

2. Therapeutic Methods

Another aspect of the invention pertains to therapeutic methods of modulating an immune response, e.g., by modulating the interaction between a PD-1 ligand and a B7 polypeptide. For example, modulation of the interaction between PD-1 and a PD-1 ligand, or between a PD-1 ligand and a B7 polypeptide, results in modulation of the immune response. The interaction between a B7 polypeptide and a PD-1 ligand also prevents the PD-1 ligand from binding to PD-1 and, thus, inhibits delivery of an inhibitory immune signal. Thus, in one embodiment, agents which block the interaction between PD-1 and the PD-1 ligand can prevent inhibitory signaling. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand polypeptide allow PD-1 ligand to bind PD-1 and provide an inhibitory signal to an immune cell. PD-1 ligand, by binding to a B7 polypeptide, also reduces the B7 polypeptide binding to the inhibitory receptor CTLA4. In one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand allow the B7 polypeptide to bind CTLA4, and thus provide an inhibitory signal to an immune cell. In another embodiment, a PD-1 ligand, by binding to a B7 polypeptide, also reduces the B7 polypeptide binding to the costimulatory receptor CD28. Thus, in one embodiment, agents that block the binding of a B7 polypeptide to a PD-1 ligand allow the B7 polypeptide to bind CD28, and thus provide a costimulatory signal to an immune cell.

An exemplary agent that modulates the interaction between a PD-1 ligand and PD-1, a B7 polypeptideand CTLA4, a B7 polypeptide and CD28, or a PD-1 ligand and a B7 polypeptide, includes such agents as described herein, e.g. antibodies against PD-1, PD-1 ligand, CTLA4, CD28, or a B7 polypeptide; fragments or peptides derived from PD-1, PD-1 ligand, CD28, CTLA4, or a B7 polypeptide; fusion proteins of PD-1, PD-1 ligand, CD28, CTLA4, or a B7 polypeptide; and small molecules that modulate the interaction of PD-1 with PD-1 ligand, a B7 polypeptide with CD28, a B7 polypeptide with CTLA4, or a PD-1 ligand with a B7 polypeptide.

These modulatory agents can be administered *in vitro* (*e.g.*, by contacting the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention relates to methods of treating an individual

afflicted with a disease or disorder that would benefit from modulation of an immune response, e.g., by modulation of the interaction between a PD-1 ligand and PD-1, or a B7 polypeptide.

3. Downregulation of Immune Responses by Modulation

There are numerous embodiments of the invention for upregulating the inhibitory function or downregulating the costimulatory function of a PD-1 ligand to thereby downregulate immune responses. Downregulation can be in the form of inhibiting or blocking an immune response already in progress, or may involve preventing the induction of an immune response. The functions of activated immune cells can be inhibited by down-regulating immune cell responses, or by inducing specific anergy in immune cells, or both.

For example, the immune response can be downmodulated using: PD-1 ligand polypeptides (e.g., soluble, monomeric forms of a PD-1 ligand polypeptide such as PD-1 ligand-Ig) and/or anti-PD-1 ligand antibodies that block the interaction of PD-1 ligand with a B7 polypeptide (e.g., while not affecting or increasing the interaction between PD-L1 and PD-1) or which promote the binding of a PD-1 ligand with PD-1, (e.g., while not affecting or while inhibiting the interaction between a B7 polypeptide and the PD-1 ligand). Other exemplary agents which can block these interactions include anti-B7 polypeptide, a B7 polypeptide, or a blocking small molecule.

Agents that promote binding of a PD-1 ligand to PD-1 or a B7 polypeptide to CTLA4, while not affecting or reducing the binding of the PD-1 ligand to the B7 polypeptide, can also be used to down modulate the immune response. Exemplary agents include PD-1 peptide mimetics, identified by the methods described herein.

In one embodiment of the invention, tolerance is induced against specific antigens by co-administering an antigen with an agent (*e.g.* antibody, peptide, fusion protein, or small molecule) which blocks the interaction between a PD-1 ligand and a B7 polypeptide. For example, tolerance can be induced to specific proteins. In one embodiment, immune responses to allergens, or to foreign proteins to which an immune response is undesirable, can be inhibited. For example, patients that receive Factor VIII frequently generate antibodies against this clotting factor. Co-administration of an agent

that blocks a PD-1 ligand-mediated costimulatory signal or an agent that stimulates a PD-1 mediated inhibitory signal in combination with recombinant factor VIII (or by physically linked to Factor VIII, e.g., by cross-linking) can result in downmodulation.

In one embodiment, fusion proteins comprising a first PD-1 ligand peptide fused to a second peptide can be used to block the interaction of the PD-1 ligand with a B7 polypeptide on an immune cell, to thereby downmodulate immune responses. In one embodiment, the second peptide blocks an activity of another B lymphocyte antigen (e.g., B7-1, B7-2, or B7-3) to further downmodulate immune responses. Alternatively, two separate agents that downmodulate immune responses can be combined as a single composition or administered separately (simultaneously or sequentially) to more effectively downregulate immune cell mediated immune responses in a subject. For instance, a PD-1 ligand can be combined with a B7 polypeptide, or with a combination of blocking antibodies (e.g., antibodies against a PD-1 ligand polypeptide with anti-B7-1 and/or anti-B7-2 monoclonal antibodies). Furthermore, a therapeutically active amount of one or more of the subject agents, can be used in conjunction with other downmodulating reagents to influence immune responses. Examples of other immunomodulating reagents include, without limitation, antibodies that block a costimulatory signal, (e.g., against CD28 or ICOS), antibodies that act as agonists of CTLA4, and/or antibodies against other immune cell markers (e.g., against CD40, against CD40 ligand, or against cytokines), fusion proteins (e.g., CTLA4-Fc), and immunosuppressive drugs, (e.g., rapamycin, cyclosporine A or FK506).

Downregulating or preventing a PD-1 ligand interaction with a B7 polypeptide, or promoting an interaction between a PD-1 ligand and PD-1 (for example, without modulating, or by additionally enhancing) the interaction between the PD-1 ligand and the B7 polypeptide (*e.g.* by stimulation of the negative signaling function of PD-1) is useful to downmodulate the immune response, e.g., in situations of tissue, skin and organ transplantation, in graft-versus-host disease (GVHD), or in autoimmune diseases such as systemic lupus erythematosus, and multiple sclerosis. For example, blockage of immune cell function results in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by immune cells, followed by an immune reaction that destroys

the transplant. The administration of a polypeptide which inhibits or blocks interaction of a PD-1 ligand with a B7 polypeptide (such as a soluble, monomeric form of the PD-1 ligand or PD-1), alone or in conjunction with another downmodulatory agent, prior to or at the time of transplantation can promote the generation of an inhibitory signal. Moreover, inhibition of PD-1 ligand costimulatory signals, or promotion of a PD-1 ligand or PD-1 inhibitory signals, may also be sufficient to anergize the immune cells, thereby inducing tolerance in a subject. Induction of long-term tolerance by blocking a PD-1 ligand mediated costimulatory signal may avoid the necessity of repeated administration of these blocking reagents.

To achieve sufficient immunosuppression or tolerance in a subject, it may also be desirable to block the costimulatory function of other polypeptides. For example, it may be desirable to block the function of B7-1, B7-2, or B7-1 and B7-2 by administering a soluble form of a combination of peptides having an activity of each of these antigens, blocking antibodies against these antigens or blocking small molecules (separately or together in a single composition) prior to or at the time of transplantation. Alternatively, it may be desirable to promote inhibitory activity of a PD-1 ligand or PD-1 and inhibit a costimulatory activity of B7-1 and/or B7-2. Other downmodulatory agents that can be used in connection with the downmodulatory methods of the invention include, for example, agents that transmit an inhibitory signal via CTLA4, soluble forms of CTLA4, antibodies that activate an inhibitory signal via CTLA4, blocking antibodies against other immune cell markers or soluble forms of other receptor ligand pairs (e.g., agents that disrupt the interaction between CD40 and CD40 ligand (e.g., anti CD40 ligand antibodies)), antibodies against cytokines, or immunosuppressive drugs.

Downmodulation of immune responses are also useful in treating autoimmune disease. Many autoimmune disorders are the result of inappropriate activation of immune cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive immune cells may reduce or eliminate disease symptoms. Administration of reagents which block costimulation of immune cells by disrupting interactions between PD-1 ligand and B7 polypeptides, or by promoting the interaction

between PD-1 ligand and PD-1, without modulating or while downmodulating the interaction between PD-1 ligand and a B7 polypeptide, are useful for inhibiting immune cell activation and preventing production of autoantibodies or cytokines which may be involved in the disease process. Additionally, agents that promote an inhibitory function of a PD-1 ligand or PD-1 may induce antigen-specific tolerance of autoreactive immune cells, which could lead to long-term relief from the disease. The efficacy of reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythematosus in MRL/*lpr/lpr* mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see, e.g., Paul ed., *Fundamental Immunology*, Raven Press, New York, Third Edition 1993, chapter 30).

Inhibition of immune cell activation is useful therapeutically in the treatment of allergy and allergic reactions, e.g., by inhibiting IgE production. An agent that promotes a PD-1 ligand or PD-1 inhibitory function can be administered to an allergic subject to inhibit immune cell mediated allergic responses in the subject. Inhibition of PD-1 ligand costimulation of immune cells or stimulation of a PD-1 ligand or PD-1 inhibitory pathway can be accompanied by exposure to allergen in conjunction with appropriate MHC polypeptides. Allergic reactions can be systemic or local in nature, depending on the route of entry of the allergen and the pattern of deposition of IgE on mast cells or basophils. Thus, inhibition of immune cell mediated allergic responses locally or systemically by administration of an inhibitory form of an agent that inhibits the interaction of a PD-1 ligand with a costimulatory receptor, or an agent that promotes an inhibitory function of a PD-1 ligand or PD-1.

Inhibition of immune cell activation through blockage of the interaction of a PD-1 ligand and a B7 polypeptide, or through promotion of the interaction between a PD-1 ligand and PD-1, without modulating or while downmodulating the interaction between the PD-1 ligand and a B7 polypeptide, may also be important therapeutically in viral infections of immune cells. For example, in the acquired immune deficiency syndrome

(AIDS), viral replication is stimulated by immune cell activation. Modulation of these interactions may result in inhibition of viral replication and thereby ameliorate the course of AIDS. Modulation of these interactions may also be useful in promoting the maintenance of pregnancy. PD-1 ligand is normally highly expressed in placental trophoblasts, the layer of cells that forms the interface between mother and fetus and may play a role in preventing maternal rejection of the fetus. Females at risk for spontaneous abortion (*e.g.*, those who have previously had a spontaneous abortion or those who have had difficulty conceiving) because of immunologic rejection of the embryo or fetus can be treated with agents that modulate these interactions.

Downregulation of an immune response by modulation of PD-1 ligand / B7 polypeptide, binding or by modulation of PD-1 ligand / PD-1 binding may also be useful in treating an autoimmune attack of autologous tissues. For example, PD-1 ligand is normally highly expressed in the heart and may protect the heart from autoimmune attack. This is evidenced by the fact that the Balb/c PD-1 knockout mouse exhibits massive autoimmune attack on the heart with thrombosis. Thus, conditions that are caused or exacerbated by autoimmune attack (e.g., in this example, heart disease, myocardial infarction or atherosclerosis) may be ameliorated or improved by modulation of these interactions. It is therefore within the scope of the invention to modulate conditions exacerbated by autoimmune attack, such as autoimmune disorders (as well as conditions such as heart disease, myocardial infarction, and atherosclerosi).

4. Upregulation of Immune Responses

Also useful therapeutically is the blockage of the interaction of a PD-1 ligand with PD-1, and/or a B7 polypeptide with CTLA4, without modulating or while upregulating the interaction between the B7 polypeptide and the PD-1 ligand, or by promoting the interaction of the PD-1 ligand with the B7 polypeptide (e.g., while not affecting or while inhibiting the interaction between the PD-1 ligand and PD-1) as a means of upregulating an immune response. Blocking the interaction between a B7 polypeptide and a PD-1 ligand to thereby increase the interaction between the B7 polypeptide and CD28, is also useful to upregulate immune responses. Upregulation of immune responses can be in the form of enhancing an existing immune response or

eliciting an initial immune response. For instance, enhancing an immune response using the subject compositions and methods is useful in cases of infections with microbes (e.g., bacteria, viruses, or parasites). In one embodiment, an agent that blocks the interaction of a PD-1 ligand with PD-1, without modulating or while upregulating the interaction between a B7 polypeptide and the PD-1 ligand, or by promoting the interaction of the PD-1 ligand with the B7 polypeptide, is used to enhance the immune response. Such an agent (e.g., a non-activating antibody that blocks PD-L1 binding to PD-1) is therapeutically useful in situations where upregulation of antibody and cell-mediated responses would be beneficial. In a preferred embodiment, the agent inhibits the interaction between PD-1 and a PD-1 ligand, without inhibiting the interaction between the PD-1 ligand and a B7 polypeptide (e.g., an interaction which prevents PD-L1 from binding to PD-1). Exemplary disorders include viral skin diseases, such as Herpes or shingles, in which case such an agent can be delivered topically to the skin. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by systemic administration of such agents.

Alternatively, immune responses can be enhanced in an infected patient through an *ex vivo* approach, for instance, by removing immune cells from the patient, contacting immune cells *in vitro* with an agent that blocks the interaction of a PD-1 ligand with PD-1, without modulating or while upmodulating the interaction between a B7 polypeptide and the PD-1 ligand, or by promoting the interaction of the PD-1 ligand with the B7 polypeptide, and reintroducing the *in vitro* stimulated immune cells into the patient.

In certain instances, it may be desirable to further administer other agents that upregulate immune responses, for example, forms of other B7 family members that transduce signals via costimulatory receptors, in order to further augment the immune response.

An agent that blocks the interaction of a PD-1 ligand with PD-1 (e.g., without modulating or while upmodulating the interaction between a B7 polypeptide and the PD-1 ligand or by enhancing the interaction of the PD-1 ligand with the B7 polypeptide) can be used prophylactically in vaccines against various polypeptides (e.g., polypeptides derived from pathogens). Immunity against a pathogen (e.g., a virus) can be induced by vaccinating with a viral protein along with an agent that blocks the interaction of a PD-1

ligand with PD-1, without modulating or while upmodulating the interaction between a B7 polypeptide and the PD-1 ligand, or by promoting the interaction of the PD-1 ligand with the B7 polypeptide, in an appropriate adjuvant.

In another embodiment, upregulation or enhancement of an immune response function, as described herein, is useful in the induction of tumor immunity

In another embodiment, the immune response can be stimulated by the methods described herein, such that preexisting tolerance is overcome. For example, immune responses against antigens to which a subject cannot mount a significant immune response, e.g., to an autologous antigen, such as a tumor specific antigens can be induced by administering an agent that blocks the interaction of a PD-1 ligand with PD-1 (e.g., without modulating or while upmodulating the interaction between a B7 polypeptide and the PD-1 ligand or by promoting the interaction of the PD-1 ligand with the B7 polypeptide). In one embodiment, a soluble PD-1 or a soluble PD-1 ligand that inhibits the interaction of a PD-1 ligand with PD-1, without modulating or while upmodulating the interaction between a B7 polypeptide and the PD-1 ligand, or by promoting the interaction of the PD-1 ligand with the B7 polypeptide, can be used to enhance an immune response (e.g., to a tumor cell). In one embodiment, an autologous antigen, such as a tumor-specific antigen can be coadministered. In another embodiment, an immune response can be stimulated against an antigen (e.g., an autologous antigen) to treat a neurological disorder. In another embodiment, the subject agents can be used as adjuvants to boost responses to foreign antigens in the process of active immunization.

In one embodiment, immune cells are obtained from a subject and cultured *ex vivo* in the presence of an agent as described herein, to expand the population of immune cells and/or to enhance immune cell activation. In a further embodiment the immune cells are then administered to a subject. Immune cells can be stimulated *in vitro* by, for example, providing to the immune cells a primary activation signal and a costimulatory signal, as is known in the art. Various agents can also be used to costimulate proliferation of immune cells. In one embodiment immune cells are cultured *ex vivo* according to the method described in PCT Application No. WO 94/29436. The

costimulatory polypeptide can be soluble, attached to a cell membrane, or attached to a solid surface, such as a bead.

V. Administration of Agents

The immune modulating agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo,* to either enhance or suppress immune cell mediated immune responses. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term "subject" is intended to include living organisms in which an immune response can be elicited, *e.g.*, mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of an agent as described herein can be in any pharmacological form including a therapeutically active amount of an agent alone or in combination with a pharmaceutically acceptable carrier.

Administration of a therapeutically active amount of the therapeutic composition of the present invention is defined as an amount effective, at dosages and for periods of time necessary, to achieve the desired result. For example, a therapeutically active amount of an anti-PD-1 ligand modulating agent may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The agents or the invention described herein can be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound can be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. For example, for administration of agents, by other than parenteral administration, it may be desirable to coat the agent with, or co-administer the agent with, a material to prevent its inactivation.

An agent can be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Sterna *et al.* (1984) *J. Neuroimmunol.* 7:27).

The agent may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions of agents suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the composition will preferably be sterile and must be fluid to the extent that easy syringeability exists. It will preferably be stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about

by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating an agent of the invention (*e.g.*, an antibody, peptide, fusion protein or small molecule) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the agent plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the agent is suitably protected, as described above, the protein can be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. 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 the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form", as used herein, refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by, and directly dependent on, (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

In one embodiment, an agent of the invention is an antibody. As defined herein, a therapeutically effective amount of antibody (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result from the results of diagnostic assays.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures, are incorporated herein by reference.

EXAMPLES

Example 1: Identification of PD-1 Ligand and B7 as Binding Partners

In order to identify new B7-1 binding partners other than CD28 and CTLA4, a cDNA library from CD28/CTLA4 deficient mice was screened. A murine cDNA library in the pAXEF mammalian expression vector was made from RNA prepared from CD4+ T cells of CD28 deficient, CTLA4 deficient 129 strain mice. CD4+ T cells were purified by MACS, with purity greater than 95% confirmed by flow cytometry. The T cells were activated with anti-CD3 mAb plus antigen presenting cells (APC). APCs

from T-depleted splenocytes of CD28 deficient, CTLA4 deficient 129 strain mice were stimulated overnight with 5 μg/ml of the anti-CD40 mAb 3/23, then treated with mitomycin C (50 μg/ml for 40 minutes at 37°C), washed, and used to stimulate T cells. RNA was prepared after 16, 24, and 40 hr and combined for preparation of the cDNA library.

For the first round of selection, 80 plates of COS cells were transfected via the DEAE-Dextran procedure with 0.2 μg of plasmid library DNA per 100 mm dish. Cells were trypsinized and replated the next day and after 45 hr, cells were harvested with 0.5 mM EDTA, 0.02% sodium azide in PBS. Panning plates were prepared by incubating a 100mm petri dish with 10 ml of 10 μg/ml goat anti-mouse IgG2a antibody in 50 mM Tris, pH 9.5 for 1.5 hours at room temperature. The plate was washed 3 times with PBS and blocked overnight in PBS plus 5 mg/ml BSA. The plate was then incubated 1 hr with 3 ml of 10 μg/ml murine B7-1-IgG2a fusion protein, washed three times with PBS, 2% FCS. Transfected COS cells from 80 plates were incubated on 8 panning plates. After 2 hours at room temperature, the plates were washed 3 times with 0.5mM EDTA, 0.02% sodiun azide, 2% FCS in PBS and then twice with 0.5mM EDTA, 10 mM HEPES, pH 7.4, 1% FCS in 0.15 M NaCl. Episomal DNA was prepared from adherent cells, re-introduced into *E. Coli* DH10B/P3 by electroporation, transfected into COS cells by polyethylene glycol-mediated fusion of spheroplasts and the panning repeated. Episomal DNA was prepared from adherent cells, re-introduced into E. Coli DH10B/P3 by electroporation, transfected into COS cells by polyethylene glycol-mediated fusion of spheroplasts and the panning was repeated a third time. Individual plasmid DNAs were prepared and sequenced. All of the plasmids (of 6 sequenced) were found to contain a cDNA encoding murine PD-L1. The cDNA clones varied slightly in the length of the 5' untranslated region, indicating that the PD-L1 gene was independently isolated multiple times, thus excluding the possibility of accidental isolation of a clone previously generated in the lab. These murine PD-L1 cDNA clones were transfected into COS cells via the DEAE-Dextran procedure (4 μg per 100 mm dish) and analyzed after 72 hr for cell surface binding of murine B7-1-IgG2a fusion protein by indirect immunofluorescence and flow cytometry.

Example 2: Binding of PD-1 Ligand Molecules to B7 Molecules

Mouse pre-B cells (300.19) were transfected with either vector DNA (pcDNAI), or an expression plasmid containing the murine PD-L1 cDNA in the pAXEF vector, together with a plasmid encoding puromycin resistance. Cells were selected in media containing 10 μg/ml puromycin, stained with PD-1-IgG2a fusion protein, sorted, and subcloned. A clone expressing high levels of PD-L1 was selected for further analysis.

300.19 transfectants (5 x 10^6 cells in 2.5 ml media) were labeled with 2.5 μl of BCECF-AM (Molecular Probes, 5 mg/ml in DMSO, 2', 7'-(bis-2-carboxyethyl)-5-(and-6)-carboxyfluorescein)) for 15 minutes at 37°. Cells were washed twice and 50,000 cells in 50 μl used per well.

Linbro 96 well microtiter plates (not tissue culture treated) were coated with 100 μl of 10 μg/ml goat anti-mouse IgG2a antibody in PBS overnight at 4 °. Plates were aspirated and blocked for 2 hr with 1% BSA in PBS. Wells were then incubated with 0.1 ml of 10 μg/ml control Ig, B7-1 Ig, B7-2 Ig, or PD-1 Ig. 50,000 BCECF-AM-labeled transfected cells were added to the plates in the presence or absence of a blocker. The blockers tested were 10 μg/ml control Ig, CTLA4 Ig, PD-1 Ig, the 1G10 antibody (which binds to murine B7-1 and blocks its interaction with CTLA4), the 16-10A1 antibody (which binds to murine B7-1 and blocks its interaction with CTLA4), the 10F.9G2 antibody (which binds to murine PD-L1 and blocks its interaction with PD-1), and the 10F.2H11 antibody (which binds to murine PD-L1 but does not block its interaction with PD-1). The plates were centrifuged 10 sec at 700 rpm and incubated at room temperature for 30 minutes. The fluorescence in each well (indicating the number of cells) was measured on a fluorescence plate reader. The plates were washed 1X by submerging the plate in a large volume of 1% BSA/PBS in a dish, gently inverting the plate, and allowing non-adherent cells to fall at 1g for 30 min at room temperature. The wash was then repeated. The plate was then righted and the fluorescence in each well (indicating the number of cells) was measured on a fluorescence plate reader. The percent of cells bound to the plate was determined. The PD-L1 expressing cells bound to the B7-1 Ig plates, but not to the B7-2 Ig plates. Neither control Ig or CTLA4 Ig competed in this binding interaction (Figure 1). In contrast, the 1G10, 10F.9G2, and

10F.2H11 antibodies and PD-1 Ig each competed for binding with the PD-L1 / B7-1 Ig interaction and lead to a decrease in cell binding (Figure 1). In a control experiment, PD-L1 expressing cells were shown to bind to plates coated with PD-1 Ig (Figure 2). Control Ig, PD-1 Ig, and the 10F.2H11 antibody all failed to compete for binding in this assay (Figure 2). Only the 10F.9G2 antibody competed for the PD-L1 / PD-1 interaction and decreased cell binding (Figure 2).

Example 3: FACS Analysis of PD-1 Ligand Molecules

300.19 cells were transfected with either vector DNA (pcDNAI), or an expression plasmid containing the ICOS ligand, mPD-L1 or mPD-L2 cDNA.

Cells were incubated with mIgG2a, mCTLA4-IgG2a, mB7-1-IgG2a, mB7-2-IgG2a, or mPD-1-IgG2a (0.1 ml of 10 μg/ml) for 30 minutes at 4°C. Cells were washed with FACS buffer (PBS plus 0.02% sodium azide and 2 % FBS) and incubated with PE labeled goat anti-mIgG2a antibody (0.1 ml of 10 μg/ml) (Southern Biotech Associates). Cells were analyzed for immunoflourescence using FACS analysis and the results are shown in Figure 4 (the numbers in Figure 4 indicate the mean fluorescence intensity). There was no binding to the ICOS ligand or control cells. The PD-L1 cells bound to mPD-1-IgG2a and mB7-1-IgG2a, but not to mCTLA4-IgG2a or mB7-2-IgG2a. The PD-L2 cells bound to mPD-1-IgG2a and mB7-1-IgG2a (slightly), but not to mCTLA4-IgG2a or mB7-2-IgG2a (Figure 4).

CLAIMS

1. A method for identifying an agent that modulates an immune response, comprising:

a) identifying agents which inhibit the interaction between a PD-1 ligand and a PD-1 polypeptide, from test agents; and

b) determining whether the agents identified in step a) inhibit the interaction between a PD-1 ligand and a B7 polypeptide;

wherein an agent of step b) which does not inhibit the interaction between a PD-1 ligand and a B7 polypeptide, is identified as an agent that modulates an immune response.

2. A method for identifying an agent that modulates an immune response, comprising:

a) identifying agents which inhibit the interaction between a PD-1 ligand and a B7 polypeptide, from test agents; and

b) determining whether the agents identified in step a) inhibit the interaction between a PD-1 ligand and a PD-1 polypeptide;

wherein an agent of step b) which does not inhibit the interaction between a PD-1 ligand and a PD-1 polypeptide, is identified as an agent that modulates an immune response.

3. A method for inhibiting the interaction between a B7 polypeptide and a PD-1 ligand, comprising:

a) contacting an immune cell bearing a PD-1 ligand; or

b) contacting an immune cell bearing a B7 polypeptide;

with an agent that inhibits the interaction between the PD-1 ligand and the B7 polypeptide.

4. The method of claim 3, wherein the agent is an anti-PD-1 ligand antibody.

5. The method of claim 3, wherein the agent is a small molecule.

6. The method of claim 3, wherein the PD-1 ligand is PD-L1.

7. The method of claim 3, wherein the PD-1 ligand is PD-L2.

8. The method of claim 3, wherein the B7 polypeptide is B7-1.

9. A method for modulating an immune response, comprising:

a) contacting an immune cell bearing the PD-1 ligand; or

b) contacting an immune cell bearing the PD-1 polypeptide;

with an agent that inhibits interactions between the PD-1 ligand and the PD-1 polypeptide, without inhibiting interactions between the PD-1 ligand and a B7 polypeptide, to thereby modulate an immune response.

10. The method of claim 9, wherein the agent is an anti-PD-1 ligand antibody.

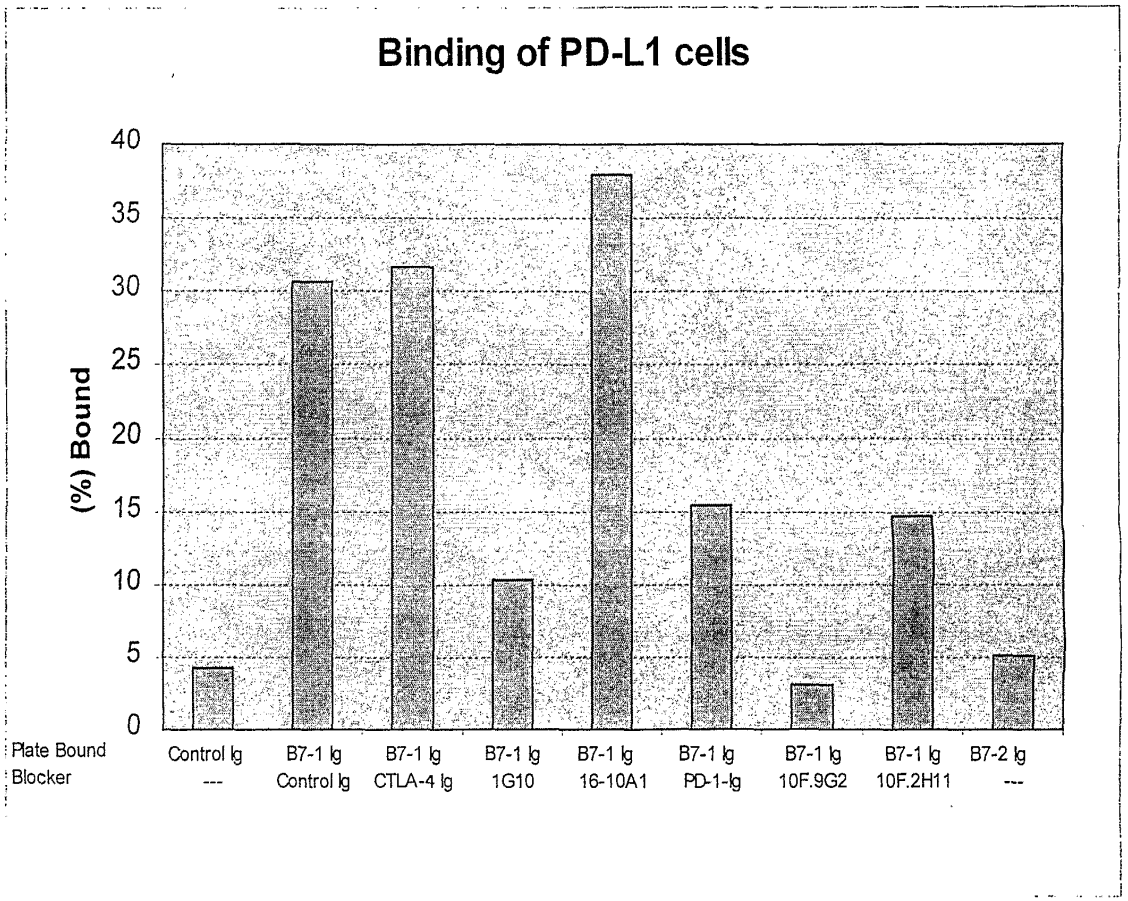
11. The method of claim 9, wherein the agent is a small molecule.

12. The method of claim 9, wherein the PD-1 ligand is PD-L1.

13. The method of claim 9, wherein the PD-1 ligand is PD-L2.

14. The method of claim 9, wherein the B7 polypeptide is B7-1.

Figure 1


Binding of PD-L1 cells
(%) Bound
40
35
30
25
20
15
10
5
0
Plate Bound
Blocker
Control Ig

B7-1 Ig
Control Ig
B7-1 Ig
CTLA-4 Ig
B7-1 Ig
1G10
B7-1 Ig
16-10A1
B7-1 Ig
PD-1-Ig
B7-1 Ig
10F.9G2
B7-1 Ig
10F.2H11
B7-2 Ig

Figure 2

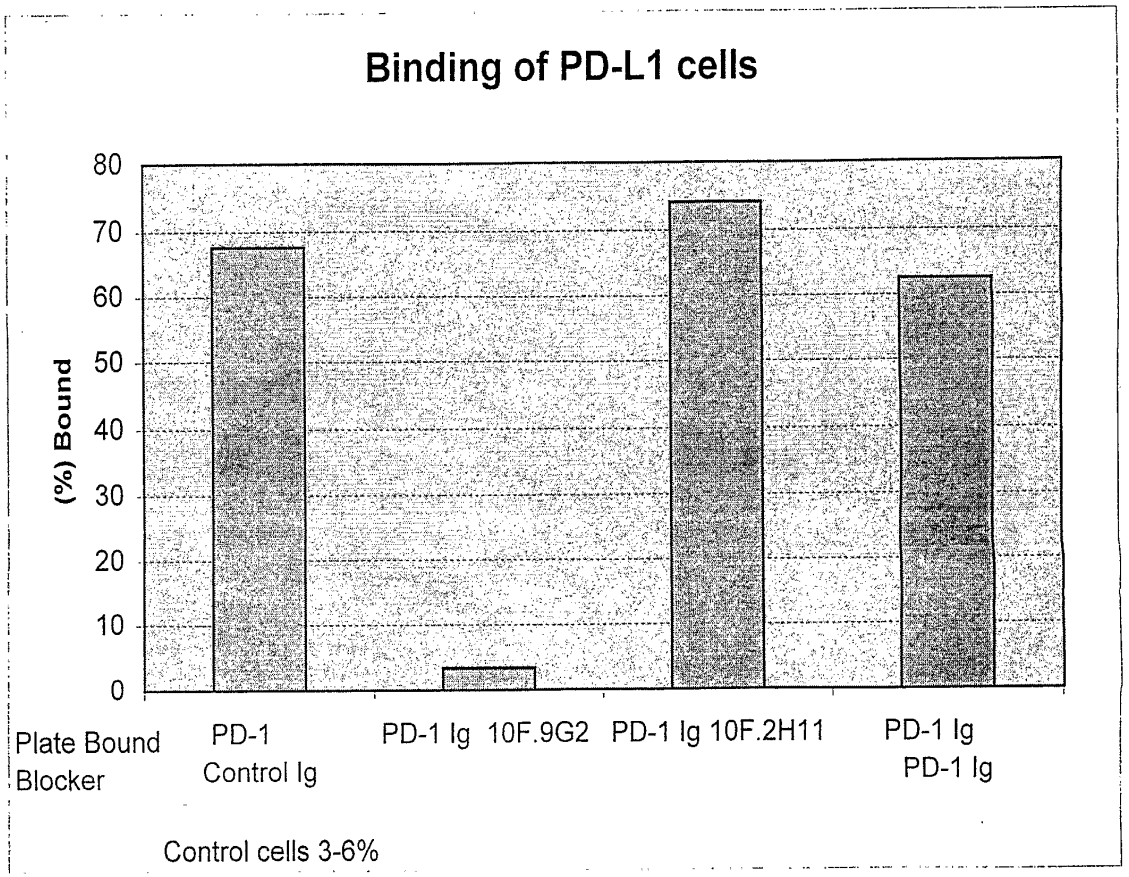
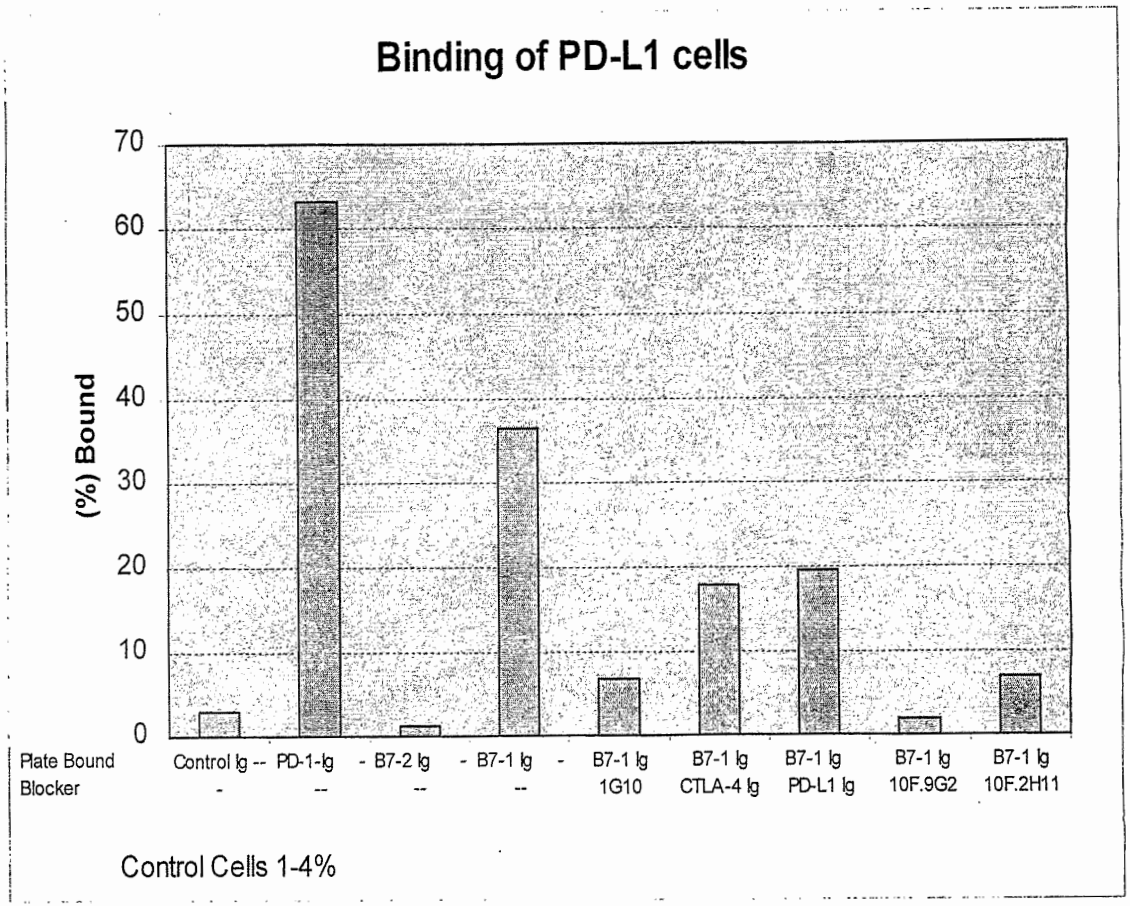

Binding of PD-L1 cells
(%) Bound
80
70
60
50
40
30
20
10
0
Plate Bound
Blocker
PD-1
Control Ig
PD-1 Ig 10F.9G2
PD-1 Ig 10F.2H11
PD-1 Ig
PD-1 Ig
Control cells 3-6%

Figure 3


Binding of PD-L1 cells
(%) Bound
70
60
50
40
30
20
10
0
Plate Bound
Blocker
Control Ig -- PD-1-Ig - B7-2 Ig - B7-1 Ig - B7-1 Ig 1G10 B7-1 Ig CTLA-4 Ig B7-1 Ig PD-L1 Ig B7-1 Ig 10F.9G2 B7-1 Ig 10F.2H11
- -- -- -- -- -- -- -- --
Control Cells 1-4%

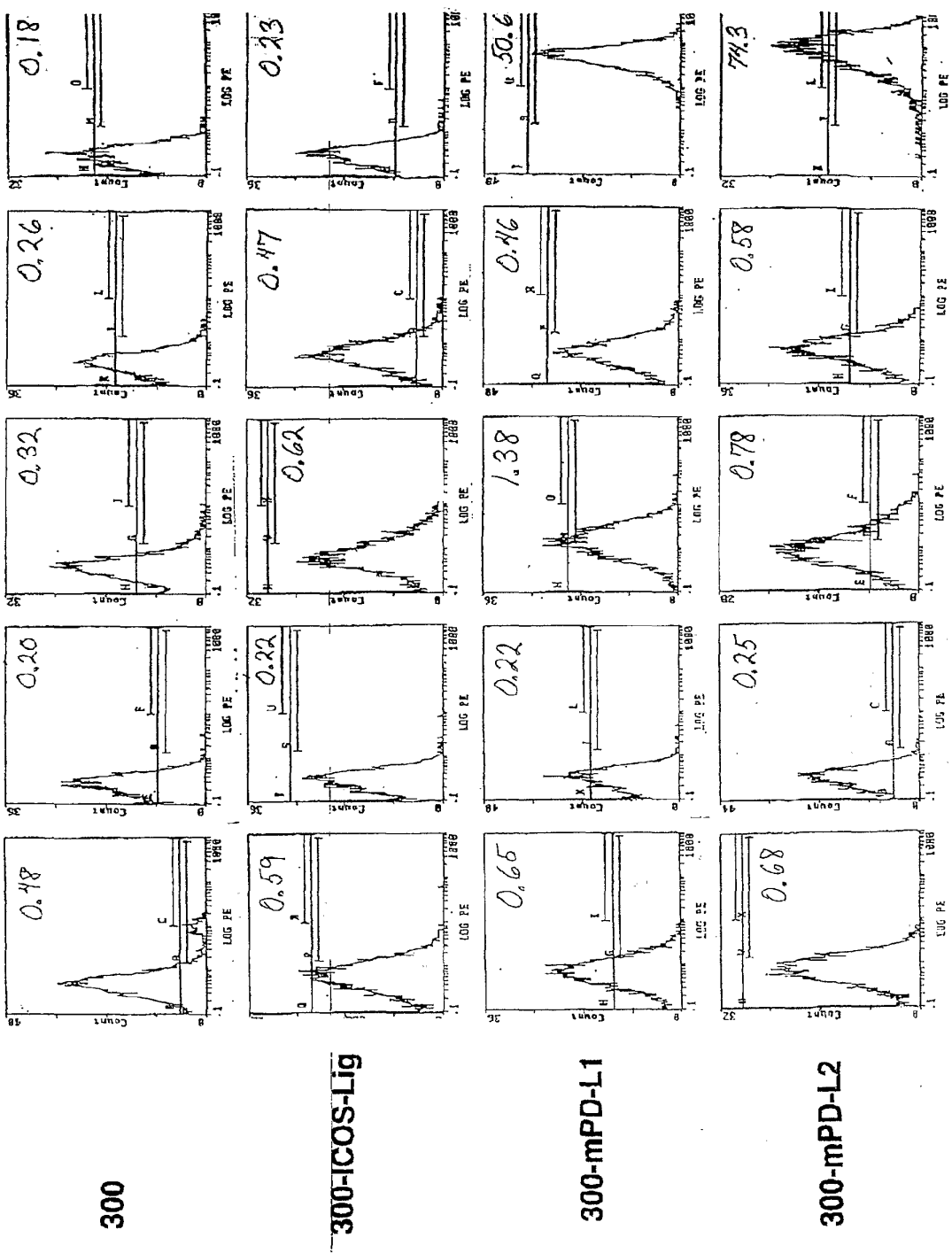


Figure 4

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Electronic Acknowledgement Receipt

EFS ID:	4073948
Application Number:	11913217
International Application Number:	
Confirmation Number:	1207
Title of Invention:	Human Monoclonal Antibodies To Programmed Death 1(PD-1) And Methods For Treating Cancer Using Anti-PD-1 Antibodies Alone or in Combination with Other Immunotherapeutics
First Named Inventor/Applicant Name:	Alan J Korman
Customer Number:	63367
Filer:	Steven Phillip Lendaris/Amanda Brown
Filer Authorized By:	Steven Phillip Lendaris
Attorney Docket Number:	077375.0499
Receipt Date:	07-OCT-2008
Filing Date:	
Time Stamp:	16:34:57
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		NYC_ECOPY13.pdf	317295 b7a62cb3f1c0e827985731de09f39329e7402b3e	yes	6

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Information Disclosure Statement Letter		1	2	
	Information Disclosure Statement (IDS) Filed (SB/08)		3	6	
Warnings:					
Information:					
2	NPL Documents	Binder1.pdf	19443813 7008f429d590f7c9eec8fb8e42b0b222fe41e1d7	no	107
Warnings:					
Information:					
3	NPL Documents	Untitled.pdf	15093339 44ca03b4df9b24b4379c54a151dd74e7e2955ccd	no	113
Warnings:					
Information:					
4	Foreign Reference	WO200101457.pdf	7891948 688ab7f987f8029ea80d46bfe2d288f98412d0e6	no	168
Warnings:					
Information:					
5	Foreign Reference	WO200207999.pdf	10498390 a7d85636f395f73aec39c5e06fdddef3de0b3060	no	213
Warnings:					
Information:					
6	Foreign Reference	WO200304202.pdf	4501893 9058197e82e33bc296c31c3927e1d9e39b45bcbd	no	72
Warnings:					
Information:					
		Total Files Size (in bytes):	57746678		

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 a 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.

1492

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET (FOR USE WITH FORM PTO-875)	SERIAL NO. 11/913217	FILING DATE
	APPLICANT(S)	

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
1	1		1			
2		1				
3		1				
4		1				
5		1				
6		1				
7		1				
8	1		1			
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13		1				
14		1				
15		1				
16	1					
17	1					
18	1					
19	1					
20	1					
21	1					
22	1					
23		1				
24		1				
25		1				
26		1				
27	1		1			
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29		1				
30		1				
31		1				
32		1				
33		1				
34		1				
35	1		1			
36		1				
37		1				
38		1				
39		1				
40		1				
41		1				
42		1				
43		36				
44		36				
45		36				
46		36				
47		36				
48		36				
49		36				
50		36		1		
TOTAL IND.	14					
TOTAL DEP.	886					
TOTAL CLAIMS	900					

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
51		36		1		36
52		36		1		36
53		36		1		36
54		36		1		36
55		36		1		8
56		36		1		
57		36		1		
58	1			1		
59		1		1		
60		1		1		
61		1		1		
62		1		1		
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92		1		1		
93		1		1		
94		1		1		
95		1		1		
96		8		1		
97		8		1		
98		8		1		
99		8		1		
100		8		1		
TOTAL IND.			6			
TOTAL DEP.			2			
TOTAL CLAIMS			2			

U.S. DEPARTMENT of COMMERCE

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2 of 2

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET (FOR USE WITH FORM PTO-875)	SERIAL NO. 11/913217	FILING DATE
	APPLICANT(S)	

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
1		6		1		
2		8		=		
3		8		=		
4		8		=		
5	1	0	1			
6						
7						
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9						
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41						
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45						
46						
47						
48						
49						
50						
TOTAL IND.	1	⬇	1	⬇		⬇
TOTAL DEP.	32	⬅	1	⬅		⬅
TOTAL CLAIMS	33		2			

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
51						
52						
53						
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91						
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93						
94						
95						
96						
97						
98						
99						
100						
TOTAL IND.		⬇		⬇		⬇
TOTAL DEP.		⬅		⬅		⬅
TOTAL CLAIMS						

U.S. DEPARTMENT of COMMERCE

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PATENT APPLICATION FEE DETERMINATION RECORD

Effective December 8, 2004

Application or Docket Number: 11/913217

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)	SMALL ENTITY RATE	SMALL ENTITY FEE	OR	LARGE ENTITY RATE	LARGE ENTITY FEE
U.S. NATIONAL STAGE FEES							
BASIC FEE			BASIC FEE		OR	BASIC FEE	310
EXAMINATION FEE			EXAM. FEE			EXAM. FEE	210
SEARCH FEE			SEARCH FEE			SEARCH FEE	410
FEE FOR EXTRA SPEC. PGS.	minus 100 =	/ 50 =	X $ 125 =			X $ 250 =	260
TOTAL CHARGEABLE CLAIMS	30 minus 20 =	10	X $ 25 =		OR	X $ 50 =	550
INDEPENDENT CLAIMS	7 minus 3 =	4	X $ 100 =		OR	X $ 200 =	810
MULTIPLE DEPENDENT CLAIM PRESENT		☐	+ $ 180 =		OR	+ $ 360 =	
* If the difference in column 1 is less than zero, enter "0" in column 2			TOTAL		OR	TOTAL	

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(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
Total	*	Minus	**	=	X $ 25 =		OR	X $ 50 =	
Independent	*	Minus	***	=	X $ 100 =		OR	X $ 200 =	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐					+ $ 180 =		OR	+ $ 360 =	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B

	(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
Total	*	Minus	**	=	X $ 25 =		OR	X $ 50 =	
Independent	*	Minus	***	=	X $ 100 =		OR	X $ 200 =	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐					+ $ 180 =		OR	+ $ 360 =	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. 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 number found in the appropriate box in column 1.

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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

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
11/913,217	Alan J Korman	077375.0499

63367
BAKER BOTTS L.L.P.
30 ROCKEFELLER PLAZA
44th Floor
NEW YORK, NY 10112-4498

INTERNATIONAL APPLICATION NO.	
PCT/JP2006/309606	
I.A. FILING DATE	PRIORITY DATE
05/02/2006	05/09/2005

CONFIRMATION NO. 1207
371 FORMALITIES LETTER

OC000000033464198

Date Mailed: 12/08/2008

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as an Elected Office (37 CFR 1.495):

- Priority Document
- Copy of the International Application filed on 10/31/2007
- Copy of the International Search Report filed on 10/31/2007
- Preliminary Amendments filed on 03/03/2008
- Information Disclosure Statements filed on 10/07/2008
- Biochemical Sequence Diskette filed on 03/03/2008
- Biochemical Sequence Listing filed on 03/03/2008
- U.S. Basic National Fees filed on 10/31/2007
- Priority Documents filed on 10/31/2007
- Specification filed on 10/31/2007
- Claims filed on 10/31/2007
- Abstracts filed on 10/31/2007
- Drawings filed on 10/31/2007
- Paper nucleotide sequence listings filed on 10/31/2007

The applicant needs to satisfy supplemental fees problems indicated below.

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.
- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.492(h) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**400** for a Large Entity:

- $**130** Surcharge.
- The fee for electronically submitting a specification and drawing containing more than 100 pages has not been paid in full. Applicant owes $**270** for **46** pages in excess of **150** pages for a large entity.

FORM PCT/DO/EO/905 (371 Formalities Notice)

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 32 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

VONDA M WALLACE

Telephone: (703) 308-9140 EXT 225

FORM PCT/DO/EO/905 (371 Formalities Notice)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	: Korman, *et al.*	Examiner	: TBD
Serial No.	: 11/913,217	Confirmation No.	: 1207
Filed	: 10/31/2007	Group Art Unit	: TBD
For	: HUMAN MONOCLONAL ANTIBOIDES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES ALONE OR IN COMBINATION WITH OTHER IMMUNOTHERAPEUTICS		

RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371

FILED VIA EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This paper is submitted in response to the Notification of Missing Requirements under 35 U.S.C. § 371 issued in connection with the above-identified application on December 8, 2008.

Applicants submit herewith a Combined Declaration and Power of Attorney in compliance with 37 C.F.R. § 1.497(a) and (b). Payment for the late oath or declaration surcharge is also enclosed.

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If any additional fee is due, or if any overpayment has been made, in connection with the filing of this response, the Commissioner is authorized to charge any such fee or credit any overpayment, to our Deposit Account No. 02-4377.

Respectfully submitted,

BAKER BOTTS L.L.P.

January 29, 2009
Date

Lisa D. Tyner
Patent Office Reg. No. 51,619

30 Rockefeller Plaza
44th Floor
New York, NY 10012-4498
Attorney for Applicant(s)
212-408-2500

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COMBINED DECLARATION AND POWER OF ATTORNEY

(Original, Design, National Stage of PCT, Divisional, Continuation or C-I-P Application)

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES ALONE OR IN COMBINATION WITH OTHER IMMUNOTHERAPEUTICS

This declaration is of the following type:

- ☐ Original
- ☐ Design
- ☒ National Stage of PCT/JP2006/309606 filed May 2, 2006
- ☐ Divisional
- ☐ Continuation
- ☐ Continuation-in-part (C-I-P)

the specification of which: *(complete (a), (b), or (c))*

(a) ☐ is attached hereto.
(b) ☒ was filed as United States Patent Application Serial No. 11/913,217 on 10/31/2007 and was amended on _____ *(if applicable).*
(c) ☐ was described and claimed in PCT International Application No. _____ filed on _____ and was amended on _____ *(if applicable).*

Acknowledgement of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

☐ In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.98.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT International Application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International Application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is claimed.

(complete (d) or (e))

(d) ☒ no such applications have been filed.
(e) ☐ such applications have been filed as follows:

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
COUNTRY APPLICATION NO.	DATE OF FILING (month, day, year)	DATE OF ISSUE (month, day, year)	PRIORITY CLAIMED UNDER 35 USC 119
ALL FOREIGN APPLICATION[S], IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date
60/679,466	05/09/2005
60/738,434	11/21/2005
60/748,919	12/08/2005

Claim for Benefit of Earlier U.S./PCT Application(s) under 35 U.S.C. 120

(complete this part only if this is a divisional, continuation or C-I-P application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status

Power of Attorney

As a named inventor, I hereby appoint the practitioners at Customer Number **63367** of the firm of BAKER BOTTS L.L.P., with offices at 30 Rockefeller Plaza, New York, New York 10112, as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO:
BAKER BOTTS L.L.P. 30 ROCKEFELLER PLAZA, NEW YORK, N.Y. 10112 CUSTOMER NUMBER: **63367**	BAKER BOTTS L.L.P. (212) 408-2500

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF FIRST INVENTOR	LAST NAME Korman	FIRST NAME Alan	MIDDLE NAME J.	
RESIDENCE & CITIZENSHIP	CITY Piedmont	STATE or FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 521 Cottonwood Drive	CITY Milpitas	STATE or COUNTRY California	ZIP CODE 95035
DATE 1/22/09	SIGNATURE OF INVENTOR Alan J. Korman 1/22/09 AK 1/22/09			
FULL NAME OF SECOND INVENTOR	LAST NAME Srinivasan	FIRST NAME Mohan	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY San Jose	STATE or FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP India	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 521 Cottonwood Drive	CITY Milpitas	STATE or COUNTRY California	ZIP CODE 95035
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF THIRD INVENTOR	LAST NAME Wang	FIRST NAME Changyu	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY Fremont	STATE or FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 521 Cottonwood Drive	CITY Milpitas	STATE or COUNTRY California	ZIP CODE 95035
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF FOURTH INVENTOR	LAST NAME Selby	FIRST NAME Mark	MIDDLE NAME J.	
RESIDENCE & CITIZENSHIP	CITY San Francisco	STATE or FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 521 Cottonwood Drive	CITY Milpitas	STATE or COUNTRY California	ZIP CODE 95035
DATE	SIGNATURE OF INVENTOR			
FULL NAME OF FIFTH INVENTOR	LAST NAME Chen	FIRST NAME Bing	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY Alameda	STATE or FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 521 Cottonwood Drive	CITY Milpitas	STATE or COUNTRY California	ZIP CODE 95035
DATE	SIGNATURE OF INVENTOR			

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF FIRST INVENTOR	LAST NAME: Korman	FIRST NAME: Alan	MIDDLE NAME: J.	
RESIDENCE & CITIZENSHIP	CITY: Piedmont	STATE or FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: United States	
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DATE	SIGNATURE OF INVENTOR			
FULL NAME OF SECOND INVENTOR	LAST NAME: Srinivasan	FIRST NAME: Mohan	MIDDLE NAME:	
RESIDENCE & CITIZENSHIP	CITY: San Jose	STATE or FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: India	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 521 Cottonwood Drive	CITY: Milpitas	STATE or COUNTRY: California	ZIP CODE: 95035
DATE: 1/14/09	SIGNATURE OF INVENTOR			
FULL NAME OF THIRD INVENTOR	LAST NAME: Wang	FIRST NAME: Changyu	MIDDLE NAME:	
RESIDENCE & CITIZENSHIP	CITY: ~~Fremont~~ Union City CW 1/13/09	STATE or FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 521 Cottonwood Drive	CITY: Milpitas	STATE or COUNTRY: California	ZIP CODE: 95035
DATE: 1/13/09	SIGNATURE OF INVENTOR			
FULL NAME OF FOURTH INVENTOR	LAST NAME: Selby	FIRST NAME: Mark	MIDDLE NAME: J.	
RESIDENCE & CITIZENSHIP	CITY: San Francisco	STATE or FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 521 Cottonwood Drive	CITY: Milpitas	STATE or COUNTRY: California	ZIP CODE: 95035
DATE: 1/13/09	SIGNATURE OF INVENTOR			
FULL NAME OF FIFTH INVENTOR	LAST NAME: Chen	FIRST NAME: ~~Bing~~ BING CHEN 1/22/09	MIDDLE NAME:	
RESIDENCE & CITIZENSHIP	CITY: Alameda	STATE or FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS: 521 Cottonwood Drive	CITY: Milpitas	STATE or COUNTRY: California	ZIP CODE: 95035
DATE: 1/22/09	SIGNATURE OF INVENTOR			

FULL NAME OF SIXTH INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	Cardarelli	Josephine	M.	
RESIDENCE & CITIZENSHIP	CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	San Carlos	California	United States	
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE or COUNTRY	ZIP CODE
	521 Cottonwood Drive	Milpitas	California	95035
DATE	SIGNATURE OF INVENTOR			
Jan 14, 2009	Josephine Cardarelli			

Check proper box(es) for any added page(s) forming a part of this declaration

☒ Signature for subsequent joint inventors. Number of pages added 1.

☐ Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. Number of pages added _____.

☐ Signature for inventor who refuses to sign, or cannot be reached, by person authorized under 37 CFR 1.47. Number of pages added _____.

Electronic Patent Application Fee Transmittal

Application Number:	11913217
Filing Date:	
Title of Invention:	Human Monoclonal Antibodies To Programmed Death 1(PD-1) And Methods For Treating Cancer Using Anti-PD-1 Antibodies Alone or in Combination with Other Immunotherapeutics
First Named Inventor/Applicant Name:	Alan J Korman
Filer:	Lisa Tyner/Amanda Brown
Attorney Docket Number:	077375.0499

Filed as Large Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Oath/decl > 30 months from priority date	1617	1	130	130
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**130**

Electronic Acknowledgement Receipt

EFS ID:	4701296
Application Number:	11913217
International Application Number:	
Confirmation Number:	1207
Title of Invention:	Human Monoclonal Antibodies To Programmed Death 1(PD-1) And Methods For Treating Cancer Using Anti-PD-1 Antibodies Alone or in Combination with Other Immunotherapeutics
First Named Inventor/Applicant Name:	Alan J Korman
Customer Number:	63367
Filer:	Lisa Tyner/Amanda Brown
Filer Authorized By:	Lisa Tyner
Attorney Docket Number:	077375.0499
Receipt Date:	29-JAN-2009
Filing Date:	
Time Stamp:	17:13:11
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$130
RAM confirmation Number	2757
Deposit Account	024377
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1		NYC_ECOPY17.pdf	530071 65e19e8b47ed84a6389d120949143dd78562cf58	yes	9

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Miscellaneous Incoming Letter	1	2
	Applicant Response to Pre-Exam Formalities Notice	3	4
	Oath or Declaration filed	5	9

Warnings:

Information:

2	Fee Worksheet (PTO-06)	fee-info.pdf	30229 693c237051e8cc6c959902103071b70152b31e85	no	2

Warnings:

Information:

Total Files Size (in bytes):	560300

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a 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.

PTO/SB/21 (10-07)
Approved for use through 10/31/2007. OMB 0651-0031
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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	11/913,217
Filing Date	10/31/2007
First Named Inventor	Korman, et al.
Art Unit	
Examiner Name	
Attorney Docket Number	077375.0499

ENCLOSURES *(Check all that apply)*

- [x] Fee Transmittal Form
  - [x] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [x] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):
  -Combined Declaration and Power of Attorney

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Baker Botts L.L.P.		
Signature			
Printed name	Lisa D. Tyner		
Date	01/29/2009	Reg. No.	51,619

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

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This collection of information is required by 37 CFR 1.5. 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 and1.14. This collection is estimated to 2 hours 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.**

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FEE TRANSMITTAL for FY 2007

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 130

Complete if Known	
Application Number	11/913,217
Filing Date	10/31/2007
First Named Inventor	Korman, et al.
Examiner Name	
Art Unit	
Attorney Docket No.	077375.0499

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☑ Deposit Account:

Deposit Account Number: 02-4377

Deposit Account Name: Baker Botts L.L.P.

The Director is authorized to: *(check all that apply)*

☑ Charge fee(s) indicated below ☑ Credit any overpayments

☑ Charge any additional fee(s) or any underpayment of fee(s)

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FEE CALCULATION

Extra Claim Fees

	Extra Claims		Fee		Fee Paid
Total Claims		x	52	=	$0
Independent Claims		x	220	=	$0
Multiple Dependent				=	$0
		SUBTOTAL			$0

Fee Description	Large Entity	Small Entity
Claims in excess of 20	52	26
Independent claims in excess of 3	220	110
Multiple dependent claim, if not paid	390	195

FEE CALCULATION (continued)

ADDITIONAL FEES

☑	Surcharge - late oath or filing fee	$130
☐	Non-English Specification	
☐	Extension for reply within first month	
☐	Extension for reply within second month	
☐	Extension for reply within third month	
☐	Extension for reply within fourth month	
☐	Extension for reply within fifth month	
☐	Notice of Appeal	
☐	Filing a brief in support of an appeal	
☐	Petition to revive - unavoidable	
☐	Petition to revive - unintentional	
☐	Utility Issue Fee	
☐	Design Issue Fee	
☐	Publication Fee	
☐	Petitions to the Commissioner	
☐	Request for Continued Examination (RCE)	
☐	Information Disclosure Statement (IDS)	
	Other fee -	
	SUBTOTAL	($) 130

SUBMITTED BY (Complete *(if applicable)*)

Name (Print/Type)	Lisa D. Tyner	Registration No. (Attorney/Agent)	51,619	Telephone	212-408-2500
Signature				Date	01/29/2009

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This collection of information is required by 37 CFR 1.17 and 1.27. 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 1.14. This collection is estimated to take 12 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.**

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	: Korman *et al.*	Customer No.	: 63367
Serial No.	: 11/913,217	Confirmation No.	: 1207
Filing Date	: 10/31/2007	Group Art Unit	: 1656
Examiner	: TBD		
For	: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1(PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES ALONE OR IN COMBINATION WITH OTHER IMMUNOTHERAPEUTICS		

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

FILED VIA EFS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Pursuant to 37 C.F.R. §§ 1.56 and 1.97(b)(3), applicants bring to the attention of the Examiner the documents listed on the attached Form PTO 1449 and respectfully request that the listed documents be considered by the Examiner and made of record in the above-captioned application.

This submission does not represent that a search has been made or that no better art exists and does not constitute an admission that the listed documents are material or constitute "prior art." If the Examiner applies the documents as prior art against any claim in the application and applicants determine that the cited documents do not constitute "prior art" under United States law, applicants reserve the right to present to the Office the relevant facts and law regarding the appropriate status of the documents.

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Applicants further reserve the right to take appropriate action to establish the patentability of the disclosed invention over the listed documents, should the documents be applied against the claims of the present application.

Applicants respectfully submit that each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement.

Applicants believe no fee is due in connection with this submission. If any additional fee is due, or if any overpayment has been made, the Commissioner is authorized to charge any such fee or credit any overpayment, to our Deposit Account No. 02-4377.

Respectfully submitted,

BAKER BOTTS L.L.P.

April 24, 2008
Date

Lisa D. Tyner
Patent Office Reg. No. 51,619

Lisa B. Kole
Patent Office Reg. No. 35,225

30 Rockefeller Plaza
44th Floor
New York, NY 10012-4498
Attorney for Applicant(s)
212-408-2500

Substitute for form 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** **(Use as many sheets as necessary)**		
	Application Number	11/913,217
	Filing Date	10/31/2007
	Inventor(s)	Korman, et al.
	Art Unit	1656
	Examiner Name	TBD
	Attorney Docket Number	077375.0499

FOREIGN PATENT DOCUMENTS

Exam Initial	No.	Document No.	Publication Date	Applicant(s)
	1.	WO/1997/007671	03/06/1997	KIRIN BEER KABUSHIKI KAISHA, *et al.* (English Abstract)
	2.	JP 07291996 (A)	11/07/1995	HONSHIYO YUU *et al.* (English Abstract)

Exam Initial	No.	**OTHER DOCUMENTS (including Author, Title, Date, Pertinent Pages, Etc.)**
	3.	WONG RM *et al.*, "Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs," *Int Immunol.* (2007), 19(10):1223-34.

Examiner Signature		Date Considered	

NY02:653999.1

* Examiner: Initial citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

(19)日本国特許庁（ＪＰ）　(12) **公開特許公報（Ａ）**　(11)特許出願公開番号

特開平7－291996

(43)公開日　平成７年(1995)11月７日

(51)Int.Cl.[6]	識別記号	庁内整理番号	ＦＩ		技術表示箇所
Ｃ０７Ｋ　14/52		8318－4H			
Ａ６１Ｋ　38/00	ＡＥＤ				
Ｃ０７Ｋ　16/24					
			Ａ６１Ｋ　37/ 02	ＡＥＤ	
		9281－4B	Ｃ１２Ｎ　15/ 00	ＺＮＡ　Ａ	

審査請求　未請求　請求項の数10　ＦＤ　（全 11 頁）　最終頁に続く

(21)出願番号　特願平7－61533

(22)出願日　平成７年(1995)２月27日

(31)優先権主張番号　特願平6－55224

(32)優先日　平６(1994)３月１日

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(74)代理人　弁理士　大家　邦久

最終頁に続く

(54)【発明の名称】　ヒトにおけるプログラムされた細胞死に関連したポリペプチド、それをコードするＤＮＡ、そのＤＮＡからなるベクター、そのベクターで形質転換された宿主細胞、そのポリペプチドの抗体、

(57)【要約】

【構成】　ヒトのプログラムされた細胞死に関連した新規な膜結合ポリペプチド（ＰＤ－１）、そのポリペプチドをコードするＤＮＡ、そのＤＮＡからなる複製及び発現ベクター、そのベクターで形質転換された宿主細胞、そのポリペプチドの抗体、及びそのポリペプチド又はその抗体を含有する薬学的組成物。

【効果】　ヒトＰＤ－１は、感染症、免疫機能の低下又は亢進、腫瘍等の治療剤等として用いることができる。ＰＤ－１をコードするＤＮＡはＰＤ－１を生産する際の必須の鋳型として用いられ、そのＤＮＡ又は断片を用いてＰＤ－１遺伝子の検出ができ、その遺伝子と生体防御機能、免疫機能、腫瘍等の疾患との関係の研究、疾患の診断等に利用できる。またＰＤ－１の抗体はＰＤ－１関連疾患の診断或いは治療剤としてに利用できる。

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【特許請求の範囲】

【請求項１】　実質的に純粋な形である配列番号１で示されるアミノ酸配列からなるポリペプチド、そのホモローグ、そのフラグメントまたはそのフラグメントのホモローグ。

【請求項２】　配列番号１で示されるアミノ酸配列からなる請求項１記載のポリペプチド。

【請求項３】　請求項１に記載されたポリペプチドをコードするＤＮＡ。

【請求項４】　配列番号２で示される塩基配列を有する請求項３記載のＤＮＡ、またはその配列に選択的にハイブリダイズするフラグメント。

【請求項５】　配列番号３で示される塩基配列を有する請求項３記載のＤＮＡ、またはその配列に選択的にハイブリダイズするフラグメント。

【請求項６】　請求項３から５のいずれかの項に記載のＤＮＡからなる複製または発現ベクター。

【請求項７】　請求項６記載の複製または発現ベクターで形質転換された宿主細胞。

【請求項８】　請求項１または２に記載されたポリペプチドを発現させるための条件下で請求項７記載の宿主細胞を培養することからなる該ポリペプチドの製造方法。

【請求項９】　請求項１または２に記載されたポリペプチドのモノクローナルまたはポリクローナル抗体。

【請求項１０】　請求項１または２に記載されたポリペプチドまたは請求項９記載の抗体および薬学的に許容される賦形剤および／または担体を含有することを特徴とする薬学的組成物。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明は細胞死に関連したポリペプチドに関する。さらに詳しくいえば、本発明は、ヒトにおけるプログラムされた細胞死に関連した新規なポリペプチド、そのポリペプチドをコードするＤＮＡ、そのＤＮＡからなるベクター、そのベクターで形質転換された宿主細胞、そのポリペプチドの抗体、およびそのポリペプチドまたはその抗体を含有する薬学的組成物に関する。

【０００２】

【発明の背景】発生学的に、また生理学的にコントロールされた細胞の死は、種々の動物のほとんどあらゆる組織で観察することができる。そのような細胞の死は一般的に「プログラムされた細胞死」と呼ばれ、病理学的メカニズムによって起こる「偶然に起こる細胞死」とは区別されている。動物における、種々のタイプのプログラムされた細胞死を一律に定義することは不可能であるが、死をプログラムされた細胞のほとんどは、死ぬためには新たにＲＮＡまたは蛋白のドゥノボ（de novo ）合成が必要であることが示されている。これらすべての事実は、何らかの遺伝子が（もしかしたら細胞死に特定の

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遺伝子ではないかもしれないが）、プログラムされた細胞死を起こすために発現されなければならないことを示している。

【０００３】一方、あるクラスの細胞死の形態学的特徴を表わすのに「アポトーシス」ということばが好んで用いられる。アポトーシスを受けた細胞では、染色体は核の周辺部に凝集してくるが、ミトコンドリアや他の細胞内小器官は大きく変化しない。生化学的には、アポトーシスによって死につつある細胞は、染色体は不規則にいくつかのヌクレオソームを単位としたＤＮＡ断片に分断されている。哺乳動物において、死をプログラムされたほとんどの細胞は、アポトーシスの形態学的および生化学的状態を示すようであるが、明らかにアポトーシス状態を示さない細胞もある。さらに、いかなる蛋白合成が行なわれなくてもアポトーシスによる細胞死が引き起こされることもある。このように、アポトーシスはプログラムされた細胞死と同義語でないことを理解しておくことは重要なことであろう。最近になって、発ガン遺伝子のひとつであるｂｃｌ－２は、細胞死を防ぐことによりＢ細胞を不死化していることが明らかにされ、細胞死の制御の重要性が示された。

【０００４】

【従来の技術】これまでに、プログラムされた細胞死に関連したポリペプチドが数種類報告されている。代表的なものとしてＦａｓ抗原が知られている［Itoh, N. et al.,Cell, 66, 233 (1991)］。ヒトのＦａｓ抗原は３３５個のアミノ酸からなるポリペプチドで、Ｎ末端には１６個の疎水性アミノ酸よりなるシグナルペプチドを有し、成熟蛋白はＮ末端側より細胞外領域（１５７アミノ酸）、膜貫通領域（１７アミノ酸）および細胞質領域（１４５アミノ酸）に区分されるような構造を有すると推定されている。そしてＦａｓ抗原は、細胞死を誘導する因子（リガンド）の受容体として機能していると考えられている。

【０００５】

【発明の目的】本発明は、Ｆａｓ抗原に代表される既知のポリペプチドとはまったく別個の、新規な哺乳動物のプログラムされた細胞死に深く関わるポリペプチドおよびそれをコードするＤＮＡを提供することを目的とする。本発明の他の目的は、前記ＤＮＡからなる複製および発現ベクター、前記ベクターで形質転換された宿主細胞、前記ポリペプチドの製造方法を提供することにある。さらに、本発明の他の目的は、前記ポリペプチドのモノクローナルおよびポリクローナル抗体、および前記ポリペプチドまたはその抗体および薬学的に許容される賦形剤および／または担体を含有する薬学的組成物を提供することにある。本発明者等は、プログラムされた細胞死に深く関わる遺伝子を単離し、その塩基配列を決定し、アミノ酸配列を推定した。その結果、まったく新規なポリペプチドおよびそれをコードするＤＮＡを見出す

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ことに成功し、本発明を完成した。

【０００６】ヒトにおけるプログラムされた細胞死に深く関わる遺伝子を単離するために、本発明者らは、マウスのＴ細胞ハイブリドーマ、２Ｂ 4.11 から得たマウスＰＤ－１（特開平5-336973号）をプローブとして用いた。本発明で同定された、プログラムされた細胞死に関連したポリペプチドのアミノ酸配列を、ナショナルバイオメディカルリサーチファンデーションのデータベースに登録されている既知のポリペプチドのアミノ酸配列と比較してみたが、同一または類似のアミノ酸配列を有しているものは、マウスＰＤ－１以外にはなかった。もちろん、該ポリペプチドはＦａｓ抗原とはまったく相同性のないことも確認された。

【０００７】

【発明の構成】本発明は、ヒトにおける、プログラムされた細胞死に深く関わるポリペプチド（以下、ヒトＰＤ－１という。）に関する。本発明は、実質的に純粋な形である配列番号１で示されるアミノ酸配列からなるポリペプチド、そのホモローグ、その配列のフラグメント、およびそのホモローグに関する。本発明はさらにそれらのポリペプチドをコードするＤＮＡに関する。より具体的には、配列番号２または３で示される塩基配列を有するＤＮＡ、および配列番号２または３で示される塩基配列に選択的にハイブリダイズするフラグメントを有するＤＮＡに関する。

【０００８】特に本発明は、（１）配列番号１で示されるアミノ酸配列からなるポリペプチド、（２）前記（１）に記載したポリペプチドをコードするＤＮＡ、（３）配列番号２で示される塩基配列を有するＤＮＡ、および（４）配列番号３で示される塩基配列を有するＤＮＡに関する。

【０００９】実質的に純粋な形である配列番号１で示されるアミノ酸配列を有するポリペプチドとは、一般に、生産時のポリペプチドの９０％以上、例えば９５、９８または９９％が配列番号１で示されるアミノ酸配列を有するポリペプチドであることを意味する。

【００１０】配列番号１で示されるアミノ酸配列からなるポリペプチドのホモローグとは、一般に少なくとも２０個、好ましくは少なくとも３０個、例えば４０、６０または１００個の連続したアミノ酸領域で、少なくとも７０％、好ましくは少なくとも８０または９０％、より好ましくは９５％以上相同性のあるものであり、そのようなホモローグは、以後本発明のポリペプチドとして記載される。さらに、配列番号１で示されるアミノ酸配列からなるポリペプチドのフラグメント、またはそれらのホモローグのフラグメントとは、少なくとも１０アミノ酸、好ましくは少なくとも１５アミノ酸、例えば２０、２５、３０、４０、５０または６０アミノ酸部分を意味する。

【００１１】配列番号２または３で示される塩基配列を

有するＤＮＡに選択的にハイブリダイズするＤＮＡとは、一般に、少なくとも２０個、好ましくは少なくとも３０個、例えば４０、６０または１００個の連続した塩基配列領域で、少なくとも７０％、好ましくは少なくとも８０または９０％、より好ましくは９５％以上の相同性のあるものであり、そのようなＤＮＡは、以後本発明のＤＮＡとして記載される。配列番号２または３で示される塩基配列を有するＤＮＡのフラグメントとは、少なくとも１０塩基、好ましくは少なくとも１５塩基、例えば２０、２５、３０または４０塩基部分を意味し、そのようなフラグメントも本発明のＤＮＡに含まれる。

【００１２】さらに、本発明には、本発明のＤＮＡからなる複製または発現ベクターが含まれる。ベクターとしては、例えば、ｏｒｉ領域と、必要により上記ＤＮＡの発現のためのプロモーター、プロモーターの制御因子などからなるプラスミド、ウィルスまたはファージベクターが挙げられる。ベクターはひとつまたはそれ以上の選択的マーカー遺伝子、例えばアンピシリン耐性遺伝子を含んでいてもよい。ベクターは、イン・ビトロ（in vitro）において、例えば、ＤＮＡに対応するＲＮＡの製造、宿主細胞の形質転換に用いることができる。

【００１３】さらに、本発明には、配列番号２または３で示される塩基配列、またはそれらのオープンリーディングフレームを有するＤＮＡを含む本発明のＤＮＡを複製または発現させるためのベクターで形質転換された宿主細胞も含まれる。細胞としては、例えば細菌、酵母、昆虫細胞または哺乳動物細胞が挙げられる。

【００１４】さらに、本発明には、本発明のポリペプチドを発現させるための条件下で、本発明の宿主細胞を培養することからなる本発明のポリペプチドの製造方法も含まれる。培養は、本発明のポリペプチドが発現し、宿主細胞より製造される条件下で行われることが好ましい。本発明のＤＮＡは、上記のようなベクターのアンチセンス領域に挿入することでアンチセンスＲＮＡを製造することもできる。このようなアンチセンスＲＮＡは、細胞中の本発明のポリペプチドのレベルを制御することに用いることもできる。

【００１５】本発明は、本発明におけるポリペプチドのモノクローナルまたはポリクローナル抗体も含む。さらに本発明におけるポリペプチドのモノクローナルまたはポリクローナル抗体の製造方法も含む。モノクローナル抗体は、本発明のペプチドまたは、その断片を抗原として用い、通常のハイブリドーマの技術により製造することができる。ポリクローナル抗体は、宿主動物（例えば、ラットやウサギ等）に本発明のポリペプチドを接種し、免疫血清を回収する、通常の方法により製造することができる。

【００１６】本発明には、本発明のポリペプチド、その抗体と薬学的に許容される賦形剤および／または担体を含有する薬学的組成物も含まれる。本発明のポリペプチ

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ドとしては、配列番号１で示されたアミノ酸配列を有するもの以外に、その一部が欠損したもの（例えば、配列番号１中、生物活性の発現に必須な部分だけから成るポリペプチド）、その一部が他のアミノ酸と置換したもの（例えば、物性の類似したアミノ酸に置換したもの）、およびその一部に他のアミノ酸が付加または挿入されたものも含まれる。

【００１７】よく知られているように、ひとつのアミノ酸をコードするコドンは１～６種類（例えば、Ｍｅｔは１種類、Ｌｅｕは６種類）知られている。従って、ポリペプチドのアミノ酸配列を変えることなくＤＮＡの塩基配列を変えることができる。（２）で特定される本発明のＤＮＡには、（１）の配列番号１で示されるポリペプチドをコードするすべての塩基配列群が含まれる。塩基配列を変えることによって、ポリペプチドの生産性が向上することがある。

【００１８】（３）の配列番号２で特定されるＤＮＡは、（２）で示されるＤＮＡの一態様であり、天然型配列を表わす。（４）の配列番号３で示されるＤＮＡは、（３）で特定されるＤＮＡに天然の非翻訳部分を加えた配列を示す。

【００１９】本発明のＤＮＡは、遺伝子組換え法、合成法あるいは当業者に公知の方法によって取得することができる。ヒトＰＤ－１には、Ｆａｓ抗原とはその構造的特徴のまったく異なる一連の（種々の哺乳動物に共通の）ポリペプチド群が含まれる。すなわち、本発明によってそのアミノ酸配列が明らかにされたヒトＰＤ－１をはじめとし、これらと高いホモロジー（ヒトＰＤ－１の抗体と交差反応を生じるというような免疫学的同等性を意味する）を有する他の哺乳動物のＰＤ－１が含まれる。

【００２０】本発明のヒトＰＤ－１の構造的特徴は以下のとおりである。ヒトＰＤ－１は２８８個のアミノ酸からなる膜結合型蛋白であると考えられる。Ｎ末端部分と中間部分にふたつの疎水性領域を有しており、それらはそれぞれシグナルペプチと細胞膜貫通領域に相当すると思われる。ヒトＰＤ－１のＮ末端のシークエンスを、典型的シグナルペプチドの切断部位と較べてみると、ヒトＰＤ－１のシグナルペプチドは、１番目のＭｅｔから２０番目のＡｒｇまでと推定される。従って、成熟蛋白部分は２１番目のＰｒｏから２８８番目のＬｅｕまでの２６８アミノ酸よりなり、細胞外領域（１４７アミノ酸）、膜貫通領域（２５アミノ酸）および細胞質領域（９６アミノ酸）で構成されているものと考えられる。また細胞外領域には４ヶ所のＮ－グリコシレーションサイトが存在する可能性がある。

【００２１】ヒトＰＤ－１のアミノ酸配列をナショナルバイオメディカルリサーチファンデーションのデータベースに登録されたすべてのアミノ酸配列と照合してみると、ヒトＰＤ－１の細胞外領域は、既知の免疫グロブリンスーパーファミリーに属する数種類のポリペプチドとホモロジーを有していることがわかる。免疫グロブリンドメインは、保持されたアミノ酸パターンと逆行性β－ストランドの数に基づいて分類されているＶ、Ｃ１およびＣ２セットを有する。５４番目のＣｙｓと１２３番目のＣｙｓに挟まれた６８個のアミノ酸配列はジスルフィドで結合されたＶセット配列に類似している。また、４個のアミノ酸残基（９４Ａｒｇ，９５Ｐｈｅ，１１７Ａｓｐおよび１１９Ｇｌｙ）は多くの免疫グロブリンのＶセット配列に特徴的なものである。

【００２２】細胞質領域は、既知のいかなる配列とも明白なホモロジーを有していないが、注意深く調べると、抗原受容体やＦｃ受容体に関連する数種のポリペプチドが有する細胞質領域の尾部にみられる共通の配列（Asp/Glu-X8-Asp/Glu-X2-Tyr-X2-Leu/Ile-X7-Tyr-X2- Leu/Ile）の変形した配列を含んでいることがわかる。最近、この共通配列が１単位あれば、シグナルを伝えるのに十分であることが示されている。他の哺乳動物のＰＤ－１も配列中のアミノ酸の数や種類に多少の違いはあるが、その構造上の特徴はヒトＰＤ－１に類似したものであると考えられる。

【００２３】本発明のヒトＰＤ－１ポリペプチドをコードするＤＮＡは、以下の方法により作製することができる。ＰＤ－１をコードするＤＮＡの塩基配列が、一旦確定されると、その後は、化学合成によって、あるいは該塩基配列の断片を化学合成し、またはＰＣＲ法によりこれをプローブとしてハイブリダイズさせることにより、本発明のＤＮＡを得ることができる。さらに、本ＤＮＡを含有するベクターＤＮＡを適当な宿主に導入し、これを増殖させることによって、目的とする本発明ＤＮＡを必要量得ることができる。

【００２４】本発明のＰＤ－１ポリペプチドを取得する方法としては、（１）生体または培養細胞から精製単離する方法、（２）ペプチド合成する方法、または（３）遺伝子組換え技術を用いて生産する方法、などが挙げられるが、工業的には（３）に記載した方法が好ましい。遺伝子組換え技術を用いてポリペプチドを生産するための発現系（宿主－ベクター系）としては、例えば、細菌、酵母、昆虫細胞および哺乳動物細胞の発現系が挙げられる。

【００２５】例えば、大腸菌で発現させる場合には、成熟蛋白部分をコードするＤＮＡの５′末端に開始コドン（ＡＴＧ）を付加し、得られたＤＮＡを、適当なプロモーター（例えば、ｔｒｐプロモーター、ｌａｃプロモーター、λＰＬプロモーター、Ｔ７プロモーター等）の下流に接続し、大腸菌内で機能するベクター（例えば、ｐＢＲ３２２、ｐＵＣ１８、ｐＵＣ１９等）に挿入して発現ベクターを作製する。次に、この発現ベクターで形質転換した大腸菌（例えば、E.ColiＤＨ１、E.ColiＪＭ１０９、E.ColiＨＢ１０１株等）を適当な培地で培養し

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て、その菌体より目的とするポリペプチドを得ることができる。

【００２６】また、バクテリアのシグナルペプチド（例えば、ｐｅｌＢのシグナルペプチド）を利用すれば、ペリプラズム中に目的とするポリペプチドを分泌することもできる。さらに、他のポリペプチドとのフュージョン・プロテイン（fusion protein）を生産することもできる。また、哺乳動物細胞で発現させる場合には、例えば、ＰＤ－１の全長をコードするＤＮＡを適当なベクター（例えば、レトロウイルスベクター、パピローマウイルスベクター、ワクシニアウイルスベクター、ＳＶ４０系ベクター等）中の適当なプロモーター（例えば、ＳＶ４０プロモーター、ＬＴＲプロモーター、メタロチオネインプロモーター等）の下流に挿入して発現ベクターを作製する。

【００２７】次に、得られた発現ベクターで適当な哺乳動物細胞（例えば、サルＣＯＳ－７細胞、チャイニーズハムスターＣＨＯ細胞、マウスＬ細胞等）を形質転換し、形質転換体を適当な培地で培養することによって、その培養液中に目的とするポリペプチドが分泌される。以上のようにして得られたポリペプチドは、一般的な生化学的方法によって単離精製することができる。本発明によって得られるＰＤ－１遺伝子をコードするＤＮＡは、これをプローブとして他の動物のＰＤ－１遺伝子を分離することができる。配列番号３で示される塩基配列を有するｃＤＮＡの作製は、以下の方法に従って行なわれる。

【００２８】すなわち、(i) 本発明のポリペプチドを産生する細胞、例えば、ヒト食道癌セルラインからｍＲＮＡを分離し、(ii)該ｍＲＮＡからファーストストランド（１本鎖ｃＤＮＡ）、次いでセカンドストランド（２本鎖ｃＤＮＡ）を合成し（ｃＤＮＡの合成）、(iii) 該ｃＤＮＡを適当なファージベクターに組込み、(iv) 得られた組換えファージを宿主細胞に感染させ（ｃＤＮＡライブラリーの作製）、(v) 得られたｃＤＮＡライブラリーをマウスＰＤ－１　ｃＤＮＡをプローブに用いてプラークハイブリダイゼーションによりスクリーニングし、(vi)得られたポジティブクローンよりファージＤＮＡを調製し、切り出したｃＤＮＡをプラスミドベクターにサブクローニングし、制限酵素地図を作成し、(vii) 各制限酵素断片の塩基配列を決定し、連結させることで全長をシークエンスする事によって作成することができる。

【００２９】より詳細に説明すると、工程(i) は、ヒトセルラインを適当な刺激剤（例えば、ＩＬ－１等）で刺激するか、または無刺激のままで、オカヤマ（Okayama, H.）等の方法（Method in Enzymology, 154 , 3 (1987)　参照）に従って行なわれる。本ポリペプチドを産生する細胞としては、好ましくはヒトＹＴＣ３セルラインが挙げられる。工程(ii)、(iii) および(iv)は、ｃＤＮＡライブラリー作製の工程であり、改変した Gubler & Hoffman法 (Gene, 25, 263 (1983)参照) に準じて行なわれる。　工程(iii) で用いられるベクターとしては、プラスミドベクター（例えば、ｐＢＲ３２２、pBluescript II 等）やファージベクター（例えば、λｇｔ１０、λＤＡＳＨ II 等）が多数知られているが、好適にはファージベクターλｇｔ１０（43.3 kbp；Stratagene社より販売）が用いられる。

【００３０】工程(iv)で用いられる細胞宿主としては、好ましくは、大腸菌ＮＭ５１４（Stratagene社より販売）が用いられる。工程(v) および(vi)は、Molecular Cloning （Sambrook, J., Fritsh, E. F.,および Maniatis, T, Cold Spring Harbor Laboratory Press (1989)）に記載の方法に準じて行なわれる。工程(vii) のシークエンシングは、マキサム－ギルバート(Maxam-Gilbert) 法やジデオキシターミネーター法により行なわれる。

【００３１】この様にして得られたｃＤＮＡは、該ｃＤＮＡが全長またはほぼ全長であることを確認する必要がある。この確認は、該ｃＤＮＡをプローブとして、ノーザン（Northern）解析により行なわれる（前記 Molecular Cloning　参照）。ハイブリダイズしたバンドから得られるｍＲＮＡのサイズと該ｃＤＮＡのサイズを比較し、ほぼ同じであれば該ｃＤＮＡはほぼ全長であると考えられる。それらのＰＤ－１遺伝子をコードするＤＮＡあるいはＤＮＡ断片はプローブあるいはプライマーとしてＰＤ－１遺伝子の検出ができ、これによって該遺伝子と生体防御機能、免疫機能あるいは腫瘍等の疾患との関係の研究あるいは疾患の診断等に利用することができる。

【００３２】また、それらのＤＮＡは通常の遺伝子組換え法によって多大な有用性が期待されるＰＤ－１ポリペプチド、ポリペプチド断片あるいはその誘導体を生産する際の必須の鋳型として用いることができる。そのようにして生産されたポリペプチド、ポリペプチド断片あるいは修飾ポリペプチドは感染症、免疫機能の低下または亢進あるいは腫瘍等の治療剤として用いることが期待される。

【００３３】さらに、本発明のポリペプチドあるいはその断片を抗原として常法によりポリクローナル抗体およびモノクローナル抗体を作製できるので、それらを用いて該ポリペプチドを定量することによって該ポリペプチドと疾患との関係の研究あるいは疾患の診断等に利用することができる。また、該モノクローナル抗体はそのままの形あるいはヒト抗体とのキメラ抗体の形で治療剤として用いることができる。

【００３４】

【医薬品への適用】本発明の目的のために本発明のポリペプチドは通常、全身的又は局所的に、一般的には経口または非経口の形で投与される。好ましくは、経口投与、静脈内投与および脳室内投与である。投与量は、年

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齢、体重、症状、治療効果、投与方法、処理時間等により異なるが、通常、成人一人あたり、一回につき、１００μｇから１００ｍｇの範囲で、一日一回から数回経口投与されるかまたは、成人一人当り、一回につき、１０μｇから１００ｍｇの範囲で、一日一回から数回非経口投与される。もちろん前記したように、投与量は、種々の条件により変動するので、上記投与量より少ない量で十分な場合もあるし、また範囲を越えて必要な場合もある。

【００３５】本発明化合物を投与する際には、経口投与のための固体組成物、液体組成物およびその他の組成物、非経口投与のための注射剤、外用剤、座剤等として用いられる。経口投与のための固体組成物には、錠剤、丸剤、カプセル剤、散剤、顆粒剤等が含まれる。カプセルには、ソフトカプセルおよびハードカプセルが含まれる。このような個体組成物においては、一つまたはそれ以上の活性物質が、少なくとも一つの不活性な希釈剤（例えば、ラクトース、マンニトール、グルコース、ヒドロキシプロピルセルロース、微結晶セルロース、デンプン、ポリビニルピロリドン、メタケイ酸アルミン酸マグネシウム等）と混合される。組成物は、常法に従って、不活性な希釈剤以外の添加物、例えば、潤滑剤（ステアリン酸マグネシウム等）、崩壊剤（繊維素グリコール酸カルシウム等）、安定化剤（ヒト血清アルブミン、ラクトース等）、溶解補助剤（アルギニン、アスパラギン酸等）を含有していてもよい。

【００３６】錠剤または丸剤は、必要により白糖、ゼラチン、ヒドロキシプロピルセルロース、ヒドロキシプロピルメチルセルロースフタレート等の胃溶性あるいは腸溶性のフィルムで皮膜してもよいし、また２以上の層で皮膜してもよい。さらにゼラチンのような吸収されうる物質のカプセルも包含される。経口投与のための液体組成物は、薬学的に許容される乳濁剤、溶液剤、懸濁剤、シロップ剤、エリキシル剤等を含み、一般に用いられる不活性な希釈剤（例えば、精製水、エタノール等）を含んでいてもよい。この様な組成物は、不活性な希釈剤以外に湿潤剤、懸濁剤のような補助剤、甘味剤、風味剤、芳香剤、防腐剤を含有していてもよい。

【００３７】経口投与のためのその他の組成物としては、ひとつまたはそれ以上の活性物質を含み、それ自体公知の方法により処方されるスプレー剤が含まれる。この組成物は不活性な希釈剤以外に亜硫酸水素ナトリウムのような安定剤と等張性を与えるような安定化剤、塩化ナトリウム、クエン酸ナトリウムあるいはクエン酸のような等張剤を含有していてもよい。スプレー剤の製造方法は、例えば米国特許第2,868,691 号および同第3,095,355 号明細書（参考のために記載した）に詳しく記載されている。

【００３８】本発明による非経口投与のための注射剤としては、無菌の水性または非水性の溶液剤、懸濁剤、乳濁剤を包含する。水性または非水性の溶液剤、懸濁剤としては、一つまたはそれ以上の活性物質が、少なくとも一つの不活性な希釈剤と混合される。水性の希釈剤としては、例えば注射用蒸留水および生理食塩水が挙げられる。非水性の希釈剤としては、例えばプロピレングリコール、ポリエチレングリコール、オリーブ油のような植物油、エタノールのようなアルコール類、ポリソルベート８０（登録商標）等が挙げられる。

【００３９】このような組成物は、さらに防腐剤、湿潤剤、乳化剤、分散剤、安定化剤（例えば、ヒト血清アルブミン、ラクトース等）、溶解補助剤（例えば、アルギニン、アスパラギン酸等）のような補助剤を含んでいてもよい。これらはバクテリア保留フィルターを通す濾過、殺菌剤の配合または照射によって無菌化される。これらはまた無菌の固体組成物を製造し（例えば、凍結乾燥法等により）、使用前に無菌の注射用蒸留水または他の溶媒に溶解して使用することもできる。非経口投与のためのその他の組成物としては、ひとつまたはそれ以上の活性物質を含み、常法により処方される外用液剤、軟膏、塗布剤、直腸内投与のための坐剤およびペッサリー等が含まれる。

【００４０】

【実施例】以下に実施例を挙げて本発明をより具体的に説明するが、これらは本発明の範囲を制限するものではない。

【００４１】実施例１：細胞の培養

ヒトセルライン（CESS、HPB-ALL 、Jarkat、YTC3、CCRF-CEM、ＪＭおよびMOLT-4F ）を、１０％熱非働化ウシ胎児血清、２ｍＭグルタミン、５０μＭ　２－メルカプトエタノール、１００Ｕ／ｍｌペニシリンおよび１００μｇ／ｍｌストレプトマイシンを添加したＲＰＭＩ 1640（Gibco 社製）中で培養した。

【００４２】実施例２：ノザンブロッティング

グアニジウム・イソシアネート法（前記 molecular cloning 参照）によりそれぞれのセルラインより全ＲＮＡを調製した。次にオリゴテックスｄＴ３０スーパー（oligotex－ｄＴ３０「ｓｕｐｅｒ」）（第一化学薬品）により、全ＲＮＡからpoly（Ａ）$^{+}$ ＲＮＡのみを分離した。３μｇ のpoly（Ａ）$^{+}$ ＲＮＡを 1.2%ホルムアルデヒド－アガロースゲル上で電気泳動により分離した後、ナイロン膜フィルター（Biodyne Ａ，日本ジェネティック社）に移した。フィルターは、８０℃で２時間焼き付けた。ランダムプライミング法によって、^{32}ＰでラベルされたマウスｐＤ－１のコーディング領域を含むＥｃｏＲＩ断片（１ｋｂ）をプローブとして用いた。このプローブの比活性は、１×１０^{9} d.p.m./μｇであった。ハイブリダイゼーションは、１０×Denhardt′s、１Ｍ　ＮａＣｌ、５０ｍＭ　Ｔｒｉｓ（ｐＨ 7.5）、１０ｍＭ　ＥＤＴＡ、１％ＳＤＳおよび１ｍｇ／ｍｌの超音波処理されたサケ精子ＤＮＡを含む溶液中、６０℃で１５時間

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行った。次にフィルターを、１×ＳＳＣ、0.1％ＳＤＳ中、６０℃で１０分間洗浄した。オートラジオグラフィーの結果、リンパ系セルラインＹＴＣ３より、約 2.3ｋｂのハイブリダイズ信号が得られた。

【００４３】実施例３：ｃＤＮＡライブラリーの作成およびヒトＰＤ－１　ｃＤＮＡのクローニング

ＹＴＣ３細胞株から抽出された５μｇのpoly（Ａ）$^+$ ＲＮＡから、タイム・セイバー・ｃＤＮＡ合成キット（Time Saver cDNA Synthesis Kit）（Pharmacia社製）を用いて、ｃＤＮＡのライブラリーを作成した。ファーストストランドｃＤＮＡの合成には、Oligo（ｄＴ）プライマーを用いた。ＥｃｏＲＩ－ＮｏｔＩアダプターをつないだ二本鎖ｃＤＮＡをλｇｔ１０ベクターにクローニングし、パッケージングを行った（GIGAPACK II GOLD, Stratagene社製）。これを E.ColiNM５１４を蒔いたプレートに重層した。ファージＤＮＡを各プレートにつき、２枚ずつのフィルターに移しとり、８０℃で２時間焼きつけた後、６０℃で１５時間ハイブリダイズした。Bluescript　ＳＫプラスミドベクター（Stratagene社製）から、ＥｃｏＲＩで切り出したマウスＰＤ－１コード領域（１ｋｂ）をプローブとして用いた。フィルターは、１×ＳＳＣ、0.1％ＳＤＳで６０℃、１０分間洗浄した。オートラジオグラフィーの結果、1.2×10^6 ファージのうち、５１個がポジティブな信号を与えた。単一になるまで精製した後、２３クローンを選び、さらに解析をした結果、最長のｃＤＮＡはインサートは、 2.1ｋｂであった。この結果は、サザンブロッティングの結果とよく一致した。

【００４４】実施例４：ＤＮＡのシークエンシング

ヒトｃＤＮＡライブラリーから分離されたファージのｃＤＮＡ挿入部を Bluescript ＳＫプラスミドベクター（Stratagene社製）にサブクローニングし、修飾Ｔ７ＤＮＡポリメラーゼ（United States Biochemical 社製）および［α－３２Ｐ］ｄＣＴＰ（3000Ｃｉ/mmol, Amersham 社製）を用いたジデオキシヌクレオチドチェーンターミネーション法（Sangerら, 1977）によりシークエンシングを行った。シークエンシングプライマーには、Bluescript　プラスミドに特異的なプライマーを用いた。ヌクレオチド配列決定は、ｃＤＮＡの両鎖を完全解析することにより行った。これにより、配列番号２に示されるヌクレオチド配列を得た。全長のヌクレオチド配列を配列番号３に示す。配列番号２のヌクレオチド配列より、配列番号１で示されるペプチド配列を決定した。推定されるアミノ酸の総数は２８８個であり、マウスＰＤ－１と同数であった。また、そのホモロジーは、約６０％であった。また、配列番号３で示される全長のヌクレオチド配列とそれによってコードされる推定アミノ酸を併せて配列番号４に示す。

【００４５】実施例５：サザンブロッティング

常法（前記のmolecular cloning 参照）により、種々の動物細胞からゲノムＤＮＡを分離した。ＤＮＡをＥｃｏＲＩ、ＢａｍＨＩまたはＨｉｎｄ IIIによって、マニュファクチュアーズレコメンデーションに従い消化し、電気泳動（１００Ｖ、 0.8％アガロースゲル、ＴＥＡ緩衝液）により、分離した。ＤＮＡ断片を、0.25Ｎ　ＨＣｌで１０分間洗浄し、 0.2Ｎ　ＮａＯＨ／ 0.6Ｍ　ＮａＣｌで３０分間変性し、 0.6Ｍ　ＮａＣｌ／ 0.2Ｍトリス（ｐＨ 7.5）で１時間中和したのち、通常のサザンブロッティングに使用されるナイロン膜（ Bidyne Ａ）に移した。フィルターは、８０℃で２時間焼きつけた。

【００４６】ヒトＰＤ－１のコード領域を含むEcoRI-StuI断片（９００ｂｐ）を、ランダムプライミング法によって、^{32}Ｐでラベルし、プローブとして用いた。このプローブの比活性は、9×10^8 d.p.m./ μｇ であった。ハイブリダイゼーションは、１０×Denhardt′ｓ　１Ｍ　ＮａＣｌ、５０mMトリス（ｐＨ 7.5）、１０mMＥＤＴＡ、１％ＳＤＳおよび１ｍｇ／ｍｌの超音波処理されたサケ精子ＤＮＡ中で６５℃で１５時間行った。フィルターは、 0.1×ＳＳＣ、 0.1％ＳＤＳ中、６５℃で１０分間洗浄した。オートラジオグラフィーの結果、ヒトゲノムＤＮＡをいずれの酵素で切断した場合も１つのバンドが得られたので、ヒトＰＤ－１遺伝子は、１コピーで存在することが判った。

【００４７】次に、マウスＰＤ－１のコード領域を含むＥｃｏＲＩ断片（１ｋｂ）をプローブとして用い、実施例２で記載したハイブリダイゼーションおよび洗浄の条件で種々の動物のゲノムＤＮＡとサザンブロッティングを行った。マウスＰＤ－１とハイブリダイズしたのは、マウスとヒトのみであり、ショウジョウバエ、ツメガエル、ウサギのＤＮＡはハイブリダイズしなかった。

【００４８】実施例６：ヒトＰＤ－１ゲノムクローンの分離

食道癌のセルラインのゲノムをＳａｕ３ＡＩで部分消化し、λＤＡＳＨ II ベクターのＢａｍＨＩサイトにつないで、構築したＤＮＡゲノムライブラリー（入手先：京都大学医学部第一病理学教室、西山博士）を用いた。ヒトＰＤ－１遺伝子は、Bluescript　ＳＫベクターからＥｃｏＲＩで切断したヒトＰＤ－１　ｃＤＮＡとのハイブリダイゼーションによって、このライブラリーから分離した。プローブは、ランダムプライミングにより^{32}Ｐでラベルした。1×10^6 ファージからポジティブなクローン２個を分離し、プラーク精製によって単一にし、制限酵素により消化した後、同じプローブを用いて、サザンハイブリダイゼーションにより解析した。この結果、ＣＩＳＳ（chromosomal in situ suppression）により、ヒトＰＤ－１遺伝子は、２ｑ37.3上にマップされることが判明した。

【００３６】

【配列表】

配列番号:1

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13　　14

配列の長さ:288　　＊トポロジー: 直鎖状

配列の型: アミノ酸　　＊　配列の種類: 蛋白質

配列

```
Met Gln Ile Pro Gln Ala Pro Trp Pro Val Val Trp Ala Val Leu Gln
-20                 -15                 -10                  -5
Leu Gly Trp Arg Pro Gly Trp Phe Leu Asp Ser Pro Asp Arg Pro Trp
                  1               5                  10
Asn Pro Pro Thr Phe Ser Pro Ala Leu Leu Val Val Thr Glu Gly Asp
          15                  20                  25
Asn Ala Thr Phe Thr Cys Ser Phe Ser Asn Thr Ser Glu Ser Phe Val
      30                  35                  40
Leu Asn Trp Tyr Arg Met Ser Pro Ser Asn Gln Thr Asp Lys Leu Ala
  45                  50                  55                  60
Ala Phe Pro Glu Asp Arg Ser Gln Pro Gly Gln Asp Cys Arg Phe Arg
                  65                  70                  75
Val Thr Gln Leu Pro Asn Gly Arg Asp Phe His Met Ser Val Val Arg
              80                  85                  90
Ala Arg Arg Asn Asp Ser Gly Thr Tyr Leu Cys Gly Ala Ile Ser Leu
          95                  100                 105
Ala Pro Lys Ala Gln Ile Lys Glu Ser Leu Arg Ala Glu Leu Arg Val
     110                 115                 120
Thr Glu Arg Arg Ala Glu Val Pro Thr Ala His Pro Ser Pro Ser Pro
125                 130                 135                 140
Arg Ser Ala Gly Gln Phe Gln Thr Leu Val Val Gly Val Val Gly Gly
                145                 150                 155
Leu Leu Gly Ser Leu Val Leu Leu Val Trp Val Leu Ala Val Ile Cys
            160                 165                 170
Ser Arg Ala Ala Arg Gly Thr Ile Gly Ala Arg Arg Thr Gly Gln Pro
        175                 180                 185
Leu Lys Glu Asp Pro Ser Ala Val Pro Val Phe Ser Val Asp Tyr Gly
    190                 195                 200
Glu Leu Asp Phe Gln Trp Arg Glu Lys Thr Pro Glu Pro Pro Val Pro
205                 210                 215                 220
Cys Val Pro Glu Gln Thr Glu Tyr Ala Thr Ile Val Phe Pro Ser Gly
                225                 230                 235
Met Gly Thr Ser Ser Pro Ala Arg Arg Gly Ser Ala Asp Gly Pro Arg
            240                 245                 250
Ser Ala Gln Pro Leu Arg Pro Glu Asp Gly His Cys Ser Trp Pro Leu
        255                 260                 265
```

【００３６】配列番号：２　　40　鎖の数：一本鎖

配列の長さ：８６４　　トポロジー：直鎖状

配列の型：核酸　　配列の種類：cDNA to mRNA

配列

```
ATGCAGATCC CACAGGCGCC CTGGCCAGTC GTCTGGGCGG TGCTACAACT GGGCTGGCGG   60
CCAGGATGGT TCTTAGACTC CCCAGACAGG CCCTGGAACC CCCCCACCTT CTCCCCAGCC  120
CTGCTCGTGG TGACCGAAGG GGACAACGCC ACCTTCACCT GCAGCTTCTC CAACACATCG  180
GAGAGCTTCG TGCTAAACTG GTACCGCATG AGCCCCAGCA ACCAGACGGA CAAGCTGGCC  240
GCCTTCCCCG AGGACCGCAG CCAGCCCGGC CAGGACTGCC GCTTCCGTGT CACACAACTG  300
CCCAACGGGC GTGACTTCCA CATGAGCGTG GTCAGGGCCC GGCGCAATGA CAGCGGCACC  360
TACCTCTGTG GGGCCATCTC CCTGGCCCCC AAGGCGCAGA TCAAAGAGAG CCTGCGGGCA  420
```


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GAGCTCAGGG TGACAGAGAG AAGGGCAGAA GTGCCCACAG CCCACCCCAG CCCCTCACCC 480
AGGTCAGCCG GCCAGTTCCA AACCCTGGTG GTTGGTGTCG TGGGCGGCCT GCTGGGCAGC 540
CTGGTGCTGC TAGTCTGGGT CCTGGCCGTC ATCTGCTCCC GGGCCGCACG AGGGACAATA 600
GGAGCCAGGC GCACCGGCCA GCCCCTGAAG GAGGACCCCT CAGCCGTGCC TGTGTTCTCT 660
GTGGACTATG GGGAGCTGGA TTTCCAGTGG CGAGAGAAGA CCCCGGAGCC CCCCGTGCCC 720
TGTGTCCCTG AGCAGACGGA GTATGCCACC ATTGTCTTTC CTAGCGGAAT GGGCACCTCA 780
TCCCCCGCCC GCAGGGGCTC AGCTGACGGC CCTCGGAGTG CCCAGCCACT GAGGCCTGAG 840
GATGGACACT GCTCTTGGCC CCTC 864

【００３６】配列番号：３
配列の長さ：９２１
配列の型：核酸
＊鎖の数：一本鎖
トポロジー：直鎖状
＊配列の種類：cDNA to mRNA

配列

CACTCTGGTG GGGCTGCTCC AGGCATGCAG ATCCCACAGG CGCCCTGGCC AGTCGTCTGG 60
GCGGTGCTAC AACTGGGCTG GCGGCCAGGA TGGTTCTTAG ACTCCCCAGA CAGGCCCTGG 120
AACCCCCCCA CCTTCTCCCC AGCCCTGCTC GTGGTGACCG AAGGGGACAA CGCCACCTTC 180
ACCTGCAGCT TCTCCAACAC ATCGGAGAGC TTCGTGCTAA ACTGGTACCG CATGAGCCCC 240
AGCAACCAGA CGGACAAGCT GGCCGCCTTC CCCGAGGACC GCAGCCAGCC CGGCCAGGAC 300
TGCCGCTTCC GTGTCACACA ACTGCCCAAC GGGCGTGACT TCCACATGAG CGTGGTCAGG 360
GCCCGGCGCA ATGACAGCGG CACCTACCTC TGTGGGGCCA TCTCCCTGGC CCCCAAGGCG 420
CAGATCAAAG AGAGCCTGCG GGCAGAGCTC AGGGTGACAG AGAGAAGGGC AGAAGTGCCC 480
ACAGCCCACC CCAGCCCCTC ACCCAGGTCA GCCGGCCAGT TCCAAACCCT GGTGGTTGGT 540
GTCGTGGGCG GCCTGCTGGG CAGCCTGGTG CTGCTAGTCT GGGTCCTGGC CGTCATCTGC 600
TCCCGGGCCG CACGAGGGAC AATAGGAGCC AGGCGCACCG GCCAGCCCCT GAAGGAGGAC 660
CCCTCAGCCG TGCCTGTGTT CTCTGTGGAC TATGGGGAGC TGGATTTCCA GTGGCGAGAG 720
AAGACCCCGG AGCCCCCCGT GCCCTGTGTC CCTGAGCAGA CGGAGTATGC CACCATTGTC 780
TTTCCTAGCG GAATGGGCAC CTCATCCCCC GCCCGCAGGG GCTCAGCTGA CGGCCCTCGG 840
AGTGCCCAGC CACTGAGGCC TGAGGATGGA CACTGCTCTT GGCCCCTCTG ACCGGCTTCC 900
TTGGCCACCA GTGTTCTGCA G 921

【００３６】配列番号：４
配列の長さ：９２１
配列の型：核酸
鎖の数：一本鎖
トポロジー：直鎖状
配列の種類：cDNA to mRNA
起源
生物名　：Homo Sapiens
セルライン：ＹＴＣ３
配列の特徴
特徴を表す記号：ＣＤＳ
存在位置：25..888
特徴を決定した方法：Ｐ
特徴を表す記号：sig peptide
存在位置：25..84
特徴を決定した方法：Ｓ
特徴を表す記号：mat peptide
存在位置：85..888
特徴を決定した方法：Ｓ

配列

CACTCTGGTG GGGCTGCTCC AGGC ATG CAG ATC CCA CAG GCG CCC TGG CCA GTC 54
Met Gln Ile Pro Gln Ala Pro Trp Pro Val
-20 -15
GTC TGG GCG GTG CTA CAA CTG GGC TGG CGG CCA GGA TGG TTC TTA GAC 102
Val Trp Ala Val Leu Gln Leu Gly Trp Arg Pro Gly Trp Phe Leu Asp
-10 -5 1 5
TCC CCA GAC AGG CCC TGG AAC CCC CCC ACC TTC TCC CCA GCC CTG CTC 150
Ser Pro Asp Arg Pro Trp Asn Pro Pro Thr Phe Ser Pro Ala Leu Leu
10 15 20
GTG GTG ACC GAA GGG GAC AAC GCC ACC TTC ACC TGC AGC TTC TCC AAC 198
Val Val Thr Glu Gly Asp Asn Ala Thr Phe Thr Cys Ser Phe Ser Asn

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17 *18*

```
      25                  30                  35
ACA TCG GAG AGC TTC GTG CTA AAC TGG TAC CGC ATG AGC CCC AGC AAC    246
Thr Ser Glu Ser Phe Val Leu Asn Trp Tyr Arg Met Ser Pro Ser Asn
   40                  45                  50
CAG ACG GAC AAG CTG GCC GCC TTC CCC GAG GAC CGC AGC CAG CCC GGC    294
Gln Thr Asp Lys Leu Ala Ala Phe Pro Glu Asp Arg Ser Gln Pro Gly
55                  60                  65                  70
CAG GAC TGC CGC TTC CGT GTC ACA CAA CTG CCC AAC GGG CGT GAC TTC    342
Gln Asp Cys Arg Phe Arg Val Thr Gln Leu Pro Asn Gly Arg Asp Phe
               75                  80                  85
CAC ATG AGC GTG GTC AGG GCC CGG CGC AAT GAC AGC GGC ACC TAC CTC    390
His Met Ser Val Val Arg Ala Arg Arg Asn Asp Ser Gly Thr Tyr Leu
            90                  95                 100
TGT GGG GCC ATC TCC CTG GCC CCC AAG GCG CAG ATC AAA GAG AGC CTG    438
Cys Gly Ala Ile Ser Leu Ala Pro Lys Ala Gln Ile Lys Glu Ser Leu
      105                 110                 115
CGG GCA GAG CTC AGG GTG ACA GAG AGA AGG GCA GAA GTG CCC ACA GCC    486
Arg Ala Glu Leu Arg Val Thr Glu Arg Arg Ala Glu Val Pro Thr Ala
   120                 125                 130
CAC CCC AGC CCC TCA CCC AGG TCA GCC GGC CAG TTC CAA ACC CTG GTG    534
His Pro Ser Pro Ser Pro Arg Ser Ala Gly Gln Phe Gln Thr Leu Val
135                 140                 145                 150
GTT GGT GTC GTG GGC GGC CTG CTG GGC AGC CTG GTG CTG CTA GTC TGG    582
Val Gly Val Val Gly Gly Leu Leu Gly Ser Leu Val Leu Leu Val Trp
               155                 160                 165
GTC CTG GCC GTC ATC TGC TCC CGG GCC GCA CGA GGG ACA ATA GGA GCC    630
Val Leu Ala Val Ile Cys Ser Arg Ala Ala Arg Gly Thr Ile Gly Ala
           170                 175                 180
AGG CGC ACC GGC CAG CCC CTG AAG GAG GAC CCC TCA GCC GTG CCT GTG    678
Arg Arg Thr Gly Gln Pro Leu Lys Glu Asp Pro Ser Ala Val Pro Val
      185                 190                 195
TTC TCT GTG GAC TAT GGG GAG CTG GAT TTC CAG TGG CGA GAG AAG ACC    726
Phe Ser Val Asp Tyr Gly Glu Leu Asp Phe Gln Trp Arg Glu Lys Thr
   200                 205                 210
CCG GAG CCC CCC GTG CCC TGT GTC CCT GAG CAG ACG GAG TAT GCC ACC    774
Pro Glu Pro Pro Val Pro Cys Val Pro Glu Gln Thr Glu Tyr Ala Thr
215                 220                 225                 230
ATT GTC TTT CCT AGC GGA ATG GGC ACC TCA TCC CCC GCC CGC AGG GGC    822
Ile Val Phe Pro Ser Gly Met Gly Thr Ser Ser Pro Ala Arg Arg Gly
               235                 240                 245
TCA GCT GAC GGC CCT CGG AGT GCC CAG CCA CTG AGG CCT GAG GAT GGA    870
Ser Ala Asp Gly Pro Arg Ser Ala Gln Pro Leu Arg Pro Glu Asp Gly
           250                 255                 260
CAC TGC TCT TGG CCC CTC TGAC CGGCTTCCTT GGCCACCAGT GTTCTGCAG      921
His Cys Ser Trp Pro Leu
      265
```


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Ｃ１２Ｎ	1/21		8828－4B			
	15/09	ＺＮＡ				
Ｃ１２Ｐ	21/02		Ｃ 9282－4B			
	21/08		9161－4B			
// Ａ６１Ｋ	39/395		Ｎ			
Ｃ１２Ｎ	5/10					
（Ｃ１２Ｎ	1/21					
Ｃ１２Ｒ	1:19）					
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Ｃ１２Ｒ	1:19）					
			7729－4B	Ｃ１２Ｎ	5/00	Ｂ

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(54)【発明の名称】　ヒトにおけるプログラムされた細胞死に関連したポリペプチド、それをコードするＤＮＡ、そのＤＮＡからなるベクター、そのベクターで形質転換された宿主細胞、そのポリペプチドの抗体、およびそのポリペプチドまたはその抗体を含有する薬学的組成物

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Search result: 1 of 1

(WO/1997/007671) CHIMERIC ANIMAL AND METHOD FOR CONSTRUCTING THE SAME

Latest bibliographic data on file with the International Bureau

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Publication Date: 06.03.1997 **International Filing Date:** 29.08.1996
Chapter 2 Demand Filed: 31.01.1997

IPC: ***A01K 67/027*** (2006.01), ***C07K 16/00*** (2006.01), ***C07K 16/18*** (2006.01), ***C12N 15/90*** (2006.01)

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ISHIDA, Isao [JP/JP]; (JP) *(US Only)*.

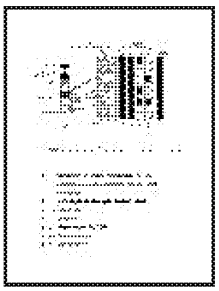
Inventors: **TOMIZUKA, Kazuma**; (JP).
YOSHIDA, Hitoshi; (JP).
HANAOKA, Kazunori; (JP).
OSHIMURA, Mitsuo; (JP).
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Priority Data: 7/242340 29.08.1995 JP
8/27940 15.02.1996 JP

Title: CHIMERIC ANIMAL AND METHOD FOR CONSTRUCTING THE SAME

Abstract: A method for constructing a chimeric nonhuman animal which comprises preparing microcells containing one or more foreign chromosomes or fragments of the same, and transferring the foreign chromosome(s) or fragments of the same into cells having differentiative pluripotency via the fusion with the microcells; a chimeric nonhuman animal which can be constructed by the above method and offsprings of the same; tissues and cells originating in the same; a method for utilizing the tissues and cells; cells which contain one or more foreign chromosomes or fragments of the same and sustain differentiative pluripotency; a method for consiructing the cells; and a method for utilizing the cells.



Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN.
African Regional Intellectual Property Org. (ARIPO) (KE, LS, MW, SD, SZ, UG)
Eurasian Patent Organization (EAPO) (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM)
European Patent Office (EPO) (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE)
African Intellectual Property Organization (OAPI) (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Publication Language:	Japanese (JA)
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国際事務局

PCT

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(30) 優先権データ

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(81) 指定国 AL, AM, AU, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO特許 (KE, LS, MW, SD, SZ, UG), ユーラシア特許 (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), 欧州特許 (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI特許 (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

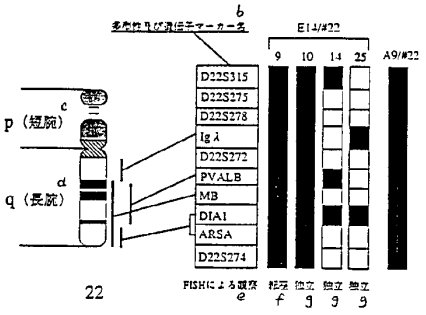
添付公開書類
国際調査報告書

(54)Title: **CHIMERIC ANIMAL AND METHOD FOR CONSTRUCTING THE SAME**

(54)発明の名称 キメラ動物およびその作製法

(57) Abstract

A method for constructing a chimeric nonhuman animal which comprises preparing microcells containing one or more foreign chromosomes or fragments of the same, and transferring the foreign chromosome(s) or fragments of the same into cells having differentiative pluripotency via the fusion with the microcells; a chimeric nonhuman animal which can be constructed by the above method and offsprings of the same; tissues and cells originating in the same; a method for utilizing the tissues and cells; cells which contain one or more foreign chromosomes or fragments of the same and sustain differentiative pluripotency; a method for constructing the cells; and a method for utilizing the cells.



a
E14耐性株におけるヒト22番染色体（断片）の保持（PCR解析）

a ... Retention of human chromosome No. 22 (fragment) in E14-tolerant strain (PCT analysis)
b ... polymorphism and gene marker names
c ... short arm
d ... long arm
e ... observation by FISH
f ... translocating
g ... independent

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（５７）要約

単一または複数の外来染色体あるいはその断片を含むミクロセルを作製し、該ミクロセルとの融合により、分化多能性を保持する細胞へ前記単一または複数の外来染色体あるいはその断片を移入させることを特徴とする、キメラ非ヒト動物の作製法。前記の方法により作製することができるキメラ非ヒト動物およびその子孫、それらに由来する組織および細胞、並びに、それらの利用方法。さらに、単一または複数の外来染色体あるいはその断片を含む分化多能性を保持する細胞、その作製法および利用法も提供される。

情報としての用途のみ
ＰＣＴに基づいて公開される国際出願をパンフレット第一頁にＰＣＴ加盟国を同定するために使用されるコード

AL	アルバニア	DE	ドイツ	LI	リヒテンシュタイン	PL	ポーランド
AM	アルメニア	DK	デンマーク	LC	セントルシア	PT	ポルトガル
AT	オーストリア	EE	エストニア	LK	スリランカ	RO	ルーマニア
AU	オーストラリア	ES	スペイン	LR	リベリア	RU	ロシア連邦
AZ	アゼルバイジャン	FI	フィンランド	LS	レソト	SD	スーダン
BA	ボスニア・ヘルツェゴビナ	FR	フランス	LT	リトアニア	SE	スウェーデン
BB	バルバドス	GA	ガボン	LU	ルクセンブルグ	SG	シンガポール
BE	ベルギー	GB	イギリス	LV	ラトヴィア	SI	スロヴェニア
BF	ブルギナ・ファソ	GE	グルジア	MC	モナコ	SK	スロヴァキア
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BJ	ベナン	GR	ギリシャ	MG	マダガスカル	SN	セネガル
BR	ブラジル	HU	ハンガリー	MK	マケドニア旧ユーゴスラヴィア共和国	SZ	スワジランド
BY	ベラルーシ	IE	アイルランド	ML	マリ	TD	チャド
CA	カナダ	IL	イスラエル	MN	モンゴル	TG	トーゴ
CF	中央アフリカ共和国	IS	アイスランド	MR	モーリタニア	TJ	タジキスタン
CG	コンゴ	IT	イタリア	MW	マラウイ	TM	トルクメニスタン
CH	スイス	JP	日本	MX	メキシコ	TR	トルコ
CI	コート・ジボアール	KE	ケニア	NE	ニジェール	TT	トリニダード・トバゴ
CM	カメルーン	KG	キルギスタン	NL	オランダ	UA	ウクライナ
CN	中国	KP	朝鮮民主主義人民共和国	NO	ノールウェー	UG	ウガンダ
CU	キューバ	KR	大韓民国	NZ	ニュー・ジーランド	US	アメリカ合衆国
CZ	チェッコ共和国	KZ	カザフスタン			UZ	ウズベキスタン
						VN	ヴィェトナム

明 細 書

キメラ動物およびその作製法

技術分野

本発明は、キメラ非ヒト動物、その作製法およびその利用法に関する。本発明のキメラ非ヒト動物を用いれば、これまで不可能であった1Mb（百万塩基対）以上の外来巨大DNA断片を動物個体で保持発現させることが可能になる。従って、これを利用することにより、

・生物学的に活性な物質をコードする遺伝子の全長、例えば、ヒト抗体遺伝子全長を保持し、発現する動物個体の作製が可能になる。この動物から得られる生物学的に活性な物質、例えば、ヒト型抗体は医薬品としての利用価値がある。

・ヒト巨大遺伝子（組織適合性抗原、ジストロフィン等）の動物個体における機能解析が可能になる。

・ヒト優性遺伝病及び染色体異常症のモデル動物作製に利用できる。

背景技術

外来遺伝子を動物個体において発現させる技術、すなわちトランスジェニック動物作製技術は、その遺伝子の生体内機能に関する情報を得るために有用なばかりでなく、遺伝子発現を制御するDNA配列の同定（例えば、Magramら，Nature，315:338，1985）、ヒト疾患モデル動物の開発（山村ら，マニュアル疾患モデルマウス，中山書店；1994）、さらには家畜の育種（例えば、Mullerら，Experientia，47:923，1991）、それを用いた有用物質の生産に利用されてきた（例えば、Velanderら，P.N.A.S.，89:12003，1992）。遺伝子導入の対象としてはこれまでマウスが最も多く用いられてきた。実験動物として詳細に研究され、胚操作技術も確立されているマウスはこの目的に最も適した哺乳動物であるといえる。

マウス個体への外来遺伝子導入法は大きく分けて２つ知られている。１つは受精卵前核にDNAを注入する方法（Gordonら，P.N.A.S.，77:7380，1980）であり、もう一つは分化全能性を保持した胚幹細胞（以下ES細胞、という）にDNAを導入し、キメラマウスを作製する方法（Takahashiら，Development，102:259，1988）

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である。後者の場合、キメラマウスにおいては、ES細胞の貢献した細胞、組織においてのみ、ES細胞由来の生殖細胞を経て得られる子孫においてはすべての細胞、組織で導入遺伝子が保持される。これらの技術を利用して現在までに数多くのトランスジェニックマウスが作製されてきた。

しかし、現状では導入可能なDNAの大きさに限界があり、それがこの技術の利用範囲を大きく制限している。その限界はクローン化可能なDNAの大きさに依存し、これまで最も大きなDNA断片を導入した例の一つは、酵母人工染色体（YAC）にクローニングされた約670kbのDNA断片である（Jakobovitsら，Nature，362:255，1993）。この実験は、YACを保持する酵母とマウスES細胞を融合することにより行なわれた。YACにおいては、約2Mbまでの外来DNAをクローニングできるとされているが（Den Dunnenら，Hum. Mol.Genet.，1:19，1992 ）、出芽酵母細胞中では、相同DNA配列同士の組換え頻度が高いことが知られており、反復配列を多く有するヒトDNA断片を完全な形で安定に保持することが困難な場合がある。事実、ヒトゲノムDNAを含むYACライブラリーでは、その20～40%が何らかの組換えを起こしている（Greenら，Genomics，11:584，1991）。

もう１つの方法として、ヒト培養細胞から得られる中期染色体を顕微切断し、その断片（10Mb以上と推定される）をマウス受精卵に注入することが試みられた（Richaら，Science，245:175，1989）。この結果得られたマウス個体においてヒト特異的DNA配列（Alu 配列）が検出されたが、ヒト遺伝子発現については確認されていない。また、ここで用いられた染色体調製法では染色体をスライドグラスに固定する際、酢酸メタノールを使用するために、DNA自体が小さく断片化してしまうことが避けられず、注入したDNAが一続きのDNAとして存在している可能性は低い。

いずれにせよ、現在まで1Mb以上の一続きの外来DNA断片をマウス個体へ導入、発現させた例はないといえる。

マウスに導入することが望まれる、有用かつ興味深いヒト遺伝子：抗体（例えば、Cookら，Nature Genetics，7:162，1994）、T細胞受容体（Hoodら，Cold Spring Harbor Symposia on Quantitative Biology，Vol. LVIII，339，1993 ）、組織適合性抗原（Carrolら，P.N.A.S.，84:8535，1987）、ジストロフィン（Den

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Dunnenら，前記）等は、そのコード領域自体が1Mbを越える大きさを持つことが知られている。とりわけ、ヒト型抗体の医薬品としての重要性から、ヒトの免疫グロブリン重鎖（～1.5Mb、Cookら，前記）、軽鎖κ（～3Mb、Zachau, Gene, 13 5:167, 1993)、軽鎖λ（～1.5Mb、Frippiatら，Hum. Mol. Genet., 4:983, 1995）の遺伝子全長を保持し、発現するようなマウスの作製が望まれているがそれは現状の技術では不可能である（日経バイオテク，1993.7.5.）。

また、ヒトの優性遺伝疾患、先天異常を引き起こす染色体異常症（ダウン症等）の原因遺伝子の多くはクローン化されておらず、染色体上の大まかな位置情報のみ利用可能である。例えば、中期染色体をギムザ染色することによって得られるGバンドは、通常数Mb～10Mb以上の大きさを有している。従って、ある原因遺伝子が特定のGバンド上に存在することが明らかとなっても、これらの異常形質をマウスに導入するためには、原因遺伝子周辺の染色体断片（数Mb以上）を導入することが必要であるが、これも現状の技術では不可能である。

ここに従来技術の限界である1Mbを越える外来DNAをマウス個体に導入し、発現させる技術の開発が望まれている。

動物培養細胞においては、従来技術により上述の限界を越える大きさのDNAを導入することが可能である。これは主に、染色体（ヒトの場合約50～300Mbの大きさを持つ）を媒体として行なわれる。細胞への染色体移入法はいくつか報告されているが（例えば、McBrideら，P.N.A.S.,70:1258, 1973）、その中でも望みの染色体を1本だけ導入する方法として最適と考えられているのがミクロセル法である（Koiら，Jpn. J. Cancer Res., 80:413, 1989）。ミクロセルと呼ばれる構造体は１本～数本の染色体が核膜、形質膜に包まれたものである。ある種の細胞において紡垂体形成を阻害する薬剤により誘導されるミクロセルを分離し、受容細胞と融合することにより、少数（多くの場合は１本）の染色体を導入することが可能である。この方法により得られた、ヒト染色体を1本だけ保持するモノクロモソーム雑種細胞のライブラリーは既知遺伝子のマッピングや、未知の癌抑制遺伝子、細胞老化遺伝子等の存在する染色体を特定するために利用されてきた（例えば、Saxonら，EMBO J., 5:3461, 1986 ）。さらに、ミクロセルにガンマ線を照射することにより、染色体を断片化しその一部を導入することも可能であ

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る（Koiら，Science，260:361，1993）。すなわち、ミクロセル法は、1Mb以上のDNAを動物培養細胞に導入する方法として適していると考えられる。

培養細胞からマウス個体を作り出すことができないかという期待は、分化全能性を安定に保持するES細胞の発見（Evansら，Nature，292:154，1981）により確実に現実のものとなった。このES細胞には外来遺伝子、種々の突然変異、さらには、標的遺伝子組換えによる変異が導入可能となり、マウス個体レベルの遺伝的改変が自在に行なえるようになった（例えば、Mansourら，Nature，336:348，1988）。一方、巨大DNAの導入という意味では前述のYACベクターにクローニング可能な外来DNA断片の大きさが限界と考えられてきた。その大きさを越えるDNAを培養細胞に導入可能な染色体移入という従来技術がマウス個体レベルへの遺伝子導入に利用されたことはなく、またその達成は困難であると考えられてきた（村松ら，トランスジェニックバイオロジー，講談社サイエンティフィク，p143-，1989）。

理由としては、以下のようなものが挙げられる。

・受容細胞を正常核型のマウスES細胞としてこれにヒト染色体導入を行なう場合、それは染色体異常そのものを導入することである。これまで、顕微鏡によって識別可能な染色体レベルの大きな遺伝子異常はマウスの発生にとって多くの場合致死的であると考えられてきた（Groppら，J.Exp.Zool.，228:253，1983；相沢慎一，バイオマニュアルシリーズ 8 ジーンターゲティング，羊土社，1995）。

・我々が得ることのできるヒト染色体は通常、有限増殖の正常繊維芽細胞、あるいは癌細胞等の分化した体細胞のものであり、そのような体細胞由来の染色体が未分化なES細胞に導入された場合、ES細胞を分化させたり（Mullerら，Nature，311:438，1984）、老化させてしまうのではないか（Sugawara，Science，247:707，1990）と考えられた。

・体細胞由来の染色体が初期胚に導入された場合、胚発生過程で生殖細胞由来のそれと同様に正しく機能し、多様な組織、細胞における特異的遺伝子発現を担うことができるかという問題についての研究は非常に少ない。この2者における大きな違いの一つは染色体DNAのメチル化状態と想像される。これは細胞の分化に伴って変化し、組織特異的な遺伝子発現における重要な役割が示唆されている

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（Ceder, Cell, 53:3, 1988）。例えば、抗体遺伝子の活性化に不可欠な部位特異的DNA組換え反応について、メチル化した基質をB細胞に導入した場合、メチル化の状態は複製後も保持され、組換え反応を阻害するという報告がある（Hsiehら, EMBO J., 11:315, 1992）。また、株化された細胞中では生体内と比較して、de novoのメチル化が起こりやすいとされている（Antequeraら, Cell, 62:503, 1990）。よってこれまでの研究から、繊維芽細胞や、ヒトーマウス雑種細胞のおそらく高度にメチル化されているであろう抗体遺伝子がマウスのB細胞で正常に発現すると想像することは容易ではなかった。

ここで、関連するものとしてIllmenseeらの２つの報告（P.N.A.S.,75;1914, 1978; P.N.A.S.,76: 879, 1979 ）に注目しなければならない。１つはヒト肉腫細胞とマウスEC細胞、もう１つはラット肝癌細胞とマウスEC細胞の全細胞融合により得られた融合株からキメラマウスを作製したという報告である。これらの報告においてはその実験結果に数多くの疑問点が指摘され、その信憑性は低いと考えられている（野口ら, マウスのテラトーマ, 理工学社, 5節, 1987）。また、１日も早い追試が望まれていたにも関わらず、報告から17年を経た現在までこの実験を再現できたという報告は存在しない。従って、これらの報告によっては、マウス個体において外来染色体を保持させ、該染色体上の遺伝子を発現させ得たということはできないと考えられてきた。

こうした状況において、染色体断片ほどの巨大DNAをマウスをはじめとする動物個体に導入し、発現させることは困難とされ、実際この問題について検討がなされたことは上記のIllmenseeらの報告以来ない。

従って、本発明は、外来染色体またはその断片を保持し、該染色体またはその断片上の遺伝子を発現するキメラ非ヒト動物およびその子孫、並びに前記のキメラ非ヒト動物の作製法を提供することを目的とする。

また、本発明は、前記のキメラ非ヒト動物およびその子孫に由来する組織および細胞を提供することを目的とする。

さらに、本発明は、前記のキメラ非ヒト動物およびその子孫に由来する細胞とミエローマ細胞との融合により作製されたハイブリドーマを提供することも目的とする。

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さらにまた、本発明は、非ヒト動物の個体、組織または細胞を用いて、外来染色体またはその断片上の遺伝子の発現産物である生物学的に活性な物質を製造する方法を提供することを目的とする。

発明の開示

発明者は前記課題を解決するために鋭意研究を行なった結果、ヒト正常繊維芽細胞由来染色体あるいはその部分断片をミクロセル法によりマウスES細胞に導入し、それを安定に保持する株を得ることに成功した。さらにこのES細胞株から、その正常組織においてヒト染色体を保持し、ヒト抗体重鎖遺伝子を含む複数のヒト遺伝子を発現するキメラマウスを得た。我々はこの一連の方法により、これまで不可能であった巨大DNA断片の個体における保持、及び該DNA 断片上の遺伝子の発現を可能にした。

本発明の要旨は以下の通りである。

(1)単一または複数の外来染色体あるいはその断片を含むミクロセルを作製し、該ミクロセルとの融合により、分化多能性を保持する細胞へ前記単一または複数の外来染色体あるいはその断片を移入させることを特徴とする、キメラ非ヒト動物の作製法。

(2)単一または複数の外来染色体あるいはその断片を含むミクロセルを作製し、該ミクロセルとの融合により、分化多能性を保持する細胞へ前記単一または複数の外来染色体あるいはその断片を移入させることを特徴とする、単一または複数の外来染色体あるいはその断片を含む分化多能性を保持する細胞の作製法。

(3)前記(1)の方法により作製することができ、単一または複数の外来染色体あるいはその断片を保持し、該染色体あるいはその断片上の遺伝子を発現するキメラ非ヒト動物、および前記の単一または複数の外来染色体あるいはその断片を保持し、該染色体あるいはその断片上の遺伝子を発現するその子孫。

(4)前記(2)の方法により作製することができ、単一または複数の外来染色体あるいはその断片を含む分化多能性を保持する細胞。

(5)キメラ非ヒト動物を作製するための前記(4)の分化多能性を保持する細胞の使用。

(6)前記(3)のキメラ非ヒト動物またはその子孫の交配により得られる、単一または

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複数の外来染色体あるいはその断片を保持し、該染色体あるいはその断片上の遺伝子を発現する非ヒト動物およびその子孫。

(7)前記(3)のキメラ非ヒト動物もしくはその子孫または前記(6)の非ヒト動物もしくはその子孫に由来する組織。

(8)前記(3)のキメラ非ヒト動物もしくはその子孫または前記(6)の非ヒト動物もしくはその子孫に由来する細胞。

(9)前記(8)の細胞とミエローマ細胞との融合により作製されたハイブリドーマ。

(10)前記(3)のキメラ非ヒト動物もしくはその子孫または前記(6)の非ヒト動物もしくはその子孫を、前記の染色体あるいはその断片上の遺伝子と同じあるいは相同の遺伝子が欠損している系統の非ヒト動物と交配させることにより作製された非ヒト動物およびその子孫。

(11)前記(3)のキメラ非ヒト動物もしくはその子孫または前記(6)の非ヒト動物もしくはその子孫の個体、組織または細胞において、単一または複数の外来染色体あるいはその断片上の遺伝子を発現させ、その産物としての生物学的に活性な物質を回収することを特徴とする、生物学的に活性な物質の製造法。

(12)前記(3)のキメラ非ヒト動物もしくはその子孫または前記(6)の非ヒト動物もしくはその子孫を、前記の染色体あるいはその断片上の遺伝子と同じあるいは相同の遺伝子が欠損している系統の非ヒト動物と交配させ、誕生した子動物の個体、組織または細胞において、前記の単一または複数の外来染色体あるいはその断片上の遺伝子を発現させ、その産物としての生物学的に活性な物質を回収することを特徴とする、生物学的に活性な物質の製造法。

図面の簡単な説明

第1図は、ヒト2番染色体（断片）を保持するA9細胞の解析（PCR解析）の結果が示されている。

第2図は、E14耐性株においてヒト22番染色体（断片）が保持されていることが示されている（PCR解析）。

第3図は、22番染色体導入ES細胞由来キメラマウスにおいてヒトL1配列が保持されていることを示す電気泳動写真である（サザン解析）。

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第4図は、ヒト22番染色体導入キメラマウス臓器におけるヒト染色体の保持の様子を示す電気泳動写真である（PCR解析）。

第5図は、ヒト22番染色体導入キメラマウスにおけるヒト遺伝子発現（RT-PCR）の結果を示す電気泳動写真である。

第6図は、ヒト22番染色体導入キメラマウス臓器におけるヒト遺伝子発現（RT-PCR）の結果を示す電気泳動写真である。

第7図は、E14耐性株においてヒト4番染色体（断片）が保持されていることが示されている（PCR解析）。

第8図は、ヒト4番染色体導入E14細胞株におけるヒトL1配列の検出（サザン解析）の結果を示す電気泳動写真である。

第9図は、ヒト4番染色体導入ES細胞由来キメラマウスにおいてヒトL1配列が保持されていることを示す電気泳動写真である（サザン解析）。

第10図は、TT2耐性株においてヒト14番染色体（断片）が保持されていることが示されている（PCR解析）。

第11図は、ヒト14番染色体導入ES細胞由来キメラマウス臓器におけるヒト染色体の保持の様子を示す電気泳動写真である（PCR解析）。

第12図は、尻尾由来繊維芽細胞G418耐性テストの結果が示されている。

第13図は、ヒト血清アルブミン免疫キメラマウス血清中のヒト抗体IgM濃度（ELISA）が示されている。

第14図は、ヒト血清アルブミン免疫キメラマウス血清中のヒト抗体IgG濃度（ELISA）が示されている。

第15図は、ヒトIgM産生ハイブリドーマクローンH4B7の解析（ELISA）の結果が示されている。

第16図は、ヒト2番染色体部分断片及び14番染色体部分断片を保持するマウスES細胞株（TT2細胞株PG15）のFISH解析の結果を示す染色体の形態の写真である。

第17図は、ヒト血清アルブミン免疫キメラマウス血清中の抗ヒト血清アルブミンヒトIgG抗体価の増加が示されている。

第18図は、ヒト血清アルブミン免疫キメラマウス血清中の抗ヒト血清アルブミンヒトIgκ抗体価の増加が示されている。

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第19図は、ヒト22番染色体導入TT2細胞株におけるヒトL1配列の検出（サザン解析）の結果を示す電気泳動写真である。

第20図は、ヒト血清アルブミン免疫キメラマウス血清中の抗ヒト血清アルブミンヒトIgλ抗体価の増加が示されている。

第21図は、ヒト2番染色体部分断片導入キメラマウスの子孫においてヒト2番染色体部分断片が保持されていることが示されている（PCR解析）。

第22図は、ヒト14番染色体導入キメラマウス脾臓において細胞表面にヒトμ鎖が発現している細胞の存在を示している（フローサイトメトリー解析）。

第23図は、LoxP-pstNEOプラスミドDNA の構造を示している。

第24図は、マウス抗体重鎖Cμのゲノムの構造を示している。

第25図は、マウス抗体軽鎖κのゲノムDNAの構造を示している。

第26図は、マウス抗体重鎖ターゲッティングベクター及び、サザンブロット用プローブの構造と相同組換え体で検出されるべきDNA断片について示している。

第27図は、マウス抗体軽鎖κターゲッティングベクター及び、サザンブロット用プローブの構造と相同組換え体で検出されるべきDNA断片について示している。

第28図は、マウス抗体重鎖相同組換え体及び相同組換え体由来高濃度G418耐性株のサザン解析の結果を示す電気泳動写真である。

発明を実施するための最良の形態

ヒト染色体またはその断片を保持し、該染色体またはその断片上の遺伝子を発現するマウス個体は、

（1）標識されたヒト染色体またはその断片を保持する染色体供与細胞の作製

（2）分化多能性を保持するマウス細胞へのミクロセル法によるヒト染色体またはその断片の移入

（3）上記マウス細胞を用いたキメラマウスの作製

（4）キメラマウスにおけるヒト染色体保持及び、ヒト遺伝子発現確認

の過程を経ることにより得ることができる。なお、ここでは、ヒト染色体またはその断片を保持し、該染色体またはその断片上の遺伝子を発現する非ヒト動物として、マウスを例にとった（以下、このマウスを「ヒト染色体導入マウス」と称

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する。）。

ここでヒト染色体とは、ヒト細胞由来の天然のDNAと蛋白質の複合体のことを指す。正常なヒト染色体は23種（雄は24種）46本存在し、それぞれ約50～300Mbの長さのDNAを含むとされるが、本発明においては独立染色体として安定に複製、分配が可能な部分断片、およびマウス染色体上に転座し、安定に保持されている部分断片も含まれる。そのDNAの大きさは通常1Mb以上だが、それ以下の場合もある。本発明の特徴は大腸菌、酵母等でのクローニングあるいは細胞からのDNA抽出処理等を行なわず、染色体そのものを媒体としてマウス個体でヒト遺伝子を保持、発現させることにある。

また、ヒト染色体導入マウスとは、その正常体細胞の全てまたは一部において、単一あるいは複数のヒト染色体あるいはその断片を保持するものを指す。さらには、その正常体細胞の全てまたは一部において、ヒト染色体上の単一あるいは複数のヒト遺伝子を発現しているものを指す。

（1）標識されたヒト染色体またはその断片を保持する染色体供与細胞の作製

染色体供与細胞としては、1）受容細胞で選別可能なマーカーで標識されたヒト染色体を保持し、2）それ以外のヒト染色体を含まない、3）ミクロセル形成能が高い細胞、が望ましい。

ヒト染色体提供の材料としては、ヒト由来のあらゆる細胞株、癌細胞、初代培養細胞を用いることが出来るが、染色体の欠失、増幅等の異常の可能性が低く、また培養が容易な点を考慮すると正常繊維芽細胞が適している。

まず1）について、ヒト細胞は薬剤（G418，ピューロマイシン，ハイグロマイシン，ブラストサイジン）耐性等のマーカー遺伝子を発現するベクターにより形質転換することができる。ここで用いるマーカー発現制御のためのプロモーターとしては、ヒト細胞のみならず、マウスES細胞のような受容細胞で効率よく働くものが望ましい。この目的にはSV40エンハンサーと単純ヘルペスウイルスチミジンキナーゼプロモーターの連結したもの（Katohら，Cell Struct.Funct.，12:575，1987）、マウスPGK-1プロモーター（Sorianoら，Cell，64:693，1991）等を用いることが出来る。エレクトロポレーション（石田ら，細胞工学実験操作入門，講談社，1992）等による形質転換を実施し、選択することにより、導入されたマ

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ーカー遺伝子が、23種46本あるヒト染色体上にランダムに挿入されたようなヒト細胞形質転換体のライブラリーを得ることが出来る。

3）については、多くのヒト正常細胞がミクロセル形成能が非常に低いので、上記の形質転換体をミクロセル形成能の高い細胞例えば、マウスA9細胞（Oshimura, M., Environ. Health Perspect., 93:57, 1991）と全細胞融合することにより、形成能を付与することが出来る。マウスーヒト雑種細胞では、ヒト染色体が選択的に消失することが知られているが、ここで得られた融合株は、先述のマーカーで選択することにより、マーキングされたヒト染色体を安定に保持することが出来る。

さらに、2）の条件を満たすために、この細胞融合株からミクロセルを取得し、マウスA9細胞と再度融合することが望ましい。この場合も、ヒト染色体上のマーカーで選択することにより、得られるミクロセル融合株の多くは、1）、2）、3）の条件を満たしたものであると考えられる。マーキングされたヒト染色体の同定は、最終段階で得られるマウスーヒトモノクロモソーム雑種細胞において、PCR（ポリメラーゼチェインリアクション、Saikiら, Science, 239:487, 1988）、サザンブロット解析（Ausubelら, Current protocols in molecular biology, John Wiley & Sons, Inc., 1994）、FISH（フルオレッセンスインサイチューハイブリダイゼーション、Lawrenceら, Cell, 52:51, 1988）解析等により調べることが出来る。ある特定の染色体導入を望む場合には、多くのヒト細胞形質転換体クローンについて、それぞれ上記の過程を繰り返して、目的染色体がマーキングされたものを探す。あるいは、ヒト細胞形質転換体クローンの混合物について上記の過程を実施し、得られる多数のマウスーヒトモノクロモソーム雑種細胞についてヒト染色体の同定を行なうことが可能である。

さらに、導入を望む染色体上のDNA配列を標的とした相同組換え(Thomas ら, Cell, 51:503, 1987）により、特定の部位にマーカー遺伝子を挿入することも可能である。

マウスーヒト雑種細胞から調製したミクロセルにガンマ線照射することにより、マーキングされたヒト染色体を断片化してマウスA9細胞に導入することも出来る。また、ミクロセルにガンマ線照射しない場合でも、ある割合で部分断片化したヒ

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ト染色体が移入されることがある。これらの場合、得られるミクロセル融合株は、マーキングされたヒト染色体の部分断片を保持している。これらは、受容細胞に染色体部分断片を導入したい場合に用いることができる。

(2) 分化多能性を保持するマウス細胞へのヒト染色体またはその断片の移入

これまでに、種々の系統のマウス由来の胚性癌腫細胞（EC細胞、Hanaokaら，Differentiation, 48:83, 1991 ）、胚性幹細胞（ES細胞、Evansら，Nature, 292:154, 1981 ）、胚性生殖細胞（EG細胞、Matsuiら，Cell, 70:841, 1992）がマウス初期胚に注入あるいは共培養することによりその正常体細胞に寄与する、すなわちキメラマウス作製可能であることが報告されている。ES、EG細胞はその能力が特に高く、多くの場合生殖細胞にも寄与し、その細胞由来の子孫を作ることができる。EC細胞は主に生殖細胞癌から、ES細胞は胚盤胞の内部細胞塊から、EG細胞は発生初期に出現する始原生殖細胞から得られる。本発明におけるヒト染色体移入の受容細胞としてはこれらの細胞株及びその変異株、さらにはマウス個体において全て、あるいは一部の正常体細胞に分化可能なあらゆる未分化細胞を用いることができる。

受容細胞へのヒト染色体移入の材料としては（1）で得られたヒト染色体供与細胞から調製されるミクロセルあるいはガンマ線照射したミクロセルを用いることができる。受容細胞への移入は<清水素行，細胞工学ハンドブック，羊土社，1992>等に記された方法で受容細胞とミクロセルを融合することにより行なう。ミクロセル供与細胞においてはあるヒト染色体またはその断片が受容細胞において選別可能なマーカーを保持している。この中から、（1）と同様PCR、サザンブロット解析、FISH解析等により、導入を目的とする遺伝子あるいは染色体あるいはその断片を保持する株を選択すれば、あらゆるヒト染色体、あるいはその断片を導入することが可能である。また、異なる選択マーカーを保持する複数の染色体、あるいはその断片を逐次導入することにより、これらを同時に保持する受容細胞を得ることもできる。さらに、すでにあるヒト染色体を導入した細胞株の中から、導入染色体数が増加したものを選抜することも可能である。これは通常、培養液中に添加する選択薬剤の濃度を高めることにより達成される。

ヒト染色体上のマーカー（G418耐性等）により選抜された受容細胞が、供与細

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胞の保持していたヒト染色体の全部あるいは一部を保持していることは、以下のようにして確認される。選抜された受容細胞から抽出したゲノムDNAを用い、プローブとしてヒト特異的繰り返し配列（L1、 Alu等、Korenbergら，Cell，53:391，1988）、ヒト遺伝子等を用いたサザン解析により検出される。また、ヒト遺伝子特異的プライマーによるPCR法、及びヒト染色体特異的プローブ（Lichterら，Human Genetics，80:224，1988）を用いたフルオレッセンスインサイチューハイブリダイゼーション（FISH）等の染色体解析により確認することができる。

（3）ヒト染色体導入ES細胞からのキメラマウス作製

（2）で得られたES細胞株からのキメラマウス作製は、＜相沢慎一，バイオマニュアルシリーズ 8 ジーンターゲティング，羊土社，1995>等に記された方法で行なう。効率的なキメラマウス作製を行なうための宿主胚の発生時期、系統等の選択については、それぞれのES細胞株についてすでに検討された条件を用いることが望ましい。例えば、CBA×C57BL/6 F1 由来のTT2細胞（野生色、Yagiら，Analytical Biochemistry，214:70，1993）についてはBalb/c （白色、日本クレア社）あるいはICR（白色、日本クレア社）由来の8細胞期胚を宿主胚として用いるのが望ましい。

（4）キメラマウスにおけるヒト染色体の保持及びヒト遺伝子発現

ES細胞株を注入した胚から誕生したマウスにおけるES細胞の貢献率は、その毛色によりおおまかに判定することができる。但し、毛色に全く貢献が見られないからといって他の組織で貢献がないとは断定できない。より詳細な、キメラマウス各組織におけるヒト染色体の保持は各組織より抽出されたゲノムDNAを用いたサザン解析、PCR等によって確認することができる。

導入ヒト染色体上の遺伝子発現は以下のようにして確認される。ヒト染色体由来mRNAの発現は各組織由来RNAを用いたRT-PCR法（Kawasakiら，P.N.A.S.，85:5698，1988）、ノーザンブロット法（Ausubelら，前記）により検出される。蛋白質レベルの発現は、マウスの相同蛋白質との交差反応性を最小にした抗ヒト蛋白質抗体による酵素免疫測定法（ELISA、富山・安東，単クローン抗体実験マニュアル，講談社サイエンティフィク，1987；石川，超高感度酵素免疫測定法，学会出版センター，1993）、ウエスタンブロット法（Ausubelら，前記）あるいは、

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電気泳動度の違いを利用したアイソザイム分析（Koi ら，Jpn. J. Cancer Res.，80:413，1989)等により検出される。さらに、キメラマウス細胞中でのヒト染色体の保持及び該染色体上の遺伝子の発現が、キメラマウス由来初代培養細胞での薬剤耐性マーカー遺伝子発現による耐性細胞の出現より確認できる。

例えば、ヒト免疫グロブリン重鎖の存在するヒト14番染色体を保持するES細胞から作製されたキメラマウスについて、キメラマウス血清中のヒトIgM、IgG、IgA等をマウス抗体との交差反応性を最小にした抗ヒトIg抗体による酵素免疫測定法により検出することができる。また、このキメラマウスをヒト由来抗原（例えばヒト血清アルブミン）により免疫し、その脾臓細胞とマウスミエローマを融合することにより得られる、ハイブリドーマ（安東・千葉，単クローン抗体実験操作入門，講談社サイエンティフィク，1991）をELISAによりスクリーニングすることにより、ヒト免疫グロブリン重鎖を産生するハイブリドーマを得ることができる。

以上、マウスを例にとり、ヒト染色体またはその断片を保持し、該染色体またはその断片上の遺伝子を発現するキメラ非ヒト動物の作製法を説明したが、本発明において、キメラ非ヒト動物へ移入される染色体またはその断片は、ヒト由来のものに限られず、広く外来染色体またはその断片を移入し、該染色体またはその断片上の遺伝子を発現させることが可能である。ここで、「外来染色体」とは、分化多能性を保持する細胞へ移入され、キメラ非ヒト動物において、該染色体またはその断片上の遺伝子が発現することを特徴とするものであり、その由来ととする生物種は特に限定されるものではない。また、本発明の方法により、キメラマウスのみならず、他のキメラ動物、例えば、ラット、ブタ等の哺乳類その他のキメラ動物も作製することができる。マウス以外の動物種におけるES細胞あるいはES様細胞の樹立はラット（Iannacconeら，Dev. Biol.，163，288-，1994）、ブタ（Wheeler ら，Reprod. Fertil. Dev.，6，563-，1994）、ウシ（Simsら，Proc. Natl. Acad. Sci. USA，91，6143-6147，1994）において報告され、さらにメダカ、ニワトリ等でも試みられている（トランスジェニック動物，蛋白質核酸酵素，1995年10月号増刊，共立出版）。また、ヒツジにおいてはES様細胞（ED細胞）さらにはそれを10代以上継代して得られる上皮様細胞由来の核を移植され

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た未受精卵が正常に発生することが知られている（Campbellら，Nature，380，64-，1996）。これらESあるいはES様細胞を受容細胞とした外来染色体移入によってマウスの場合と同様に外来染色体あるいはその断片を保持し、該染色体またはその断片上の遺伝子を発現するキメラ非ヒト動物が作製可能である。

また、本発明において、外来染色体あるいはその断片が移入される、分化多能性を保持する細胞は、前述のＥＳ細胞、ＥＣ細胞、ＥＧ細胞に限られるものではない。例えば、骨髄幹細胞に外来染色体あるいはその断片を移入することが可能であり、該骨髄幹細胞の生体への移植により、遺伝病等の治療を行うことが可能である。

キメラ非ヒト動物において、外来染色体を保持するES細胞がその生殖細胞に分化した場合、生殖により得られる子孫にも導入染色体または断片が認められ、その子孫は導入された染色体または断片上の遺伝子を発現する。

上記のようにして得られるキメラ非ヒト動物またはその子孫を利用して、外来染色体またはその断片上の遺伝子を発現させ、その産物を回収することにより、生物学的に活性な物質を製造することができる。具体的には、キメラ非ヒト動物またはその子孫の個体を外来染色体またはその断片上の遺伝子を発現しうる条件下で飼育し、その後発現産物を動物の血液、腹水などから回収することができる。あるいはまた、キメラ非ヒト動物またはその子孫の組織、細胞あるいはそれを不死化したもの（例えば、ミエローマ細胞との融合により不死化したハイブリドーマ）などを外来染色体またはその断片上の遺伝子を発現しうる条件下で培養し、その後発現産物を培養物から回収することができる。さらには、これらキメラ非ヒト動物またはその子孫の組織、細胞、あるいはそれを不死化したものから抽出した外来染色体あるいはその断片、または外来染色体またはその断片を構成するＤＮＡ、さらにはまた、キメラ非ヒト動物またはその子孫の組織、細胞、あるいはそれを不死化したものに保持された外来染色体あるいはその断片に由来するｃＤＮＡを動物細胞あるいは昆虫細胞（例えば、ＣＨＯ細胞、ＢＨＫ細胞、肝ガン細胞、ミエローマ細胞、ＳＦ９細胞等）に導入し、該細胞を外来染色体またはその断片上の遺伝子を発現しうる条件下で培養し、その後発現産物（例えば、特定の抗原特異的な抗体蛋白質等）を培養物から回収することができる。発現産物は

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遠心分離などの公知の方法に従って回収することができ、さらに、硫安分画、分配クロマトグラフィー、ゲル濾過クロマトグラフィー、吸着クロマトグラフィー、分取薄層クロマトグラフィーなどの公知の方法に従って精製することができる。生物学的に活性な物質は、外来染色体上にコードされているあらゆる物質を含み、例えば、抗体、特にヒト抗体などを挙げることができる。例えば、得られたキメラ動物の脾細胞あるいはそのハイブリドーマなどの不死化細胞から該染色体上のヒト抗体遺伝子をクローニングし、チャイニーズハムスター卵巣細胞（ＣＨＯ）やミエローマ細胞に導入してヒト抗体を生産することができる（Lynette ら, Biotechnology, 10, 1121-, 1992; Bebbingtonら, Biotechnology, 10, 169-, 1992)。

本発明により得られるヒト2番、14番、22番染色体（断片）を保持するキメラマウス及びその子孫は、ヒト抗体重鎖（14番染色体上）、軽鎖κ（2番染色体上）、軽鎖λ（22番染色体上）それぞれの遺伝子の機能的配列の大部分を保持し得る。すなわち、酵母人工染色体等を用いてヒト抗体遺伝子の一部を導入した既知のトランスジェニックマウス（Greenら, Nature Genetics, 7, 13-, 1994, Lonbergら, Nature, 368, 856-, 1994等）と比較して、ヒトにおいて観察されるものにより近い、非常に多様なヒト抗体レパートリーを発現することが可能である。また、本発明により得られる2番+14番、22番+14番等の染色体（断片）を同時に保持するキメラマウス及びその子孫、並びに、それらを交配することにより得られる2番+14番+22番等の染色体（断片）を同時に保持するマウス及びその子孫は、重鎖、軽鎖の両者がヒト由来である完全なヒト抗体を産生することが可能である。これらのマウスはヒト由来抗原に対してそれを異物とみなして免疫反応を起こし、抗原特異的ヒト抗体を産生することができる。この性質は治療用のヒトモノクローナル抗体、あるいはヒトポリクローナル抗体を得るために非常に有用である（Greenら、前記、Lonbergら、前記）。一方、特定の抗原に対して親和性の高いヒト抗体を得る効率を上げるためには、マウス抗体を産生せず、ヒト抗体のみを産生するマウスを作成することが望まれる（Greenら、前記、Lonbergら、前記）。本発明において、これは典型的には既知の手法を用いて以下の方法Aあるいは Bにより達成される。

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方法A：マウス抗体欠損ES細胞およびマウス抗体欠損キメラ宿主胚を用いる方法。
方法B：ヒト染色体導入キメラマウスよりヒト染色体を保持する子孫を得てマウス抗体遺伝子を欠損するマウス系統との交配を行う方法。

以下にA，Bそれぞれの方法の典型的な例について以下に具体的に記す。

方法Aの具体的手順

1．マウスES細胞に2コピー存在するマウス抗体重鎖遺伝子の一方を標的遺伝子相同組み換え法（Joynerら，Gene Targeting，1993，IRL PRESS）を用いて破壊する。遺伝子破壊箇所には後に部位特異的組換えにより除去可能な配列、例えばloxP配列（Creレコンビナーゼにより組み換え、Sauerら、前記、他にFLPレコンビナーゼ-FRT配列を用いたO'Gormanら，Science，251，1351-，1991の例がある）にはさまれたG418耐性遺伝子等のマーカー遺伝子を挿入する。

2．一方の抗体重鎖遺伝子が破壊された上記薬剤耐性マウスES細胞を高濃度の薬剤存在下で培養し、高濃度薬剤耐性となった株を選抜する。これらの株をスクリーニングすることにより両方の抗体重鎖遺伝子が破壊された株が得られる（相沢慎一、前記）。

3．2．で得られた両方の抗体重鎖遺伝子が破壊されたマウスES細胞に1．で薬剤耐性遺伝子の両側に挿入した組換え配列の間で部位特異的組み換え反応を起こす酵素遺伝子、例えばCreレコンビナーゼ遺伝子（Sauerら、前記）を一時的に導入し、loxP配列の間で組み換え反応が起こって両方の抗体重鎖遺伝子に挿入された薬剤耐性遺伝子が除去された薬剤感受性株を選抜する（高津聖志ら、実験医学別冊、免疫研究の基礎技術、p255-、1995、羊土社）。

4．マウス抗体軽鎖κ遺伝子について上記1～3の過程を繰り返し、最終的に抗体重鎖及びκ鎖を完全に欠損した薬剤感受性株を取得する。

5．4の株（抗体重鎖、κ鎖欠損マウスES細胞）を受容細胞としたミクロセル融合により、薬剤耐性マーカー（例えばG418耐性遺伝子）でマーキングされたヒト抗体重鎖遺伝子を含むヒト14番染色体（断片）を導入する。

6．5で得られた株を受容細胞としたミクロセル融合により、5とは異なる薬剤耐性マーカー（例えばピューロマイシン耐性遺伝子）でマーキングされたヒト抗体軽鎖遺伝子を含むヒト2番染色体（断片）あるいは22番染色体（断片）または

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その両者を導入する。

7．自らの抗体を産生することができないマウス系統（例えばRAG-2ノックアウトマウス、Shinkaiら，Cell，68，855-，1992、膜型μ鎖ノックアウトマウス、Kitamuraら，Nature，350，423-，1991）から得た胚を宿主胚として6で得られたES細胞とのキメラマウスを作成する。

8．得られるキメラマウスにおいてほとんどの機能的なBリンパ球はES細胞に由来する（高津聖志ら、実験医学別冊、免疫研究の基礎技術、p234-、羊土社、1995）。このBリンパ球においてはマウス重鎖、κ鎖が欠損しているため、主として導入染色体上の機能的なヒト抗体遺伝子よりヒトの抗体のみが産生される。

方法Bの具体的手順

1．ヒト抗体重鎖、軽鎖κまたは軽鎖λを含むヒト染色体あるいはその断片を保持するキメラマウスからそれらを安定に保持する継代可能な子孫を得る。

2．1．で得られたヒト抗体重鎖または軽鎖を発現するマウス系統あるいはそれらの交配により得られたヒト抗体重鎖および軽鎖の両者を発現するマウス系統と自らの抗体遺伝子が欠損しているマウス系統（例えば膜型μ鎖ノックアウトマウス、前記、軽鎖κノックアウトマウス、Chenら，EMBO J.，3，821-，1993）との交配により、マウス抗体重鎖、及び軽鎖κの欠損についてホモ接合体であり、なおかつヒト抗体重鎖（14番）＋軽鎖κ（2番）、抗体重鎖（14番）＋軽鎖λ（22番）あるいはヒト抗体重鎖（14番）＋軽鎖κ（2番）＋軽鎖λ（22番）を含むヒト染色体を保持するマウス系統を得る。このマウス系統においてはマウス抗体重鎖、軽鎖κ遺伝子が欠損しているため、主として導入染色体上の機能的なヒト抗体遺伝子よりヒトの抗体のみが産生される。

なお、上記のAおよびBの方法は、ヒト抗体のみならず、外来染色体上に存在するあらゆる遺伝子の産物を効率的に得るために、用いることができる。

以下に実施例を示して具体的に説明するが、本発明はこれらの実施例に限定されるものでない。

（実施例1）G418耐性標識されたヒト染色体（断片）を保持する染色体供与細胞の作製

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G418耐性遺伝子を含むプラスミドpSTneoB（Katohら、Cell Struct. Funct., 12:575, 1987; Japanese Collection of Research Biologicals (JCRB), Deposit Number: VE039）を制限酵素SalI（宝酒造）で線状化し、ヒト正常繊維芽細胞HFL-1（RIKEN Cell Bankより入手、RCB0251)へ導入した。HFL-1細胞をトリプシン処理し、$5x10^6$個／mlとなるようにダルベッコのリン酸バッファー（PBS）に懸濁してから10μgDNA存在下でジーンパルサー（バイオラッド）を用いてエレクトロポレーションを行なった（石田ら，細胞工学実験操作入門，講談社，1992）。25μFの容量で1000Vの電圧を4mm長のエレクトロポレーションセル（165-2088、バイオラッド）を用いて室温で印加した。エレクトロポレーションした細胞を15％牛胎児血清（FBS）を添加したイーグルF12培地（以下F12、という）を含む100mm組織培養用プラスチックシャーレ（コーニング）3～6枚に播種した。1日後に200μg/mlのG418（GENETICIN, シグマ）を含む15%FBSを添加したF12培地と置き換えた。2～3週間後に生じたコロニー100個程度を一つの集団として52グループにそれぞれまとめ、100 mmシャーレに再び播種し培養した。

マウスA9細胞（Oshimura, Environ. Health Perspect., 93:57, 1991, JCRB0211）を10％牛胎児血清（FBS）を添加したダルベッコ改変イーグル培地（以下DMEM、という）中、100 mm シャーレで培養した。52グループのG418耐性HFL-1 細胞を15%牛胎児血清(FBS）と200μg/mlのG418を添加したF12中で、それぞれ100mmシャーレで培養した。マウスA9細胞とHFL-1 細胞をトリプシン処理後それぞれ四分の一から半分量ずつ混合し、100mmシャーレに播種し10％牛胎児血清（FBS）を添加したDMEMと15％牛胎児血清（FBS）を添加したF12との等量混合物中で半日から一日培養した。細胞融合は（清水ら，細胞工学ハンドブック，羊土社，p.127-，1992）に記述されている方法に従った。DMEMで細胞表面を2回洗った後、2ml のPEG（1：1.4）溶液で1分間処理し、さらに、2mlのPEG（1：3）溶液に換え1分間処理した。PEG溶液を吸い取り、無血清培地（DMEM）で3回洗った後、通常の培地（10％FBS、DMEM）で1日間培養した。細胞をトリプシン処理により分散し、ウワバイン（$1x10^{-5}$M, シグマ）およびG418（800μg/ml）を含む二重選択培地（10％FBS、DMEM）に懸濁し、100mmシャーレ3枚に播種した。約3週間培養した後、生じたコロニーをトリプシン処理により細胞を分散しG418（800μg/ml）

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を含む選択培地（10%FBS、DMEM）で培養した。

トリプシン処理により細胞を分散し2グループを一つにまとめて、6本の25cm^{2}遠心用フラスコ（コースター、3025）にて細胞密度が70～80%飽和程度まで培養した。コルセミド（0.05μg/ml，デメコルシン，和光純薬）を含む培養液（20%FBS、DMEM）に交換し、2日間培養しミクロセルを形成させた。培養液を除去し、予め保温（37℃）しておいたサイトカラシンB（10μg/ml，シグマ）溶液を遠心用フラスコに満たし、アクリル製遠心容器に遠心用フラスコを挿入し、34℃、8000rpm，1時間の遠心を行なった。ミクロセルを無血清培地に懸濁し、フィルターで濾過し精製した。マウスA9細胞を80%飽和の状態まで培養した25cm^2 フラスコに精製した微小核を加えPEG溶液で融合させた。G418を含む選択培地で培養しコロニーを単離した。各クローンが保持するヒト染色体（2番、4番、14番、22番）は以下の通り同定した。上記以外のすべての実験操作及び試薬等は〈清水ら、細胞工学ハンドブック、羊土社、p.127-〉に従った。

（1）PCR解析

単離した細胞を培養し、細胞からPuregene DNA Isolation kit（Gentra System社）を用いてゲノムDNAを抽出し、このゲノムDNAを鋳型とし、ヒト染色体特異的なプライマーを用いてPCR法で2、4、14、22番ヒト染色体を保持するクローンを選抜した。PCR増幅は約0.1μgのゲノムDNAを使用し（Innisら，PCR実験マニュアル，HBJ出版局，1991，サーマルサイクラーはGeneAmp 9600，Perkin-Elmer社を使用）に従い行なった。Taqポリメラーゼは Perkin-Elmer社製を用い、反応条件は、94℃5分を1サイクル行なった後、変性94℃15秒、アニーリング54～57℃15秒（プライマーにより適宜変更）、伸長72℃20秒を35サイクル行なった。プライマーは各染色体上に存在する遺伝子（O'Brien，Genetic Maps,6th edition，Book 5，Cold Spring Harbor Laboratory Press，1993 ）及び多型性マーカー（Polymorphic STS Primer Pair，BIOS 社；Weissenbach ら，Nature 359:794，1992；Walterら，Nature Genetics，7:22，1994等）を用いた。遺伝子プライマーはGenBank、EMBL等のデータベースより入手した塩基配列をもとに作製した。多型性プライマーの名称及び、遺伝子プライマーの配列はそれぞれの染色体について以下の実施例で示した（2番：実施例1、4番：実施例6、14番：実施例9、22番：実施例

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2）。2番染色体の同定には以下に示す遺伝子マーカー及び多型性マーカー（Polymorphic STS Primer Pair, BIOS社: D2S207, D2S177, D2S156, D2S159 BIOS社）を用いた。

Cκ（immunoglobulin kappa constant）：5'-TGGAAGGTGGATAACGCCCT（配列番号1）, 5'-TCATTCTCCTCCAACATTAGCA（配列番号2）

FABP1（fatty acid binding protein-1 liver）：5'-GCAATCGGTCTGCCGGAAGA（配列番号3）, 5'-TTGGATCACTTTGGACCCAG（配列番号4）

Vk3-2（immunoglobulin kappa variable）：5'-CTCTCCTGCAGGGCCAGTCA（配列番号5）, 5'-TGCTGATGGTGAGAGTGAACTC（配列番号6）

Vk1-2（immunoglobulin kappa variable）：5'-AGTCAGGGCATTAGCAGTGC（配列番号7）, 5'-GCTGCTGATGGTGAGAGTGA（配列番号8）

（2）フルオレッセンス インサイチューハイブリダイゼーション（FISH）

FISH解析は（松原ら, FISH実験プロトコール, 秀潤社, 1994）に記された方法に従い、ヒト2番、4番、14番、22番染色体特異的プローブ（CHROMOSOME PAINTING SYSTEM, Cambio社）を用いて行なった。

例えば、2番染色体を保持するクローンは26グループ(745クローン)中10グループに1個以上のクローンを得た。このうち用いた2番染色体特異的なプライマーすべてに陽性であったのは5クローンだった。これらのクローンをFISH解析した。FISH解析は（松原ら, FISH実験プロトコール、秀潤社、1994）に記された方法に従い、ヒト2番染色体特異的プローブ（CHROMOSOME PAINTING SYSTEM, CANBIO社）を用いて行なった。すべてのプライマーに陽性な細胞ではヒト2番染色体がほぼ完全な形で観察され、1部のプライマーのみ陽性なクローンのうちいくつかのクローンではヒト2番染色体よりも小さな独立染色体が観察され、あるいはヒト2番染色体以外の染色体と融合しているような形の染色体を持つ細胞も観察された（第1図）。第1図において、横列がクローン名、縦列はPCRに使用したプライマーを示す。●は陽性を、×は陰性を示した。また、FISHにより観察されたヒト2番染色体の存在形態を最下行に示した。記載のないものは実施していない。

同様にして、ヒト4番、14番、22番染色体を保持するA9細胞を得た。

（実施例2）マウスES細胞へのミクロセル法によるヒト22番染色体導入

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染色体供与細胞として、（実施例1）で得られたヒト22番染色体を保持するマウスA9細胞株（以下A9/#22、という）を用いた。染色体受容細胞としてはマウスES細胞株E14（Martin L. Hooperより入手、Hooperら，Nature，326 :292，1987）を用いた。E14の培養法は〈相沢慎一，バイオマニュアルシリーズ 8，ジーンターゲティング，羊土社，1995〉に記された方法に従い、栄養細胞としては、マイトマイシンC（シグマ）処理したG418耐性STO細胞株（大阪大学、近藤寿人教授より入手）を用いた 。まず清水ら（細胞工学ハンドブック、羊土社、1992）の報告した方法に従い、約10^8個のA9/#22からミクロセルを調製した。得られたミクロセルは全量を5mlのDMEMに懸濁した。約10^7個のE14をトリプシンで分散させた後、DMEMで3回洗浄し、5mlのDMEMに懸濁した後、ミクロセルとあわせ、1250rpm、10分間遠心して上清を除いた。沈殿をタッピングによりよくほぐし、1:1.4PEG溶液（5g PEG1000,〈和光純薬〉，1ml DMSO〈シグマ〉を6mlDMEMに溶解）0.5mlを加えて室温で1分30秒静置した後、10mlのDMEMをゆっくりと加えた。直ちに1250rpm、10分間遠心して上清を除き、沈殿を30mlのES細胞用培地に懸濁し、あらかじめ栄養細胞をまいた直径100mmの組織培養用プラスチックシャーレ（コーニング）3枚に播種した。24時間後に300μg/mlのG418（GENETICIN，シグマ）を加えた培地と交換し、その後毎日培地交換を行なった。1週間～10日後には薬剤耐性コロニーが出現する。その出現頻度はE14細胞10^7個あたり0～5個であった。そのコロニーをピックアップし増殖させ、5×10^6個あたり1mlの保存用培地（ES細胞用培地＋10%DMSO〈シグマ〉）に懸濁し、-80℃にて凍結保存した。同時に各薬剤耐性株について10^6～10^7個の細胞からゲノムDNAをPuregene DNA Isolation Kit（Gentra System社）により調製した。

ヒト22番染色体の断片化はミクロセルにガンマ線照射することにより行なった（Koiら，Science，260:361，1993）。約10^8個のA9/#22より取得したミクロセルを5mlのDMEMに懸濁し、ガンマセル40（カナダ原子力公社製）により、氷上で60Gyのガンマ線を照射した（1.2Gy/分×50分）。ガンマ線照射したミクロセルは未照射ミクロセルと同様に融合、薬剤耐性株選抜を行なった結果、薬剤耐性株の出現頻度はE14細胞10^7個あたり1～7個であった。薬剤耐性株については未照射の場合と同様にして凍結保存、DNA取得を行なった。

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未照射ミクロセル薬剤耐性株E14/#22-9 、E14/#22-10 、ガンマ線照射ミクロセル薬剤耐性株E14/#22-14、E14/#22-25における導入染色体の保持は以下の（1）～（3)により確認した。

（1）PCR解析（第２図）

薬剤耐性株ゲノムDNAを鋳型としてヒト22番染色体上に存在する遺伝子（Genetic Maps，前記）及び多型性マーカー（Polymorphic STS Primer Pair，BIOS社：D22S315，D22S275，D22S278，D22S272，D22S274；Nature 359:794，1992）の存在をPCR法により検出した。GenBank、EMBL等のデータベースより入手した塩基配列をもとに作製した遺伝子プライマーオリゴヌクレオチドの配列を記す。

PVALB（parvalbumin）：5'-TGGTGGCTGAAAGCTAAGAA（配列番号９），5'-CCAGAAGAATGGTGTCATTA（配列番号10）

MB（myoglobin）：5'-TCCAGGTTCTGCAGAGCAAG（配列番号11），5'-TGTAGTTGGAGGCCATGTCC（配列番号12）

DIA1（cytochrome b-5 reductase）：5'-CCCCACCCATGATCCAGTAC（配列番号13），5'-GCCCTCAGAAGACGAAGCAG（配列番号14）

Igλ（immunoglobulin lambda）：5'-GAGAGTTGCAGAAGGGGTGACT（配列番号15），5'-GGAGACCACCAAACCCTCCAAA （配列番号16）

ARSA（arylsulfatase A）：5'-GGCTATGGGGACCTGGGCTG（配列番号17），5'-CAGAGACACAGGCACGTAGAAG（配列番号18）

約0.1μgのゲノムDNAを鋳型として上記の10種のプライマーについてPCR増幅（Innisら，前記）を行なった。その結果、未照射の2株は全てのプライマー、ガンマ線照射した2株については一部のプライマーについて期待される長さの増幅産物が検出された。以上の結果を第２図に示す。第２図において、左側にヒト22番染色体のGバンド像に基づく模式的な染色体地図、また、位置が明らかになっているいくつかのマーカーについてはどのバンドに位置するかを示した（O'Brien，GENETIC MAPS，6th edition，BOOK 5等）。遺伝子及び多型性マーカーの並び方は、現在までに入手出来る情報（Science，HUMAN GENETIC MAP，1994，Nature Genetics，7:22，1994，Nature 359:794，1992等）を基に、大まかな位置関係を示したもので、順序は必ずしも正確ではない。4種のG418耐性E14細胞株について、PC

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Rにより期待される増幅産物が検出されたマーカーは■で、検出されなかったマーカーを□で示した。下側にはFISH解析による観察結果を示した。A9/#22は、染色体供与細胞である。

（２）サザンブロット解析

サザンブロット解析はヒト特異的な繰り返し配列であるL1配列（ハプロイドゲノム当たり10^4～10^5コピー存在、RIKEN DNA Bankより入手、Nucleic acids research,13;7813, 1985, pUK19A由来1.4kb EcoRI-BamHI 断片）をプローブとして、制限酵素（BglII、宝酒造製）処理を行なった約2μgのゲノムDNAに対して<Ausubelら, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.,1994>に記された方法に従い行なった。その結果、各薬剤耐性株DNAにおいてヒトL1配列とハイブリダイズするバンドが多数検出された。未照射の2株についてはそのパターン及び、各バンドの濃度から推定できるヒト染色体DNAのマウスゲノムDNAに対する量

比はA9/#22のそれと同等であった。ガンマ線照射株の全体のシグナル強度はA9/#22と比較した場合、PCR解析で示された欠失の程度と相関していた。

（３）フルオレッセンスインサイチューハイブリダイゼーション（FISH）

FISH解析は（松原ら, FISH実験プロトコール, 秀潤社, 1994）に記された方法に従い、ヒト22番染色体特異的プローブ（CHROMOSOME PAINTING SYSTEM, Cambio社）を用いて行なった。その結果、観察した分裂像のほとんどにおいて、E14/#22-9 はマウス染色体に転座した形で、他の3株は独立した染色体としてヒト22番染色体が検出された。

以上の実験により、得られたG418耐性株E14/#22-9 、E14/#22-10 はヒト22番染色体の全てあるいは大部分を、E14/#22-14 、E14/#22-25 はその部分断片を保持することが確かめられた。

（実施例3）ヒト22番染色体を保持するES細胞からのキメラマウス作製

一般的なマウス胚取得、培養、ES細胞の胚への注入、仮親子宮への移植等の手技については、<相沢慎一, バイオマニュアルシリーズ8, ジーンターゲティング, 羊土社, 1995>に記された方法に従った。（実施例2）で得られ、ヒト22番染色体を保持していることが確認されたG418耐性ES細胞株E14/#22-9を凍結ストックよ

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り立ち上げ、C57BL/6×C3H F1雌マウス（日本クレア社）をC3H雄マウス（日本クレア社）と交配することにより取得した胚盤胞期胚に胚あたり10～15 個注入した。偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のES細胞注入胚を移植した。その結果を（第１表）に示す。

第１表．ヒト22番染色体（断片）を保持するE14 細胞株からのキメラマウス作製

ES細胞株/ヒト染色体	G418耐性株番号	ES細胞を注入した胚盤胞期胚数	子マウスの数	キメラマウスの数	毛色への貢献率 ～10%	10-30%	30%～
E14/#22	9	166	29	16	7	3	6

計166個の注入胚を移植した結果29匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚由来の野生色（濃茶）の中にE14細胞由来の薄灰色が認められるかどうかにより判定される。誕生した29匹のうち毛色に明らかに薄灰色の部分のある、すなわち、E14細胞の貢献の認められる個体は16匹であった。また、最高の貢献率はK22-22における約40%であった。

この結果より、ヒト22番染色体を保持するマウスES細胞株E14/#22-9はキメラ形成能、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

（実施例4）ヒト22番染色体を保持するES細胞由来キメラマウス各組織におけるヒト染色体DNAの保持確認

（実施例3）の毛色による判定に加えて、尻尾より調製したゲノムDNAを鋳型としたPCR解析により、導入染色体の保持を確認した。誕生後3週以上を経たキメラマウスから<勝木元也，発生工学実験マニュアル，講談社サイエンティフィク，1987>に記された方法に従い尻尾を取得し、Puregene DNA Isolation Kitを用いてゲノムDNAを抽出した。このゲノムDNAを鋳型として（実施例2）で使用した多型性プライマーのうちPVALB、D22S278を用いて増幅産物の確認を行なった。毛色に

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貢献の見られた個体のうち10匹について解析を行った結果、全ての個体において少なくともいずれかのプライマーによる増幅産物が確認された。

サザン解析は（実施例2）と同様に、ヒトL1配列をプローブとして用い、6個体のキメラマウス、1個体の非キメラマウスの2μgの尻尾ゲノムDNAに対して行なった。その結果、全てのキメラ個体において多数のヒトL1配列の存在が認められ、そのパターンはE14/#22-9と類似していた。マウスゲノムに対する量比は最も多いもので10%程度であった（第3図）。第3図において、各レーンとも、BglII消化した2μgのゲノムDNAを使用した。^{32}P標識ヒトL1配列をプローブとし、シグナルはイメージアナライザーBAS2000（富士写真フイルム社）により検出した。右より、キメラマウス（K22-6,7,8,9,10,11,12 ： 9は非キメラ）の尻尾由来ゲノムDNAおよびコントロールDNA(C: C は、E14/#22-9 と E14ゲノムDNAを 1:9の重量比で混合したもの）のレーンである。左側にDNA分子量、右側に各キメラ個体のキメラ率を示した。（- ： 0%、+ ： ～10%、++ ： 10～30%）

さらに、毛色に5%程度の貢献の見られたキメラ個体（K22-7）について脳、肝臓、筋肉、心臓、脾臓、胸腺、卵巣、腎臓からISOGEN（ニッポンジーン社）によりゲノムDNAを取得し、それぞれの組織について、（実施例2）で使用した遺伝子プライマーのうちMB、DIA1を用いてPCR解析を行なった。その結果2つのプライマーとも全ての組織において期待される増幅産物が確認された。DIA1プライマーによる結果を（第4図）に示す。PCR産物は2%アガロースゲルにて電気泳動した後、臭化エチジウム染色して検出した。第4図の各レーンは左からB：脳、L：肝臓、SM：骨格筋、H：心臓、Sp：脾臓、Th：胸腺、Ov：卵巣、K：腎臓、nc：非キメラマウス尻尾DNA(陰性コントロール)、pc：ヒト繊維芽細胞（HFL-1）DNA(陽性コントロール)を示す。

これらの結果より、E14/#22-9はマウス個体において種々の正常組織に貢献し、かつヒト22番染色体を保持していることが確かめられた。

（実施例5）ヒト22番染色体を保持するES細胞由来キメラマウスにおけるヒト遺伝子の発現

ヒト遺伝子発現確認のための試料としては、毛色に5%程度の貢献の見られた個体（K22-7）の尻尾を液体窒素にて凍結後粉砕したものを用いた。これは皮膚、

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骨、筋肉、血液等の組織の混合したものである。これよりISOGEN（ニッポンジーン）を使用して総RNAを抽出し、RT-PCR法により、ヒト-ミオグロビン（MB）、ヒト-チトクローム b5 レダクターゼ（DIA1）のmRNAの検出を行なった。RT-PCRは<Innisら，PCR実験マニュアル，HBJ出版局，1991>に記された方法に従って行なった。逆転写反応用プライマーは、ランダムヘキサマーオリゴヌクレオチド（終濃度100pmol、宝酒造製）を、逆転写酵素はBRL社製（スーパースクリプト）を使用した。cDNAを鋳型とした増幅に用いたプライマーを記す。

MB：5'-TTAAGGGTCACCCAGAGACT（配列番号19），5'-TGTAGTTGGAGGCCATGTCC（配列番号20）

DIA1：5'-CAAAAAGTCCAACCCTATCA（配列番号21），5'-GCCCTCAGAAGACGAAGCAG（配列番号22）

その結果、両遺伝子のmRNAに特異的な増幅産物が検出された（第５図）。RT-PCR産物は2%アガロースゲルにて電気泳動した後、臭化エチジウム染色して検出した。第５図において、M はマーカー（HindIII消化λDNA＋HaeIII消化φX174DNA，宝酒造）、MBはヒトミオグロビン、DIA1はヒトチトクロームb5レダクターセ、WTは野生型C3Hマウスを示す。

さらに同じ個体(K22-7）について、脳、心臓、胸腺、肝臓、脾臓、腎臓、卵巣、骨格筋からISOGENを使用して総RNAを抽出し、上記の2種のプライマーにより各臓器のRT-PCRを行った。その結果、DIA1は全ての臓器で、MBは心臓と骨格筋のみで期待される増幅産物が確認された（第６図）。ミオグロビンは筋細胞特異的に発現することが知られており(Bassel-Dubyら，MCB，12:5024，1992）、この結果は導入したヒト染色体上の遺伝子がマウス個体で正常な組織特異的発現制御を受け得ることを示している。PCR産物は2%アガロースゲルにて電気泳動した後、臭化エチジウム染色して検出した。第６図において、各レーンは左からB:脳、H:心臓、Th:胸腺、L:肝臓、Sp: 脾臓、K:腎臓、Ov: 卵巣、SM: 骨格筋、M:マーカー（上記）を示す。MBの結果において観察される下方のバンドは非特異的産物と考えられる。

すなわち、導入されたヒト22番染色体はキメラマウスの正常組織において機能し得ることが確かめられた。

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（実施例6）ヒト4番染色体またはその部分断片のES細胞への導入

染色体供与細胞として、（実施例1）で得られたヒト4番染色体を保持するマウスA9細胞株（以下A9/#4、という）を用いた。染色体受容細胞としてはマウスES細胞株E14（実施例2と同様）を用いた。ミクロセル融合実験およびG418耐性株の選択は（実施例2）と同様に行なった。薬剤耐性株の出現頻度はE14細胞10^7個あたり1～2個であった。薬剤耐性株の凍結保存、ゲノムDNA取得は（実施例2）と同様に行なった。薬剤耐性株E14/#4-4 、E14/#4-7 、E14#4-11におけるヒト4番染色体またはその断片の保持は以下の（1）～（3）により確認した。

（1）PCR解析（第７図）

薬剤耐性株ゲノムDNAを鋳型としてヒト4番染色体上に存在する遺伝子（O'Brien, Genetic Maps, 6th edition, Book 5, Cold Spring Harbor Laboratory Press, 1993）及び多型性マーカー（Polymorphic STS Primer Pair BIOS社：D4S395, D4S412, D4S422, D4S413, D4S418, D4S426, F11; Nature 359:794, 1992 ）の存在をPCR法により検出した。GenBank、EMBL等のデータベースより入手した塩基配列をもとに作製した遺伝子プライマーオリゴヌクレオチドの配列を記す。

HD（huntington disease）：5'-TCGTTCCTGTCGAGGATGAA（配列番号23), 5'-TCACTCCGAAGCTGCCTTTC（配列番号24）

IL-2（interleukin-2）：5'-ATGTACAGGATGCAACTCCTG（配列番号25), 5'-TCATCTGTAAATCCAGCAGT（配列番号26）

KIT（c-kit）：5'-GATCCCATCGCAGCTACCGC（配列番号27), 5'-TTCGCCGAGTAGTCGCACGG（配列番号28）

FABP2（fatty acid binding protein 2, intestinal）：5'-GATGAACTAGTCCAGGTGAGTT（配列番号29), 5'-CCTTTTGGCTTCTACTCCTTCA（配列番号30）

上記の11種のプライマーについてPCR増幅を行なった結果、3株共に、全て、あるいは一部のプライマーについて期待される増幅産物が確認された。E14/#4-4、E14/#4-7 株のように一部領域に欠失がみられるものもあった。以上の結果を第７図に示す。第７図において、左側にヒト4番染色体のGバンド像に基づく模式的な染色体地図、また、位置が明らかになっているいくつかのマーカーについてはどのバンドに位置するかを示した（実施例2参照）。遺伝子及び多型性マーカ

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ーの並び方は、現在までに入手出来る情報（実施例2参照）を基に、大まかな位置関係を示したもので、順序は必ずしも正確ではない。3種のG418耐性E14細胞株について、PCRにより期待される増幅産物が検出されたマーカーは■で、検出されなかったマーカーを□で示した。下側にはFISH解析による観察結果を示した。A9/#4は染色体供与細胞を示す。

（2）サザンブロット解析（第8図）

サザンブロット解析は（実施例2）と同様に、E14/#4-4 、E14/#4-7 より取得したゲノムDNAについてヒトL1配列をプローブとして行なった。その結果、両株DNAにおいてヒトL1 配列とハイブリダイズするバンドが多数検出された。A9/#4と比較して、全体のシグナル強度はPCR解析で示された欠失の程度と相関していた。第8図において、各レーンとも、BglII消化した2μgのゲノムDNAを使用した。^{32}P標識ヒトL1配列をプローブとし、シグナルはイメージアナライザーBAS2000（富士写真フイルム社）により検出した。第8図において、各レーンは、左から、1:A9/#4（染色体供与細胞）、2:A9/#4+A9（1:2）、3:A9/#4+A9（1:9）、4:A9、5:E14/#4-7、6:E14/#4-4を示す。2、3は2 種のDNAを括弧内に示した比率で混合したものである。左側にDNA分子量を示した。

（3）フルオレッセンスインサイチューハイブリダイゼーション（FISH）

FISH解析は（実施例2）と同様に、ヒト4番染色体特異的プローブ（CHROMOSOME PAINTING SYSTEM, Cambio社）を用いて行なった。その結果、3株すべてのほとんどの分裂像において、ヒト4番染色体あるいはその部分断片が検出された。E14/#4-4はマウス染色体に転座した形で、他の2株は独立した染色体として存在していた。観察されるヒト染色体の相対的な大きさは、PCR解析の結果から推測されるそれと一致していた。

以上の実験により、得られたG418耐性株はヒト4番染色体の全てあるいはその部分断片を保持することが明かとなった。

（実施例7）ヒト4番染色体部分断片を保持するES細胞からのキメラマウス作製

ヒト4番染色体部分断片を保持していることが確認されたG418耐性ES細胞株E14/#4-4、E14/#4-7を凍結ストックより立ち上げ、（実施例3）と同様にして取得した胚盤胞期胚に胚あたり10～15個注入した。偽妊娠処理後2.5日の仮親ICRマウ

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ス（日本クレア社）の子宮に片側の子宮あたり約10個のES細胞注入胚を移植した。その結果を（第２表）に示す。

第２表．ヒト４番染色体（断片）を保持するE14 細胞株からのキメラマウス作製

ES細胞株/ヒト染色体	G418耐性株番号	ES細胞を注入した胚盤胞期胚数	子ﾏｳｽの数	ｷﾒﾗﾏｳｽの数	毛色への貢献率 ～10%	10-30%	30%～
E14/#4	4	160	8	5	5	-	-
	7	80	5	2	1	1	-

計240個の注入胚を移植した結果13匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚由来の野生色（濃茶）の中にE14細胞由来の薄灰色が認められるかどうかにより判定される。誕生した13匹のうち毛色に明らかに薄灰色の部分のある、すなわち、E14細胞の貢献の認められる個体は7匹であった。また、最高の貢献率はE14/#4-7由来の1個体において約15%であった。

この結果より、ヒト4番染色体部分断片を保持するマウスES細胞株E14/#4-4、E14/#4-7はキメラ形成能を保持している、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

（実施例8）ヒト4番染色体部分断片を保持するES細胞由来キメラマウスにおけるヒト染色体DNAの保持及び、G418耐性遺伝子の発現確認

（1）PCR解析

（実施例7）で得られたキメラマウスのうちE14/#4-7由来の1個体（K#4-7-1：キメラ率約5%）、E14/#4-4由来の1個体（K#4-4-41：キメラ率約5%）について（実施例4）と同様に尻尾よりゲノムDNAを調製した。それを鋳型とし、（実施例6）で示した4番染色体解析用プライマーのうちE14/#4-7、E14/#4-4で検出された多型性マーカーF11についてPCR解析を行なった。その結果、2個体とも期待される増幅産物が検出された。

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（2）サザン解析（第９図）

サザン解析は（実施例2）と同様、E14/#4-7由来の1個体（K#4-7-1：キメラ率約5%）についてヒトL1配列をプローブとして用い、２μgの尻尾由来ゲノムDNAに対して行なった。その結果、多数のヒトL1配列の存在が認められ、そのパターンはE14/#4-7と類似していた。マウスゲノムに対する量比はE14/#4-7の約10%程度であった。第９図において、各レーンとも、BglII消化した2μgの尻尾由来ゲノムDNAを使用した。^{32}P標識ヒトL1配列をプローブとし、シグナルはイメージアナライザーBAS2000（富士写真フイルム社）により検出した。左側にDNA分子量を示した。各レーンは左から1：K#4-7-1、2：ブランク、3：E14/#4-7 を示す。

（3）尻尾由来繊維芽細胞のG418耐性試験

キメラマウスのうち、E14/#4-7由来の1個体（K#4-7-1：キメラ率約5%）、E14/#4-4由来の1個体（K#4-4-41：キメラ率約5%）について尻尾から以下のように繊維芽細胞を調製した。DNA調製（実施例4）と同様にキメラマウスの尻尾を5mm～10mm切断し、PBS/1mM EDTAで数回洗浄した後、メスで切れ込みをいれて表皮を除去し、内部の組織をメスで細かく切り刻む。組織細片を5mlのPBS/1mM EDTAをいれたチューブに移し、30分～1 時間室温静置する。その後、1mlのPBS/EDTAを残して上清を取り除き、1mlの0.25%トリプシン/PBSを加え、5～10分間室温でタッピングあるいはピペッティングしながら組織をよくほぐす。1000rpm,10分間遠心し、沈殿を2mlのDMEM（10%FCS）に懸濁し、35mmシャーレに播種する。7～10日の培養後、トリプシン処理により細胞をシャーレからはがし、シャーレあたり約10^4個の細胞を35mmシャーレ2枚に播種し、うち1枚に終濃度400μg/mlのG418を加え、5～7日間培養し、それぞれのシャーレの生細胞を観察する。この条件で、野生型ICRマウス由来の繊維芽細胞は、G418存在下でほぼ100%死滅する。この結果、2個体共G418耐性の繊維芽細胞の存在が認められた。

これらの結果より、E14/#4-7、E14/#4-4はマウス個体において種々の正常組織に貢献し、かつヒト4番染色体部分断片を保持していることが確認された。

（実施例9）マウスES細胞へのヒト14番染色体またはその断片の導入

染色体供与細胞として、（実施例1）で得られたヒト14番染色体を保持するマウスA9細胞株（以下A9/#14、という）を用いた。染色体受容細胞としてはマウス

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ES細胞株TT2（ライフテックオリエンタル社より購入、Yagiら,Analytical Biochem., 214:70, 1993）を用いた。TT2の培養法は<相沢慎一，バイオマニュアルシリーズ 8，ジーンターゲティング，羊土社，1995>に記された方法に従い、栄養細胞はマイトマイシンC（シグマ）処理したG418耐性初代培養細胞（ライフテックオリエンタル社より購入）を用いた。ミクロセル融合実験およびG418耐性株の選択は（実施例2）と同様に行なった。薬剤耐性株の出現頻度はTT2細胞10^7個あたり3～6個であった。薬剤耐性株の凍結保存、ゲノムDNA取得は（実施例2）と同様に行なった。

ヒト14番染色体の断片化はミクロセルにガンマ線照射することにより行なった（Koiら，Science, 260:361, 1993）。約10^8個のA9/#14より取得したミクロセルを5mlのDMEMに懸濁し、ガンマセル40（前記）により、氷上で30Gyのガンマ線を照射した（1.2Gy/分×25分）。ガンマ線照射したミクロセルを未照射ミクロセルと同様に融合、薬剤耐性株選抜を行なった結果、薬剤耐性株の出現頻度はTT2細胞10^7個あたり3個であった。薬剤耐性株については（実施例2）と同様にして凍結保存、DNA取得を行なった。

ガンマ線未照射ミクロセル移入によるG418耐性株1-4、1-5、ガンマ線（30Gy）照射ミクロセル移入によるG418耐性株3-1、3-2計4株におけるヒト14番染色体または部分断片の保持は以下の（1）、（2）により確認した。

（1）PCR解析（第10図）

薬剤耐性株ゲノムDNAを鋳型としてヒト14番染色体上に存在する遺伝子（O'Brien, Genetic Maps,6th edition, Book 5, Cold Spring Harbor Laboratory Press, 1993 ）及び多型性マーカー（Polymorphic STS Primer Pair BIOS社:D14S43, D14S51, D14S62, D14S65, D14S66, D14S67, D14S72, D14S75, D14S78, D14S81, PCI ;Nature 359:794, 1992; Nature Genetics, 7:22, 1994）の存在をPCR法により検出した。GenBank、EMBL等のデータベースより入手した塩基配列をもとに作製した遺伝子プライマーオリゴヌクレオチドの配列を記す。

NP（nucleoside phosphorylase）：5'-ATAGAGGGTACCCACTCTGG（配列番号31），5'-AACCAGGTAGGTTGATATGG（配列番号32）

TCRA（T-cell receptor alpha）：5'-AAGTTCCTGTGATGTCAAGC（配列番号33），5'-

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TCATGAGCAGATTAAACCCG（配列番号34）

MYH6（myosin heavy chain cardiac）：5'-TGTGAAGGAGGACCAGGTGT（配列番号35），5'-TGTAGGGGTTGACAGTGACA（配列番号36）

IGA2（immunoglobulin alpha-2 constant）：5'-CTGAGAGATGCCTCTGGTGC（配列番号37），5'-GGCGGTTAGTGGGGTCTTCA（配列番号38）

IGG1（immunoglobulin gamma-1 constant）：5'-GGTGTCGTGGAACTCAGGCG（配列番号39），5'-CTGGTGCAGGACGGTGAGGA（配列番号40）

IGM（immunoglobulin mu constant）：5'-GCATCCTGACCGTGTCCGAA（配列番号41），5'-GGGTCAGTAGCAGGTGCCAG（配列番号42）

IGVH3（immunoglobulin heavy variable-3 ）：5'-AGTGAGATAAGCAGTGGATG（配列番号43），5'-GTTGTGCTACTCCCATCACT（配列番号44）

薬剤耐性株4株のゲノムDNAを鋳型として、上記の18種のプライマーについて（実施例2）同様にPCR増幅を行なった結果、全て、あるいは一部のプライマーについて期待される増幅産物が確認された。ガンマ線照射したミクロセルを用いて得られた薬剤耐性株3-1、3-2は、14番染色体の一部領域を欠失している傾向が認められた。また、未照射ミクロセルを用いた場合でも1-4株のごとく欠失がみられるものもあった。以上の結果を第10図に示す。第10図において、左側にヒト14番染色体のGバンド像に基づく模式的な染色体地図、また、位置が明らかになっているいくつかのマーカーについてはどのバンドに位置するかを示した（実施例2参照）。遺伝子及び多型性マーカーの並び方は、現在までに入手出来る情報（実施例2参照）を基に、大まかな位置関係を示したもので、順序は必ずしも正確ではない。4種のG418耐性TT2細胞株について、PCRにより期待される増幅産物が検出されたマーカーは■で、検出されなかったマーカーを□で示した。A9/#14は染色体供与細胞である。右端には実施例11（1）の結果が示されている。

（2）フルオレッセンスインサイチューハイブリダイゼーション（FISH）

FISH解析は（松原ら，FISH実験プロトコール、秀潤社、1994）に記された方法に従い、ヒト14番染色体特異的プローブ（CHROMOSOME PAINTING SYSTEM, Cambio社）を用いて行なった。その結果、4株すべてについてほとんどの分裂像に、ヒト14番染色体あるいはその部分断片が、独立した染色体として観察された。観察

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されるヒト染色体の相対的な大きさは、PCR解析の結果から推測されるそれと一致していた。

以上の実験により、得られたG418耐性株1-4、1-5、3-1、3-2はヒト14番染色体の全てあるいはその部分断片を保持することが確かめられた。

（実施例10）ヒト14番染色体断片を保持するES細胞からのキメラマウス作製

（実施例9）で得られ、ヒト14番染色体を保持していることが確認されたG418耐性ES細胞株4株（1-4、3-1、3-2、1-5）を凍結ストックより立ち上げ、ICRあるいはMCH(ICR)（日本クレア社）雄雌マウスの交配により得られた8細胞期胚に胚あたり8～10個注入した。ＥＳ培地（実施例９）で一晩培養して胚盤胞に発生させた後、偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のインジェクション胚を移植した。その結果を（第３表）に示す。

第３表．ヒト14番染色体（断片）を保持するTT2 細胞株からのキメラマウス作製

ES細胞株/ヒト染色体	G418耐性株番号	ES細胞を注入した８細胞期胚数	子ﾏｳｽの数	ｷﾒﾗﾏｳｽの数	毛色への貢献率 ～20%	20-50%	50-80%
TT2/#14	1-4	98	20	1	-	-	1
	1-5	110	14	2	1	-	1
	3-1	103	11	2	1	1	-
	3-2	183	19	3	-	2	1

計494個の注入胚を移植した結果、64匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚（ICR）由来の白色の中にTT2細胞由来の野生色（濃茶）が認められるかどうかにより判定される。誕生した64匹のうち毛色に明らかに野生色の部分のある、すなわち、ES細胞の貢献の認められる個体は8匹であった。また、最高の貢献率は1-4由来の1個体における約80%であった。

この結果より、ヒト14番染色体またはその断片を保持するG418耐性ES細胞株（1-

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4、1-5、3-1、3-2）はキメラ形成能を保持している、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

（実施例11）ヒト14番染色体断片を保持するES細胞由来キメラマウスにおけるヒト14番染色体断片の保持確認

（実施例10）で得られたキメラマウスにおけるヒト14番染色体部分断片の保持は以下の（1）～（3）により確認した。

（1）各種組織由来DNAを用いたPCR解析

キメラマウスのうち3-1由来の1個体（K3-1-1：キメラ率約25%）について（実施例4）と同様に尻尾よりゲノムDNAを調製した。それを鋳型とし、（実施例9）で示した14番染色体解析用プライマーのうち3-1で検出された14種全てについてPCR解析を行なった。その結果、14種すべてについて期待される増幅産物が検出された（第10図）。

さらに、同じ個体（K3-1-1）について脳、腎臓、脾臓、心臓、肝臓、胸腺からPuregene DNA Isolation Kitによりゲノム DNAを取得し、それぞれの組織について、IGMプライマー（実施例9）を用いたPCR解析を行なった。その結果全ての組織において期待される増幅産物が確認された（第11図）。PCR産物は2%アガロースゲルにて電気泳動した後、臭化エチジウム染色して検出した。第11図において、各レーンは左からB：脳、K：腎臓、Sp：脾臓、H：心臓、L：肝臓、Th：胸腺、pc：ヒト繊維芽細胞（HFL-1）DNA（陽性コントロール）、nc：非キメラマウス尻尾DNA（陰性コントロール）、M：マーカー（HindIII消化λDNA＋HaeIII消化φX174 DNA, 宝酒造）を示す。

（2）尻尾由来繊維芽細胞のG418耐性試験

キメラマウスのうち、3-2由来の2個体（K3-2-1：キメラ率約25%, K3-2-3：キメラ率約50%）、1-4由来の1個体（K1-4-1：キメラ率約80%）について尻尾から以下のように繊維芽細胞を調製した。DNA調製（実施例4）と同様に3～6週令のキメラマウスの尻尾を5mm～10mm切断し、PBS/1mM EDTAで数回洗浄した後、メスで切れ込みをいれて表皮を除去し、内部の組織をメスで細かく切り刻む。組織細片を5mlのPBS/1mM EDTAをいれたチューブに移し、30分～1 時間室温静置する。その後、1mlのPBS/EDTAを残して上清を取り除き、1mlの0.25%トリプシン/PBSを加え、5～

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10分間室温でタッピングあるいはピペッティングしながら組織をよくほぐす。1000rpm,10分間遠心し、沈殿を2mlのDMEM（10%FCS）に懸濁し、35mmシャーレに播種する。7～10日の培養後、トリプシン処理により細胞をシャーレからはがし、シャーレあたり約10^4個の細胞を35mmシャーレ4枚に播種し、うち2枚に400μg/mlのG418を加え、5～7日間培養し、それぞれのシャーレの生胞数をカウントする。この条件で、野生型ICRマウス由来の繊維芽細胞は、G418存在下でほぼ100%死滅する。非選択培地での生細胞数に対する選択培地での生細胞数の割合は、G418耐性繊維芽細胞の増殖速度が２つの条件で同等であると仮定すれば、G418耐性ES細胞株由来繊維芽細胞の繊維芽細胞集団における貢献率を反映していると考えられる。この結果、（第12図）に示した通り、3個体共G418耐性の繊維芽細胞の存在が認められた。第12図において、耐性率はそれぞれの個体について2組の選択／非選択35mmシャーレから得られた値を平均した。ICR は野生型ICRマウスを示す。

（3）尻尾由来G418耐性繊維芽細胞のFISH解析

（実施例2）と同様な方法で、上記（2）で得られたG418耐性繊維芽細胞（K3-2-3,K1-4-1由来）のFISH解析を行なった。プローブはHFL-1細胞（実施例1）より抽出したヒト全DNAをFITC標識した（松原ら，FISH実験プロトコール，秀潤社，1994）ものを用いた。その結果、2個体共、ほとんどの分裂像に独立したヒト染色部分断片が観察された。

これらの結果より、ヒト14番染色体部分断片を保持したTT2細胞株はマウス個体において種々の正常組織に貢献し、かつヒト14番染色体部分断片を保持していることが確かめられた。

（実施例12）ヒト2番染色体部分断片のES細胞への導入

染色体供与細胞として、（実施例1）で得られたヒト2番染色体部分断片を保持するマウスA9細胞W23（以下A9/#2 W23、という）を用いた。染色体受容細胞としてはマウスES細胞株TT2（実施例9）を用いた。ミクロセル融合実験およびG418耐性株の選択は（実施例２）と同様に行なった。薬剤耐性株の出現頻度はTT2細胞10^7個あたり1～3個であった。薬剤耐性株の凍結保存、ゲノムDNA取得は（実施例２）と同様に行なった。薬剤耐性株5-1、5-2、5-3におけるヒト2番染色体部分断片の

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保持は以下の（1）、（2）により確認した。

（1）PCR解析

薬剤耐性株ゲノムDNAを鋳型としてヒト2番染色体上に存在する遺伝子（Genetic Maps,前記）のうち、染色体供与細胞A9/#2 W23において検出されたＣκ、FABP1の存在をPCR法により検出した。

各プライマーについてPCR増幅を行なった結果、3株共に、両方のプライマーについて期待される増幅産物が確認された。

（2）フルオレッセンスin situハイブリダイゼーション（FISH）

FISH解析は（実施例2）と同様な方法で、ヒト2番染色体特異的プローブ（CHROMOSOME PAINTING SYSTEM, Cambio社）を用いて行なった。その結果、3株すべてのほとんどの分裂像において、ヒト2番染色体部分断片が独立した染色体として検出された。その大きさはA9/#2 W23で観察されたものと同等であった。

以上の実験により、得られたG418耐性株はヒト2番染色体部分断片を保持することが確かめられた。

（実施例13）ヒト2番染色体断片を保持するES細胞からのキメラマウス作製

（実施例12）で得られ、ヒト2番染色体部分断片を保持していることが確認されたG418耐性ES細胞株5-1を凍結ストックより立ち上げ、 ICRあるいはMCH(ICR)（日本クレア社）雄雌マウスの交配により得られた8細胞期胚に胚あたり10～12個注入した。ＥＳ培地（実施例９）で一晩培養して胚盤胞に発生させた後、偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のインジェクション胚を移植した。

キメラ作製の結果を（第４表）に示す。

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第4表. ヒト2番染色体（断片）を保持するTT2 細胞株からのキメラマウス作製

ES細胞株/ ヒト染色体	G418耐性 株番号	ES細胞を注入した8細胞期胚数	子マウスの数	キメラマウスの数	毛色への貢献率 ～20%	20-50%	50-80%
TT2/#2 (W23)	5-1	264	51	18	7	5	6

計264個の注入胚を移植した結果、51匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚（ICR）由来の白色の中にTT2細胞由来の野生色（濃茶）が認められるかどうかにより判定される。誕生した51匹のうち毛色に明らかに野生色の部分のある、すなわち、ES細胞の貢献の認められる個体は18匹であった。また、最高の貢献率は約80%であった。

この結果より、ヒト2番染色体部分断片を保持するG418耐性ES細胞（5-1）はキメラ形成能を保持している、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

（実施例14）ヒト14番染色体断片導入キメラマウス血清におけるヒト抗体重鎖の検出

血清中のヒト抗体濃度をエンザイムリンクドイムノソルベントアッセイ(ELISA)を用いて測定した。ELISA は以下に記載されている方法に従った。富山・安東、単クローン抗体実験マニュアル、講談社、1987; 安東・千葉、単クローン抗体実験操作入門、講談社、1991; 石川、超高感度酵素免疫測定法、学会出版センター、1993 ; Ed Harlow and David Lane, Antibodies A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; A. Doyle and J.B. Griffiths, Cell & Tissue Culture: Laboratory Procedures, John Wiley & Sons Ltd., 1996 。これらの文献に記載の方法を参考にして、測定系によっては反応時間や温度を4 ℃で終夜行うなどの改良を行った。測定しようとするヒト免疫グロブリンに対する抗体あるいは抗原を、0.5 から10 μg/ml程度に(100から5000倍）に希釈し、ELISA プレ

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ートを4 ℃で一晩コーティングした。血清試料の測定では、ブロッキング、試料および標識抗体の希釈に5%マウス血清（シグマ、M5905)を添加したPBSを、ハイブリドーマ培養上清の測定には1%牛胎児血清を添加したPBSを用いた。20倍にキメラマウス血清を希釈する場合にはPBSを用いて希釈した。コーティングしたプレートを洗浄した後、ブロッキングを１時間以上行った。プレートを洗浄後、試料を加え30分以上インキュベートした。プレート洗浄後100 から5000倍に希釈した酵素標識抗ヒト免疫グロブリン抗体を加えて、１時間以上インキュベートした後、プレートを洗浄し基質液を加えて発色させた。また測定系によって、基本的には同じ操作で、ビオチン標識した抗体を用い、プレート洗浄後これにアビジン－酵素複合体を加えてインキュベートした後洗浄し基質液を加えた。マイクロプレートリーダー（バイオテック、EL312e）で吸光度を測定した。

生後29日から35日のキメラマウス（実施例10、K3-1-2, K3-2-2, K3-2-3）より採血しELISAで解析した。50mMの炭酸－炭酸水素バッファーpH9.6で希釈した抗ヒトIgMマウスモノクローナル抗体（シグマ,I6385）を96穴マイクロタイタープレートにコーティングし、マウス血清（シグマ、M5905）を加えたPBSで希釈した血清試料を加えた。次いでペルオキシダーゼ標識抗ヒトIgMヤギ抗体（Tago,2392）を加えてインキュベートした後、ABTS基質（Kirkegaard & Perry Laboratories Inc., 506200）の添加により酵素活性を405nmの吸光度で評価した。精製されたヒトIgM抗体（オルガノン・テクニカ, 6001-1590 ）またはヒトIgG抗体（シグマ, I4506)を標準とした。標準はマウス血清を添加したPBSで段階的に希釈した。またヒトIgG測定には抗ヒトIgGヤギ抗体（シグマ, I3382）をプレートに固定し、ペルオキシダーゼ標識抗ヒトIgGヤギ抗体（シグマ, A0170）で検出した。その結果を（第５表）に示す。ヒトIgMとIgGは共に検出された。

また生後27日、34日、41日の3回にわたりヒト14番染色体断片を保持したキメラマウス（実施例10、K3-1-1, K3-2-1）に、PBSに溶解したヒト血清アルブミン（HSA,シグマ,A3782）2mlをアジュバント（MPL+TDM Emulsion, RIBI Immunochem Reseach Inc.）と混合しその0.25mg/ml を0.2 mlを免疫した。このキメラマウス血清も同様にELISAによって解析した。その結果を（第13図、第14図）に示す。結果は、HSAで免疫したキメラマウス血清中のヒト抗体濃度は免疫後上昇し、個体

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K3-1-1ではヒトIgM18μg/mlとIgG2.6μg/mlが免疫後17日目の血清中に検出された。ヒト染色体を導入していないマウスの血清ではヒト抗体の力価は有意ではなかった。

第5表. キメラマウス血清中のヒト抗体濃度(ELISA)

キメラマウス	IgG (mg/l)	IgM (mg/l)
K3-1-2	0.37	3.7
K3-2-2	0.33	5.9
K3-2-3	0.51	3.4

(実施例15) ヒト14 番染色体導入キメラマウスからのヒト抗体重鎖産生ハイブリドーマ取得

(実施例14) においてヒトアルブミンで免疫したキメラマウス(K3-1-1, 実施例14) から生後44日目に脾臓を取り出し、ミエローマ細胞と細胞融合し、ハイブリドーマを作製した。ハイブリドーマの作製法は〈安東、単クローン抗体実験操作入門、講談社サイエンティフィク, 1991〉に記された方法に従い、ミエローマ細胞としては、P3X63-Ag.8.653 (大日本製薬より購入、05-565) を使用した。96穴プレート10枚にまき込み、1週間培養後培養上清をELISA法で解析した。ELISA法は抗ヒトIgMマウスモノクローナル抗体 (シグマ、I6385) をプレートに固定化して実施例14と同様に行ない、陽性のクローンを6個得た。またHSAを抗原とし50mMの炭酸－炭酸水素バッファーpH9.6で濃度5μg/mlの溶液とし、ELISAプレートの全ウエルに100μlづつ分注した。ペルオキシダーゼで標識した抗ヒトIgA+IgG+IgMヤギ抗体 (Kirkegaard & Perry Laboratories Inc., 04-10-17) を用いて検出した。プレート10枚中陽性のクローンを1つ確認した。このクローンは6個のヒトIgM陽性クローンのうちの1つであった。このクローン (H4B7) をさらに培養し、培養上清を希釈しHSAを抗原として上述のようにペルオキシダーゼ標識抗

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ヒトIgMヤギ抗体（Tago, 2392）を用いてELISAを行なったところ、培養上清の希釈率の増加にともなって吸光度の減少が認められた。一方ヒトIgM（オルガノン・テクニカ，6001-1590)を培地によって2μg/mlに希釈した試料では希釈率によらず吸光度は低かった。これはハイブリドーマH4B7が生産する抗体がHSAに特異性のある抗体であることを示唆するものである（第15図）。第15図において、横軸は培養上清試料の希釈率を縦軸は405nmにおける吸光度を示した。

（実施例16）G418耐性マーキングされたヒト2番染色体断片のピューロマイシン耐性による再マーキング

G418耐性で標識されたヒト2番染色体断片を保持するA9細胞（W23)(実施例1、第1図参照）を100mmシャーレでG418（800μg/ml）を含む選択培地（10%FBS、DMEM）で培養した。ピューロマイシン耐性遺伝子を含むプラスミドpPGKPuro （WHITEHEAD INSTITUTE, Dr. Peter W. Laird から分与）をトランスフェクション前に制限酵素SalI（宝酒造）で線状化した。細胞をトリプシン処理し、$5x10^6$ 個／mlとなるようにダルベッコのリン酸バッファー（PBS）に懸濁してから10μgDNA存在下でジーンパルサー（バイオラッド）を用いてエレクトロポレーション（実施例1参照）を行なった。25μFの容量で1000Vの電圧を4mm長のエレクトロポレーションセル（実施例1）を用いて室温で印加した。エレクトロポレーションした細胞を100mmシャーレ3～6枚に播種した。1日後に10μg/mlのピューロマイシン（シグマ, P-7255）およびG418（800μg/ml）を含む二重選択培地と置き換えた。2～3週間後に生じたコロニー200個程度を一つの集団としてまとめた。この細胞を3つの集団についてそれぞれ25cm^2フラスコ2～3本中で培養し、ミクロセルを形成させ25cm^2フラスコで培養したマウスA9細胞と実施例1と同様に融合した。100mmシャーレ2枚に移しG418とピューロマイシンを含む上記の二重選択培地で培養し、3つの集団のうち1つの集団から2つの二重薬剤耐性クローンが得られた。このクローンではヒト2番染色体断片にピューロマイシン耐性マーカーが導入された可能性が高い。

（実施例17）ヒト染色体導入ES細胞における導入ヒト染色体倍加

G418耐性遺伝子でマーキングされたヒト14番染色体断片を保持するES細胞株（E14/#14-36）を、高濃度のG418を添加した培地中で培養することにより、ヒト染

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色体が倍化したES細胞のクローンを取得した（バイオマニュアルシリーズ8、ジーンターゲティング、羊土社、1995）。G418耐性マウス初代細胞（ライフテックオリエンタルより購入）をマイトマイシン処理することなく100mmシャーレに播種し栄養細胞とした。この100mmシャーレにE14/#14-36 を播種し、半日後G418濃度16mg/mlの培地と交換した。1～2日毎に培地交換し1週間後にG418 濃度を10mg/mlとして培養を続け、生じたコロニーの中から15個を取り出し培養し、染色体をヒト14番特異的プローブ（実施例9参照）を用いてFISH解析した。結果として8クローンでヒト14番染色体断片が倍化していた。

（実施例18）ヒト2番染色体部分断片及び14番染色体部分断片を同時に保持するマウスES細胞株の取得

（実施例16）において取得した二重薬剤耐性クローンのうちPG1をミクロセル供与細胞、野性型A9細胞を受容細胞としたミクロセル移入実験により、PG1が保持するヒト2番染色体部分断片がさらにピューロマイシン耐性遺伝子によりマーキングされていることを確認した。ミクロセル取得及びA9細胞との融合は（実施例1）と同様に行なった。その結果、ミクロセル融合10日後に計59個のG418耐性コロニーが出現した。これらのコロニーについて8μg/mlのピューロマイシンを含む培地に交換後、さらに3日間培養したところ、45個（76%）のコロニーが生存した。ミクロセル法においては、1つの受容細胞に、多くの場合1本あるいは少数の染色体のみが移入されることから、両耐性遺伝子が高率に同時に移入されることは、すなわち、PG1が保持するG418耐性標識されたヒト2番染色体部分断片がピューロマイシン耐性遺伝子によりマーキングされていることを示している。さらに、ヒト2番染色体部分断片上の各マーカー遺伝子検出のため、A9/#2 W23（G418耐性のみ：実施例16）についてはpSTneoB（実施例1）、PG1についてはpPGKPuro（実施例16）をプローブとしたFISH解析を行なった（松原ら，FISH実験プロトコール，秀潤社，1994）。その結果、A9/#2 W23では（実施例12）で確認されたヒト2番染色体部分断片のそれぞれの姉妹染色分体上に1個づつ、計2個のシグナルが観察された。これは、ヒト2番染色体部分断片上の1箇所にpSTneoBが挿入されていることを示す。また、PG1では同等の大きさの染色体断片上に計4個のシグナルが観察された。pSTneoBとpPGKPuroはベクター部分で相同の配

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列を持つので、pPGKPuroプローブではpSTneoBも検出される。すなわち、PG1で観察された4個のシグナルのうち、2個はpSTneoB、一方の2個はpPGKPuro由来のシグナルと考えられる。この結果より、PG1が保持するヒト2番染色体部分断片は、G418耐性、ピューロマイシン耐性両者によりマーキングされていることが確認された。

ヒト2番染色体部分断片、14番染色体部分断片を同時に保持するマウスES細胞取得のため、染色体供与細胞としてこのPG1細胞株を用いた。染色体受容細胞としてはすでにヒト14番染色体部分断片を保持しているG418耐性TT2細胞株1-4（実施例9）を用いた。ミクロセル融合実験およびピューロマイシン耐性株の選択は0.75μg/mlのピューロマイシン濃度で他は（実施例9）のG418耐性株選択の場合と同様に行なった。この結果出現したピューロマイシン耐性株の出現頻度は1-4細胞10^7個あたり3～7個であった。これらのピューロマイシン耐性株は300μg/mlのG418存在下でも増殖することからG418耐性も同時に保持していることが確認された。二重薬剤耐性株の凍結保存、ゲノムDNA取得は（実施例2）と同様に行なった。ヒト2番染色体部分断片及び、ヒト14番染色体部分断片の保持は二重薬剤耐性株PG5、PG15、PG16については以下の（1）により、PG15についてはさらに（2）により確認した。

（1）PCR解析

二重薬剤耐性株ゲノムDNAを鋳型としてヒト2番、14番染色体上に存在する遺伝子（Genetic Maps,前記）のうち、2番染色体については（実施例12；A9/#2 W23）、14番染色体については（実施例9；TT2/#14 1-4）で存在が確認されている各プライマーについてPCR増幅を行なった結果、3株共に、全てのプライマーについて期待される増幅産物が確認された。

（2）フルオレッセンスin situハイブリダイゼーション（FISH）

FISH解析は（実施例11）と同様に、ヒト全DNAをFITC標識したものをプローブとして用いて行なった。その結果、ほとんどの分裂像において、大小2つのヒト染色体断片が確認された。大きい方は（実施例9；TT2/#14 1-4）でヒト14番染色体特異的プローブにより確認された部分断片と、小さい方は（実施例12；TT2/#2 5-1）でヒト2番染色体特異的プローブにより確認された部分断片と同等の大

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きさであった。（第16図）にその結果を示す。図中輝度の低い染色体はマウス由来のもの、FITCの蛍光により輝度の高い大小2つの染色体断片（矢印にて指示）はヒト由来のものであり、それぞれヒト14番、2番染色体部分断片と考えられる。

以上の実験により、得られた二重耐性ES細胞株はヒト2番染色体部分断片、14番染色体部分断片を同時に保持することが確かめられた。

（実施例19）ヒト2番染色体部分断片及び14番染色体部分断片を同時に保持するマウスES細胞株からのキメラマウス作製

（実施例18）で得られ、ヒト2番染色体部分断片及び14番染色体部分断片を保持していることが確認されたG418、ピューロマイシン二重耐性TT2細胞株PG5、PG15、PG16を凍結ストックより立ち上げ、 ICRまたはMCH(ICR)（日本クレア社）雄雌マウスの交配により得られた8細胞期胚に胚あたり10～12個注入した。ES細胞用培地（実施例9）で一晩培養して胚盤胞に発生させた後、偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のインジェクション胚を移植した。

キメラ作製の結果を（第６表）に示す。

第６表．ヒト2番染色体部分断片及び14番染色体部分断片を同時に保持するマウスES細胞株からのキメラマウス作製

ES細胞株/ヒト染色体	2重耐性株番号	ES細胞を注入した８細胞期胚数	子ﾏｳｽの数	ｷﾒﾗﾏｳｽの数	毛色への貢献率 ～10%	10-50%	50%～
TT2/	PG5	160	26	8	7	1	-
#14+#2	PG15	168	15	3	1	2	-
	PG16	223	32	12	3	6	3

計551個の注入胚を移植した結果、73匹の子マウスが誕生した。キメラ個体は、

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毛色において宿主胚（ICR）由来の白色の中にTT2細胞由来の野生色（濃茶）が認められるかどうかにより判定される。誕生した73匹のうち毛色に明らかに野生色の部分のある、すなわち、ES細胞の貢献の認められる個体は23匹であった。

この結果より、ヒト2番染色体部分断片及び14番染色体部分断片を保持するES細胞株（PG5、PG15、PG16）はキメラ形成能を保持している、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

（実施例20）ヒト2番染色体部分断片及び14番染色体部分断片を同時に保持するES細胞由来キメラマウス血清におけるヒト抗体の検出

（実施例19）で作製したキメラマウスのうち、KPG-15（9週齢；PG5由来、キメラ率10%）、KPG-18（5週齢；PG5由来、キメラ率10%）の2匹に対して、PBSに溶解したヒト血清アルブミン（HSA、シグマ、A3782）とアジュバント（MPL+TDM Emulsion、RIBI Immunochem Reseach Inc.）とを混合して0.25mg/mlのHSA溶液を調整し0.2mlを免疫した。免疫直前、及び8日後のキメラマウスより採血し、血清中のヒト抗体μ鎖およびヒト抗体κ鎖をELISA法を用いて検出した（実施例14参照）。50mMの炭酸ー炭酸水素バッファーpH9.6で希釈した抗ヒト抗体κ鎖ヤギ抗体（VECTOR LABORATORIES, INC.、AI-3060）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでビオチン標識抗ヒト抗体κ鎖ヤギ抗体（VECTOR LABORATORIES, INC.、BA-3060）を加えてインキュベートしさらにビオチン化ワサビペルオキシダーゼとアビジンDHの複合体（VECTOR LABORATORIES, INC.、Vectastain ABCキットPK4000）を加えてインキュベートした後、ペルオキシダーゼ基質として3,3',5,5'-テトラメチルベンチジン（TMBZ、住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。精製されたκ鎖を持つ濃度既知のヒトIgG（シグマ、I-3889）を標準としマウス血清を添加したPBSで段階的に希釈した。μ鎖については50mMの炭酸ー炭酸水素バッファーpH9.6で希釈した抗ヒトμ鎖マウスモノクローナル抗体（シグマ、I-6385）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでペルオキシダーゼ標識抗ヒトμ鎖マウス抗体（The Binding Site Limited、MP008）を加えてインキュベートした後、TMBZ（住友ベークライト、ML-1120T）の

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添加により酵素活性を450nmの吸光度で評価した。精製されたμ鎖を持つ濃度既知のヒトIgM（オルガノン・テクニカ、6001-1590）を標準としマウス血清（シグマ、M5905）を添加したPBSで段階的に希釈した。その結果、2個体とも免疫前においてヒト抗体μ鎖、κ鎖両者が検出され、その血清中濃度は、免疫後上昇した（第７表、第８表）。

第７表．キメラマウスＫＰＧ１５中のヒト抗体濃度（ＥＬＩＳＡ）

	ＩｇＭ（ｍｇ／ｌ）	Ｉｇκ（ｍｇ／ｌ）
免疫前	０．１９	１．６
免疫後８日目	０．７５	１．７

第８表．キメラマウスＫＰＧ１８中のヒト抗体濃度（ＥＬＩＳＡ）

	ＩｇＭ（ｍｇ／ｌ）	Ｉｇκ（ｍｇ／ｌ）
免疫前	０．２９	０．５７
免疫後８日目	３．４	０．８７

この結果より、ヒト2番染色体部分断片及び14番染色体部分断片を保持するES細胞由来キメラマウスにおいてヒト抗体重鎖、軽鎖遺伝子は機能することが確認された。

（実施例21）ヒト14番染色体断片導入キメラマウス血清における抗HSAヒト抗体γ鎖の検出

（実施例10）と同様にして作製したヒト14番染色体断片を保持したキメラマウス（K9、K11；共にTT2細胞株3-2由来、キメラ率はそれぞれ50%、30%）に対して、生後79日、93日、107日、133日の4回（K9）、または生後74日、88日、

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111日（K11）の3回にわたり（実施例20）と同様にHSAを免疫した。このキメラマウス血清中のヒト血清アルブミンに対するヒトγ鎖を含む抗体をELISA法によって検出した（実施例14参照）。50mMの炭酸ー炭酸水素バッファーpH9.6で希釈したHSA（シグマ、A 3782）を96穴マイクロタイタープレートにコーティングし、試料を加え、次いでペルオキシダーゼ標識抗ヒトIgGマウス抗体（ファーミンジェン、08007E）を加えてインキュベートした後、ペルオキシダーゼ基質としてOーフェニレンジアミン（OPD、住友ベークライト、ML-1130O）の添加により酵素活性を490nmの吸光度で評価した。HSAで免疫したキメラマウス血清中の抗HSAヒトIgGの力価は免疫後上昇した。対照ICRマウスでは、HSA免疫後の抗HSAヒトIgGの力価はバックグランドレベルであった。結果を（第17図）に示した。第17図において横軸はキメラマウスにHSAを免疫してからの日数を、縦軸は490nmにおける吸光度を示した。この結果より、ヒト14番染色体部分断片を保持するキメラマウスにおいて、HSA抗原刺激に対して抗原特異的ヒトIgGの抗体価上昇が起こることが確認された。

（実施例22）ヒト22番染色体断片導入キメラマウス血清におけるヒト抗体λ鎖の検出

9ヶ月齢のキメラマウス（実施例3、K22-7；キメラ率10%）より採血し、血清中のヒト抗体λ鎖をELISA法を用いて検出した（実施例14参照）。50mMの炭酸ー炭酸水素バッファーpH9.6で希釈した抗ヒト抗体λ鎖ヤギ抗体（VECTOR LABORATORIES, INC.、AI-3070）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでビオチン標識抗ヒト抗体λ鎖ヤギ抗体（VECTOR LABORATORIES, INC.、BA-3070）を加えてインキュベートしさらにビオチン化ワサビペルオキシダーゼとアビジンDHとの複合体（VECTOR LABORATORIES, INC.、Vectastain ABCキットPK4000）を加えてインキュベートした後、ペルオキシダーゼ基質としてTMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。精製されたλ鎖を持つ濃度既知のヒトIgG（シグマ、I-4014）を標準としマウス血清を添加したPBSで段階的に希釈した。180ng/mlのヒトIgGに相当する濃度のヒト抗体λ鎖がキメラマウス中に検出された。この結果より、ヒト

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22番染色体を保持するキメラマウスにおいてヒト抗体λ鎖遺伝子が機能することが確認された。

（実施例23）ヒト2番染色体断片導入キメラマウス血清におけるヒト抗体κ鎖の検出

5週齢のキメラマウス（実施例13、K2-8、キメラ率は70%）および9週齢のキメラマウス（実施例13、K2-3、K2-4、 K2-12、キメラ率はそれぞれ50%、20%、80%）より採血し、血清中のヒト抗体κ鎖をELISA法を用いて検出した（実施例14）。50mMの炭酸ー炭酸水素バッファーpH9.6で希釈した抗ヒト抗体κ鎖ヤギ抗体（VECTOR LABORATORIES, INC.、AI-3060）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでビオチン標識抗ヒト抗体κ鎖ヤギ抗体（VECTOR LABORATORIES, INC.、BA-3060）を加えてインキュベートしさらにビオチン化ワサビペルオキシダーゼとアビジンDHの複合体（VECTOR LABORATORIES, INC.、Vectastain ABCキット）を加えてインキュベートした後、TMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。精製されたκ鎖を持つ濃度既知のヒトIgG（シグマ、I-3889）を標準としマウス血清を添加したPBSで段階的に希釈した。結果を（第９表）に示した。

第９表．キメラマウス中のヒト抗体κ鎖濃度（ＥＬＩＳＡ）

キメラマウス	Igκ（ｍｇ／ｌ）
Ｋ２－３	１２４
Ｋ２－４	８５
Ｋ２－８	２５
Ｋ２－１２	５６

またヒト2番染色体断片を保持したキメラマウス（実施例13、K2-3、およびK2-4）に生後66日、80日、102日の3回にわたり（実施例20）と同様にHSAを免疫

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した。またキメラマウス（K2-12）に生後63日、77日、91日、116日の4回にわたり、このキメラマウス血清中のHSAに対するヒト抗体κ鎖をELISA法によって検出した（実施例14参照）。50mMの炭酸ー炭酸水素バッファーpH9.6で希釈したHSA（シグマ、A 3782）を96穴マイクロタイタープレートにコーティングし、試料を加え、次いでビオチン標識抗ヒト抗体κ鎖ヤギ抗体（VECTOR LABORATORIES, INC.、BA-3060）を加えてインキュベートしさらにビオチン化ワサビペルオキシダーゼとアビジンDHの複合体（VECTOR LABORATORIES, INC.、Vectastain ABCキット）を加えてインキュベートした後、ペルオキシダーゼ基質としてOPD（住友ベークライト、ML-1130O）の添加により酵素活性を490nmの吸光度で評価した。HSAで免疫したキメラマウス血清中の抗HSAヒトκ鎖の力価は免疫後上昇した。一方対照ICRマウスでは、HSA免疫後の抗HSAヒト抗体κ鎖の力価はバックグランドレベルであった。結果を（第18図）に示した。第18図において横軸はキメラマウスにHSAを初めて免疫してからの日数を、縦軸は490nmにおける吸光度を示した。これらの結果より、ヒト2番染色体部分断片を保持するキメラマウスにおいてヒト抗体κ鎖遺伝子が機能し、さらにはHSA抗原刺激に対して抗原特異的ヒトIgκの抗体価上昇が起こることが確認された。

（実施例24）ヒト14番染色体導入キメラマウスからのヒト抗体重鎖（μ鎖もしくはγ鎖）産生ハイブリドーマ取得

（実施例21）においてHSAで免疫したキメラマウスK9から生後136日目に脾臓を取り出し、ミエローマ細胞と細胞融合し、ハイブリドーマを作製した。ハイブリドーマの作製法は〈安東・千葉、単クローン抗体実験操作入門、講談社サイエンティフィク，1991〉に記された方法に従い、ミエローマ細胞としては、Sp-2/O-Ag14（大日本製薬、05-554）を使用した。培養液にORIGEN Hybridoma Cloning Factor（HCF、ボクスイ・ブラウン）を10%添加し96穴プレート8枚にまき込み、3日後に培養液中にG418を1mg/ml添加した。1～3週間培養後の培養上清をELISA法で解析した（実施例14参照）。μ鎖は50mMの炭酸ー炭酸水素バッファーpH9.6で希釈した抗ヒトμ鎖マウスモノクローナル抗体（シグマ、I-6385）を96穴マイクロタイタープレートにコーティングし、PBSで希釈した試料を加え、次

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いでペルオキシダーゼ標識抗ヒトμ鎖マウス抗体（The Binding Site Limited、MP008）を加えてインキュベートした後、基質として2,2ーアジノジー（3ーエチルベンズチアゾリン-6-スルホン酸）ジアンモニウム塩（ABTS、Kirkegaard&Perry Laboratories Inc.、04-10-17）を用いて検出し7個の陽性ウェルを得た。γ鎖については抗ヒトγ鎖マウスモノクローナル抗体（シグマ、I-6260）を96穴マイクロタイタープレートにコーティングし、PBSで希釈した試料を加え、次いでペルオキシダーゼ標識抗ヒトγ鎖マウス抗体（ファーミンジェン、 08007E）を加えてインキュベートした後、ABTS（Kirkegaard & Perry Laboratories Inc.、04-10-17）を用いて検出し2個のヒト抗体γ鎖陽性ウェルを得た。

（実施例25）ヒト2 番染色体導入キメラマウスからのヒト抗体軽鎖産生ハイブリドーマ取得

（実施例23）においてHSAで免疫したキメラマウスK2-3から生後105日目に脾臓を取り出し、ミエローマ細胞と細胞融合し、ハイブリドーマを作製した。ハイブリドーマの作製法は〈安東・千葉、単クローン抗体実験操作入門、講談社サイエンティフィク，1991〉に記された方法に従い、ミエローマ細胞としては、P3X63Ag8.653（大日本製薬、05-565）を使用した。培養液にHCF（ボクスイ・ブラウン）を10%添加し96穴プレート10枚にまき込み、3日後培養液中にG418を1mg/ml添加し、1～3週間培養しコロニーが出現したウェルの培養上清をELISA法で解析した。ELISA法は（実施例23）と同様に行ない、ヒト抗体κ鎖陽性のクローンを2個得た。

（実施例26）G418耐性マーキングされたヒト22番染色体のピューロマイシン耐性による再マーキング

G418耐性で標識されたヒト22番染色体を保持するA9細胞（A9/#22γ2；実施例1で取得）について、（実施例16）と同様な方法でヒト22番染色体のピューロマイシン耐性による再マーキングを行った。γ2細胞にpPGKPuroをエレクトロポレーションして得られた二重薬剤耐性株コロニー200個程度を一つの集団とし、3つの集団（P1、P2、P3）を供与細胞として野生型マウスA9細胞へのミクロセル移

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入を行なった。その結果、P1より6個、P2より1個、P3より3個の二重薬剤耐性クローンが得られた。取得した二重薬剤耐性クローンのうちP3由来の6-1をミクロセル供与細胞、野性型A9細胞を受容細胞としたミクロセル移入実験により、ヒト22番染色体がさらにピューロマイシン耐性遺伝子によりマーキングされていることを確認した（実施例18）。ミクロセル取得及びA9細胞との融合は（実施例1）と同様に行なった。その結果、ミクロセル移入11日後に28個のG418耐性コロニーが出現した。これらのコロニーについて8μg/mlのピューロマイシンを含む培地に交換した後、さらに3日間培養したところ、21個（75%）のコロニーが生存した。ミクロセル法においては、1つの供与細胞に、多くの場合1本あるいは少数の染色体のみが移入されることから、両耐性遺伝子が高率に同時に移入されることは、すなわち、6-1が保持するG418耐性標識されたヒト22番染色体がさらにピューロマイシン耐性遺伝子によりマーキングされていることを示している。

（実施例27）ヒト抗体重鎖を産生するハイブリドーマからのヒト抗体重鎖可変領域cDNAの取得及び塩基配列決定

（実施例15）において取得されたヒト抗体重鎖（IgM）を産生するハイブリドーマのうち、H4B7（HSA特異的）及びH8F9（非特異的）より、ISOGEN（ニッポンジーン）を使用して、総RNAを取得した。cDNAの合成は、Ready-To-Go T-primed 1st strandキット（Pharmacia社）を用い各々5μgの総RNAを使用した。得られたcDNAについて下記に示したプライマー（Larrickら，BIO/TECHNOLOGY，7，934-，1989、Wordら，Int. Immunol.，1，296-，1989を参考に作製）によりPCRを行ない、ヒト抗体重鎖可変領域を増幅した。

CM1（ヒトIgM定常領域）：5'-TTGTATTTCCAGGAGAAAGTG （配列番号４５）

CM2（同上）：5'-GGAGACGAGGGGGAAAAGGG（配列番号４６）

HS1（ヒト重鎖可変領域）：5'-ATGGACTGGACCTGGAGG(AG)TC(CT)TCT(GT)C（配列番号４７）（8種の混合物）

HS2（同上）：5'-ATGGAG(CT)TTGGGCTGA(GC)CTGG(GC)TTT(CT)T（配列番号４８）（16種の混合物）

HS3（同上）：5'-ATG(AG)A(AC)(AC)(AT)ACT(GT)TG(GT)(AT)(GCT)C(AT)(CT)(G

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C)CT(CT)CTG（配列番号４９）（6144種の混合物）
＊（ ）はその位置の塩基が（ ）内のいずれかからなる混合物であることを示す。

H4B7、H8F9共に、1回目のPCRはHS1×CM1、HS2×CM1、HS3×CM1の3種のプライマーの組み合わせについて行い（94℃ 1分、50℃ 2分、72℃ 3分、40サイクル、パーキンエルマー社、サーマルサイクラー140使用）、そのPCR産物をそれぞれHS1×CM2、HS2×CM2、HS3×CM2のプライマーで再度増幅した（温度条件は前記同様、30サイクル）。増幅産物は1.5%アガロース電気泳動後、臭化エチジウム染色することにより検出した。その結果、H4B7についてはHS3×CM2のプライマーで約490bpの増幅産物が確認された。また、H8F9については、HS3×CM2プライマーで微かなバンドが同様の位置に確認されたのでこのプライマーで再度増幅した（温度条件は前記同様、30サイクル）。その結果、増幅産物は非常に強いシグナルとして検出された。これらのPCR産物は、石田ら（遺伝子発現実験マニュアル、講談社サイエンティフィク、1995）の方法に従い、pBlueScriptII SK+（Stratagene社）ベクターのSmaI部位にクローニングした。増幅産物の挿入されたプラスミドのうち#2、#3、#4（H4B7）、#11、#13、#14（H8F9）について、自動蛍光シーケンサー（Applyed Bio System社）により、増幅産物の塩基配列を決定した。得られた塩基配列及び予想されるアミノ酸配列をすでに報告されているヒト抗体VH領域（Marksら、Eur. J. Immunol. 21, 985-, 1991）、JH領域（Ravetchら, Cell, 27, 583-, 1981）の配列と比較した結果、H4B7、H8F9共に、VH4ファミリー、JH2の組み合わせからなることが判明した。この結果は、ヒト14番染色体部分断片を保持するキメラマウスにおいて、完全な機能的ヒト抗体重鎖蛋白質が産生されていることを示している。

（実施例28）ヒト抗体κ鎖を発現するキメラマウス脾臓からのヒト抗体κ鎖cDNAの取得と塩基配列決定

（実施例13）において取得され、（実施例23）においてヒト抗体κ鎖を発現することが確認されたキメラマウスK2-8の脾臓より（実施例5）と同様な方法で合成したcDNAについて、下記のプライマー（Larrickら, BIO/TECHNOLOGY, 7, 934

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-, 1989、Whitehurstら, Nucleic Acids Res., 20, 4929-, 1992 を参考に作製）によりPCRを行ない、ヒトκ鎖可変領域を増幅した。陰性コントロールとしてK2-8より取得した肝臓由来cDNA及び、（実施例10）のTT2/#14 3-2由来キメラマウスK3-2-2より取得した脾臓由来cDNAを用いた。

KC2（ヒトIgκ鎖定常領域）：5'-CAGAGGCAGTTCCAGATTTC（配列番号５０）

KC3（同上）：5'-TGGGATAGAAGTTATTCAGC（配列番号５１）

KVMIX（ヒトIgκ鎖可変領域）：

5'-ATGGACATG(AG)(AG)(AG)(AGT)(CT)CC(ACT)(ACG)G(CT)(GT)CA(CG)CT T（配列番号５２）（3456種の混合物）

＊（ ）はその位置の塩基が（ ）内のいずれかからなる混合物であることを示す。

PCRはKVMIX×KC2、KVMIX×KC3プライマーの組み合わせで94℃15秒、55℃15秒、72℃20秒、40サイクル（パーキンエルマー社、サーマルサイクラー9600使用）の条件で行なった。増幅産物は1.5%アガロース電気泳動後、臭化エチジウム染色することにより検出した。その結果、両者の組み合わせ共に、期待される約420bps（KC2）、約450bps（KC3）の長さの増幅産物が検出された。一方、2種の陰性コントロールでは特異的増幅産物は検出されなかった。これらの増幅産物は、石田ら（遺伝子発現実験マニュアル、講談社サイエンティフィク、1995）の方法に従い、pBlueScriptII SK+（Stratagene社）ベクターのSmaIあるいはEcoRV部位にクローニングした。増幅産物の挿入されたプラスミドのうちKVMIX×KC2由来のVK-#1クローン1種類について、自動蛍光シーケンサー（Applyed Bio System社）により、増幅産物の塩基配列を決定した。得られた塩基配列はヒトIgκ鎖の開始コドンから定常領域に至るまで終始コドンを含まないので、クローニングされた増幅産物は機能的ヒトIgκ鎖可変領域をコードしていると考えられる。また、すでに報告されているヒト抗体Vκ領域（Kleinら, Eur. J. Immunol., 23, 3248-, 1993）、Jκ領域（Whitehurstら, 前記）の塩基配列と比較した結果、Vκ3ファミリー、Jκ4の組み合わせからなることが判明した。この結果は、ヒト2番染色体部分断片を保持するキメラマウスにおいて、完全な機能的ヒト抗体κ鎖蛋白質が産生されていることを示している。

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（実施例29）ヒト14番染色体断片を保持するキメラマウスの血清中のヒト抗体γ鎖サブクラスおよびμ鎖の検出、および定量

（実施例10）の生後11週齢のキメラマウス（K15AおよびK16A；1-4由来、キメラ率70、50%）より採血し、血清中のヒト抗体γ鎖サブクラス濃度を（実施例14）に従いELISA法を用いて検出した。

［ヒトIgG1の測定］抗ヒトIgG抗体(シグマ、I-6260）をPBSで希釈し、96穴マイクロタイタープレートをコーティングした。血清試料を加え、次いでペルオキシダーゼ標識した抗ヒトIgG1抗体（ファーミンジェン、08027E）を加えてインキュベートした後、TMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。精製された濃度既知のヒトIgG1（シグマ、I-3889）を標準としマウス血清を添加したPBSで段階的に希釈した。

［ヒトIgG2の測定］抗ヒトIgG2抗体（シグマ、I-9513）をPBSで希釈し、96穴マイクロタイタープレートをコーティングした。血清試料を加え、次いでペルオキシダーゼ標識した抗ヒトIgG抗体（シグマ、A-0170）を加えてインキュベートした後、TMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。精製された濃度既知のヒトIgG2（シグマ、I-4139）を標準としマウス血清を添加したPBSで段階的に希釈した。

［ヒトIgG3の測定］抗ヒトIgG3抗体（シグマ、I-7260）を100mMグリシン塩酸バッファーpH2.5で希釈し5分間室温で放置した後、100mMリン酸バッファーpH7.0で10倍に希釈し、96穴マイクロタイタープレートをコーティングした。血清試料を加え、次いでペルオキシダーゼ標識した抗ヒトIgG抗体（ファーミンジェン、08007E）を加えてインキュベートした後、TMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。精製された濃度既知のヒトIgG3（シグマ、I-4389）を標準としマウス血清を添加したPBSで段階的に希釈した。

［ヒトIgG4の測定］抗ヒトIgG4抗体（シグマ、I-7635）を100mMグリシン塩酸バッファーpH2.5で希釈し5分間室温で放置した後、100mMリン酸バッファーpH7.0で10倍に希釈し、96穴マイクロタイタープレートをコーティングした。血清試料を加え、次いでペルオキシダーゼ標識した抗ヒトIgG抗体（ファーミンジェン、

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08007E）を加えてインキュベートした後、TMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。精製された濃度既知のヒトIgG4（シグマ、I-4639）を標準としマウス血清を添加したPBSで段階的に希釈した。

［ヒトIgMの測定］μ鎖についてはPBSで希釈した抗ヒトμ鎖マウスモノクローナル抗体（シグマ、I-6385）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでペルオキシダーゼ標識抗ヒトμ鎖マウス抗体（The Binding Site Limited、MP008）を加えてインキュベートした後、ペルオキシダーゼ基質としてTMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価し、精製されたμ鎖を持つ濃度既知のヒトIgM（オルガノン・テクニカ、6001-1590）を標準としマウス血清（シグマ、M5905）を添加したPBSで段階的に希釈した。

結果を表10に示す。キメラマウスK15AとK16Aの2個体でIgG1, IgG2, IgG3, IgG4のすべてのサブクラスおよびIgMが検出された。

第10表　キメラマウス中のヒト抗体IgGサブクラスおよびIgM濃度（ELISA）

キメラマウス	IgG1	IgG2	IgG3	IgG4	IgM (mg/l)
K15A	2.25	1.96	0.17	0.43	7.09
K16A	0.30	0.69	0.10	0.07	0.87

（実施例30）ヒト22番染色体を保持するマウスES細胞株（TT2）の取得

ヒト22番染色体を保持するマウスES細胞（TT2）取得のため、染色体供与細胞として（実施例26）で取得した6-1（A9/#22, G418, ピューロマイシン耐性）細胞株を用いた。染色体受容細胞としては野生型TT2細胞株（実施例9）を用いた。ミクロセル融合実験およびピューロマイシン耐性株の選択は0.75μg/mlのピューロマイシン濃度で他は（実施例9）のG418耐性株選択の場合と同様に行なった。

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この結果出現したピューロマイシン耐性株の出現頻度はTT2細胞10^7個あたり1～2個であった。ピューロマイシン耐性株の凍結保存、ゲノムDNA取得は（実施例2）と同様に行なった。ヒト22番染色体の保持はピューロマイシン耐性株PG22-1について以下の（1）、（2）により確認した。

（1）PCR解析

ピューロマイシン耐性株ゲノムDNAを鋳型としてヒト22番染色体上に存在する遺伝子（Genetic Maps,前記）のうち、（実施例2；A9/#22）で存在が確認されている10種のプライマーについてPCR増幅を行なった結果、（実施例2；A9/#22）に存在した全てのマーカーが検出された。

（2）サザンブロット解析（実施例2）で示した方法に従い、ヒトL1配列をプローブとして用い、陰性コントロール野生型TT2、染色体供与細胞6-1、ピューロマイシン耐性TT2細胞株PG22-1由来のゲノムDNAについて行った。その結果を（第19図）に示す。図中左側にDNA分子量を示した。PG22-1におけるバンドパターンは6-1のそれと一致し、シグナル強度も同程度であることから、6-1細胞株中の22番染色体は確かにPG22-1に移入されたことが確認された。

以上の実験によりピューロマイシン耐性TT2細胞株PG22-1はヒト22番染色体の全てあるいは大部分を保持することが確認された。

（実施例31）ヒト22番染色体を保持するマウスES細胞株（TT2）からのキメラマウス作製

（実施例30）で得られ、ヒト22番染色体を保持していることが確認されたピューロマイシン耐性TT2細胞株PG22-1を凍結ストックより立ち上げ、ICRまたはMCH(ICR)（日本クレア社）雄雌マウスの交配により得られた8細胞期胚に胚あたり10～12個注入した。ES細胞用培地（実施例9）で一晩培養して胚盤胞に発生させた後、偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のインジェクション胚を移植した。

キメラ作製の結果を（第11表）に示す。計266個の注入胚を移植した結果、36匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚（ICR）由来の白色の中にTT2細胞由来の野生色（濃茶）が認められるかどうかにより判定される。誕生した36匹のうち毛色に明らかに野生色の部分のある、すなわち、ES細胞の貢

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献の認められる個体は8匹であった。

この結果より、ヒト22番染色体を保持するES細胞株（TT2由来、PG22-1）はキメラ形成能を保持している、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

第11表．ヒト22番染色体を保持するTT2細胞株からのキメラマウス作製

ES細胞株/ヒト染色体	ピューロマイシン耐性株番号	ES細胞を注入した8細胞期胚数	子マウスの数	キメラマウスの数	毛色への貢献率 ～20%	20-50%	50-80%
TT2/#22	PG22-1	266	36	8	4	1	3

（実施例32）ヒト22番染色体を保持するキメラマウスの血清中のヒト抗体λ鎖の検出、および定量

（実施例31）のキメラマウスKPG22-1～3の血清中のヒト抗体濃度を（実施例14）に従いELISA法を用いて定量した。生後２ヶ月のキメラマウスより採血し、血清中のヒト抗体λ鎖をELISA法を用いて検出した。PBSで希釈した抗ヒト免疫グロブリンλ鎖抗体（VECTOR LABORATORIES, INC.、IA-3070）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでビオチン標識した抗ヒト免疫グロブリンλ鎖抗体（VECTOR LABORATORIES, INC.、BA-3070）を加えてインキュベートしさらにビオチン化ワサビペルオキシダーゼとアビジンDHの複合体（VECTOR LABORATORIES, INC.、Vectastain ABCキット）を加えてインキュベートした後、TMBZ（住友ベークライト, ML-1120T）の添加により酵素活性を450nmの吸光度で評価し、精製されたλ鎖を持つ濃度既知のヒトIgM（大日本製薬、U1 3200）を標準としマウス血清を添加したPBSで段階的に希釈した。結果を第12表に示す。この結果より22番染色体を保持するキメラマウスにおいてヒト抗体λ鎖遺伝子が機能することが確認された。

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第12表. キメラマウス中のヒト抗体λ鎖濃度（ELISA）

キメラマウス	キメラ率%	Igλ(mg/l)
KPG22-1	50	12
KPG22-2	50	18
KPG22-3	20	24

（実施例33）ヒト22番染色体導入キメラマウス血清における抗ヒトHSAヒト体λ鎖の検出

キメラマウス（実施例31、KPG22-3）に生後79日、94日、110日の3回にわたり（実施例20）と同様にHSAを免疫した。血清中のヒト抗体λ鎖を（実施例14）に従いELISA法を用いて検出した。50mMの炭酸ー炭酸水素バッファーpH9.6で5μg/mlに希釈したHSA（シグマ、A 3782）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでビオチン標識した抗ヒト免疫グロブリンλ鎖抗体（VECTOR LABORATORIES, INC.、BA-3070）を加えてインキュベートしさらにビオチン化ワサビペルオキシダーゼとアビジンDHの複合体（VECTOR LABORATORIES, INC.、Vectastain ABCキット）を加えてインキュベートした後、TMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価した。HSAで免疫したキメラマウス血清中の抗HSAヒトλ鎖の力価は免疫後上昇した。一方対照としたICRマウスでは、HSA免疫後の抗HSAヒト抗体λ鎖の力価はバックグランドレベルであった。結果を（第20図）に示す。第20図において横軸はキメラマウスに初めてHSAを免疫してからの日数を、縦軸は450nmにおける吸光度を示した。これらの結果より、ヒト22番染色体を保持するキメラマウスにおいてヒト抗体λ鎖遺伝子が機能し、さらにはHSA抗原刺激に対して抗原特異的ヒトIgλの抗体価上昇が起こることが確認された。

（実施例34）ヒト22番染色体導入キメラマウスからのヒト抗体軽鎖産生ハイブリドーマ取得

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（実施例25）と同様に（実施例33）においてヒトアルブミンで免疫したキメラマウスKPG22-3から生後113日目に脾臓を取り出し、ミエローマ細胞と細胞融合し、ハイブリドーマを作製した。ハイブリドーマの作製法は〈安東・千葉、単クローン抗体実験操作入門、講談社サイエンティフィク，1991〉に記された方法に従い、ミエローマ細胞としては、SP-2/O-Ag14（大日本製薬、05-554）を使用した。培養液にHCF（エア・ブラウン）を10%添加し96穴プレート5枚にまき込み、1～3週間培養しコロニーが出現したウェルの培養上清をELISA法で解析した。ELISA法は（実施例33）と同様に行ない、ヒト抗体λ鎖陽性のクローンを4個得た。

（実施例35）ヒト22番染色体部分断片及び、14番染色体部分断片を同時に保持するマウスES細胞株の取得

ヒト22番染色体、14番染色体部分断片を同時に保持するマウスES細胞取得のため、染色体供与細胞として（実施例26）で取得した6-1（A9/#22，G418，ピューロマイシン耐性）細胞株を用いた。染色体受容細胞としてはすでにヒト14番染色体部分断片を保持しているG418耐性TT2細胞株1-4（実施例9）を用いた。ミクロセル融合実験およびピューロマイシン耐性株の選択は0.75μg/mlのピューロマイシン濃度で他は（実施例9）のG418耐性株選択の場合と同様に行なった。この結果出現したピューロマイシン耐性株の出現頻度は1-4細胞10^7個あたり1～2個であった。これらのピューロマイシン耐性株は300μg/mlのG418存在下でも増殖することからG418耐性も同時に保持していることが確認された。二重薬剤耐性株の凍結保存、ゲノムDNA取得は（実施例2）と同様に行なった。ヒト22番染色体及び、ヒト14番染色体部分断片の保持は二重薬剤耐性株PG22-5について以下のPCR解析により確認した。二重薬剤耐性株ゲノムDNAを鋳型としてヒト22番、14番染色体上に存在する遺伝子（Genetic Maps，前記）のうち、22番染色体については（実施例2；A9/#22）、14番染色体については（実施例9；TT2/#14 1-4）で存在が確認されている各プライマーについてPCR増幅を行なった結果、22番染色体については10種のうち3種のマーカー（D22S275，D22S315，Igλ）、14番染色体についてはTT2/#14 1-4に存在した全てのマーカーが検出された。以上の実験により、得られた二重耐性TT2細胞株はヒト22番染色体部分断片、14番染色体

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部分断片を同時に保持することが確かめられた。

（実施例36）ヒト22番染色体部分断片及び、14番染色体部分断片を同時に保持するマウスES細胞株からのキメラマウス作製

（実施例35）で得られ、ヒト22番染色体部分断片及び14番染色体部分断片を保持していることが確認されたG418、ピューロマイシン二重耐性TT2細胞株PG22-5を凍結ストックより立ち上げ、 ICRまたはMCH(ICR)（日本クレア社）雄雌マウスの交配により得られた8細胞期胚に胚あたり10～12個注入した。ES細胞用培地（実施例9）で一晩培養して胚盤胞に発生させた後、偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のインジェクション胚を移植した。

キメラ作製の結果を（第13表）に示す。計302個の注入胚を移植した結果、16匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚（ICR）由来の白色の中にTT2細胞由来の野生色（濃茶）が認められるかどうかにより判定される。誕生した16匹のうち毛色に明らかに野生色の部分のある、すなわち、ES細胞の貢献の認められる個体は5匹であった。

この結果より、ヒト22番染色体部分断片及び14番染色体部分断片を保持するES細胞株（PG22-5）はキメラ形成能を保持している、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

第13表　ヒト22番染色体と14番染色体を保持するTT2細胞株からのキメラマウス作製

ES細胞株/ヒト染色体	二重耐性株番号	ES細胞を注入した8細胞期胚数	子ﾏｳｽの数	ｷﾒﾗﾏｳｽの数	毛色への貢献率 ～20%	20-50%	50-80%
TT2/#22+#14	PG22-5	302	16	5	3	2	0

（実施例37）ヒト22番染色体部分断片及び、14番染色体部分断片を同時に保持

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するES細胞由来キメラマウス血清中のヒト抗体λ鎖およびヒト抗体μ鎖の検出・定量

（実施例36）のキメラマウス（KPG22-9、10、および12）に対して、HSAを免疫した。KPG22-9とKPG22-10には生後11週齢で免疫し、その2週間後に採血した。KPG22-12には生後7週齢と9週齢の2度免疫し、2度目の免疫の2週間後に採血した。

血清中のヒト抗体μ鎖およびヒト抗体λ鎖さらにヒトλ鎖かつヒトμ鎖を有する抗体を（実施例14）に従いELISA法を用いて検出した。

完全ヒト抗体の検出にはPBSで希釈した抗ヒト免疫グロブリンλ鎖抗体（Kirkegaard & Perry Laboratories Inc.、01-10-11）を96穴マイクロタイタープレートにコーティングし、血清試料を加え、次いでペルオキシダーゼ標識抗ヒト免疫グロブリンμ鎖抗体（The Binding Site Limited、 MP008）を加えてインキュベートした後、ペルオキシダーゼ基質としてTMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価し、精製されたλ鎖を持つ濃度既知のヒトIgM（大日本製薬、U13200）をマウス血清を添加したPBSで段階的に希釈したものと比較し血清中のヒト抗体濃度を求めた。ヒト抗体μ鎖およびヒト抗体λ鎖をELISA法を用いて（実施例29）および（実施例32）と同様に検出・定量した。結果を第14表に示す。キメラマウスではλ鎖とμ鎖がともに検出された。ヒト抗体μ鎖かつλ鎖を持つ抗体も検出された。この結果より、ヒト22番染色体部分断片及び14番染色体部分断片を保持するES細胞由来キメラマウスにおいてヒト抗体λ鎖遺伝子とヒト抗体μ鎖遺伝子は同時に機能し、一部のB細胞では重鎖と軽鎖がともにヒトである完全抗体が産生されることが確認された。

また対照として測定したヒト14番染色体のみを保持する（実施例10）のキメラマウス（K9）、ヒト22番染色体のみを保持する（実施例31）のキメラマウス（KPG22-2）の血清中のヒト抗体λ鎖かつμ鎖を持つ抗体濃度はバックグランドレベルであり、ここでの検出系はヒトλ鎖かつμ鎖を持つ完全抗体のみを検出していることが確認された。

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第14表　キメラマウス中のヒト抗体濃度(ELISA)

ESクローン	キメラマウス	キメラ 率%	IgM(mg/l)	Igλ(mg/l)	IgM, λ(mg/l)
PG22-5	KPG22-9	30	2.54	9.9	0.043
PG22-5	KPG22-10	5	4.96	21.5	0.333
PG22-5	KPG22-12	40	3.71	7.0	0.048
3-2	K9	50	6.66	-	<0.003
PG22-1	KPG22-2	50	-	17.6	<0.003

（実施例38）ヒト2番染色体部分断片及び、14番染色体部分断片を同時に保持するES細胞由来キメラマウス血清におけるヒト抗体の検出（ヒトκ鎖かつヒトμ鎖を有する抗体）

（実施例19）で作製したキメラマウスKPG-15（TT2ES クローンPG5由来、キメラ率10%）に対して、生後2から3ヶ月齢の間に、PBSに溶解したヒト血清アルブミン（HSA、シグマ、A3782）とアジュバント（MPL+TDM Emulsion, RIBI Immunochem Reseach Inc.）とを混合して0.25mg/mlのHSA溶液を調整し0.2mlを3回免疫し、採血した。また生後6週齢の（実施例19）のキメラマウスKPG-26（TT2ES クローンPG6由来、キメラ率40%）から採血した。血清中の完全ヒト抗体濃度を（実施例14）に従いELISA法で検出した。PBSで希釈した抗ヒト免疫グロブリンκ鎖抗体（Kirkegaard & Perry Laboratories Inc.、01-10-10）を96穴マイクロタイタープレートにコーティングし、マウス血清（シグマ、M5905）を加えたPBSで希釈した血清試料を加え、次いでペルオキシダーゼ標識抗ヒト免疫グロブリンμ鎖抗体（The Binding Site Limited、 MP008）を加えてインキュベートした後、ペルオキシダーゼ基質としてTMBZ（住友ベークライト、ML-1120T）の添加により酵素活性を450nmの吸光度で評価し、精製されたκ鎖を持つ濃度既知のヒトIgM（オルガノン・テクニカ、6001-1590）を標準としマウス血清を添加したPBSで段階的に希釈した。κ鎖、μ鎖については（実施例20）と同様にして濃

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度を求めた。結果を第15表に示す。ヒト抗体μ鎖かつκ鎖を持つ抗体が検出された。また対照としたヒト14番染色体のみを保持する（実施例10）のキメラマウス（K9）、ヒト2番染色体のみを保持する（実施例13）のキメラマウス（K2-9）血清中のヒト抗体でκ鎖かつμ鎖を持つ抗体濃度は0.002mg/ml以下でバックグランドレベルであった。この結果より、ヒト2番染色体部分断片及び14番染色体部分断片を保持するES細胞由来キメラマウスにおいてヒト抗体κ鎖遺伝子とヒト抗体μ鎖遺伝子は同時に機能し、一部のB細胞では重鎖と軽鎖がともにヒトである完全抗体が産生されることが確認された。

第15表　キメラマウス中のヒト抗体濃度(ELISA)

ESｸﾛｰﾝ	ｷﾒﾗﾏｳｽ	ｷﾒﾗ 率%	IgM(mg/l)	Igκ(mg/l)	IgM, κ(mg/l)
PG-5	KPG15	10	0.18	1.01	0.075
PG-6	KPG26	40	1.52	1.26	0.018
3-2	K9	50	6.66	-	0.002以下
5-1	K2-9	40	-	135	0.002以下

（実施例39）ヒト2番染色体部分断片を保持するマウスES細胞株（TT2F, XO）の取得

ヒト2番染色体部分断片を保持するマウスES細胞（XO）取得のため、染色体供与細胞として（実施例16）で取得したPG1細胞株を用いた。染色体受容細胞としては（39, XO）の染色体構成を持ち、キメラマウスにおいて効率よく卵細胞に分化することが報告されている（相沢慎一、バイオマニュアルシリーズ8, ジーンターゲティング, 羊土社, 1995）TT2F細胞株（ライフテックオリエンタル社より購入）を用いた。ミクロセル融合実験およびピューロマイシン耐性株の選択は0.75μg/mlのピューロマイシン濃度で他は（実施例9）のG418耐性株選択の場合と同様に行なった。この結果出現したピューロマイシン耐性株の出現頻度はTT2F細胞

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10^7個あたり5個であった。これらのピューロマイシン耐性株の凍結保存、ゲノムDNA取得は（実施例2）と同様に行なった。ヒト2番染色体部分断片の保持は薬剤耐性株P-20, P-21について以下のPCR解析により確認した。薬剤耐性株ゲノムDNAを鋳型としてヒト2番染色体上に存在する遺伝子（Genetic Maps,前記）のうち、（実施例１：A9/#2 w23）で存在が確認されているプライマーCκ、FABP1、Vκ1-2の3種についてPCR増幅を行なった結果、2株共に、3種全てのプライマーについて期待される増幅産物が確認された。

以上の実験により、得られたピューロマイシン耐性ES細胞株（TT2F, XO）はヒト2番染色体部分断片を保持することが確かめられた。

（実施例40）ヒト2番染色体部分断片を保持するマウスES細胞株（TT2F, XO）からのキメラマウス作製

（実施例39）で得られ、ヒト2番染色体部分断片を保持していることが確認されたピューロマイシン耐性TT2F細胞株P-21を凍結ストックより立ち上げ、ICRまたはMCH(ICR)（日本クレア社）雄雌マウスの交配により得られた8細胞期胚に胚あたり10～12個注入した。ES細胞用培地（実施例9）で一晩培養して胚盤胞に発生させた後、偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のインジェクション胚を移植した。

計141個の注入胚を移植した結果、20匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚（ICR）由来の白色の中にTT2細胞由来の野生色（濃茶）が認められるかどうかにより判定される。キメラ作製の結果を（第16表）に示す。誕生した20匹のうち毛色に明らかに野生色の部分のある、すなわち、ES細胞の貢献の認められる個体は9匹であった。うち4個体は毛色が完全に野生色の（ES細胞に由来する）キメラマウスであった。

この結果より、ヒト2番染色体部分断片を保持するES細胞株（P-21）はキメラ形成能を保持している、すなわちマウス個体の正常組織に分化する能力を保持していることが確認された。

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第16表　ヒト2番染色体部分断片を保持するTT2F細胞株からのキメラマウス作製

ES細胞株/ヒト染色体	ピューロマイシン耐性株番号	ES細胞を注入した8細胞期胚数	子マウスの数	キメラマウスの数	毛色への貢献率 ～20%	20-50%	50-90%	100%
TT2F/#2fg. P-21	141	20	9	0	2	3	4	

（実施例41）ヒト2番染色体部分断片を保持するTT2F由来キメラマウスの血清中のヒト抗体κ鎖の検出、および定量

（実施例40）の生後約1ヶ月齢のキメラマウス（P-21由来、キメラ率100%、K2-1F～4F）より採血し血清中のヒト抗体κ鎖濃度を（実施例20）と同様にELISA法を用いて定量した。

結果を第17表に示す。使用するES細胞をTT2Fとしてもヒト抗体κ鎖遺伝子がキメラマウス中で機能することが確認された。

第17表　キメラマウス中のヒト抗体κ鎖濃度（ELISA）

キメラマウス	キメラ率%	Igκ(mg/l)
K2-1F	100	66
K2-2F	100	156
K2-3F	100	99
K2-4F	100	20

（実施例42）ヒト2番染色体部分断片を保持するマウスES細胞（TT2F, XO）由

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来キメラマウスの子孫におけるヒト染色体保持の検出

（実施例40）で得られた雌キメラマウスのうちK2-1F，K2-4F（共に毛色のキメラ率100%）について雄ICRマウスとの交配によりES細胞由来の子孫が誕生するかを検討した。この交配においてICR雄マウス（アルビノ、劣性）由来精子により受精したキメラマウス中のTT2F細胞（野生色：優性）由来の卵子からは野生色、ICR由来卵子からは白色の子マウスが誕生する。それぞれ1回の交配によって得られた生存可能な子マウス（K2-1F：10匹、K2-4F：5匹）のすべてがES細胞由来の毛色である野生色を示した。これらの子マウスの尻尾より調製したゲノムDNAについてヒト染色体断片の保持をPCR法により検討した。P-21（実施例39）で存在が確認されている3種のプライマーについてPCR増幅を行なった結果、10匹中4匹（K2-1F）、5匹中2匹（K2-4F）においてP-21で検出された3種のマーカーの存在が確認された。15匹の子マウスのPCRの結果を（第21図）に示す。図中右側にマーカー（φX174，HaeIII断片，ニッポンジーン）および主なバンドのDNA分子量を、左側にそれぞれのプライマーによって期待される増幅産物の長さを矢印で示した。右側に陽性コントロールとして親キメラK2-1F、K2-4Fの尻尾由来DNAを用いた結果も示す。これらの結果は、ヒト2番染色体部分断片を保持するTT2F細胞株P-21がキメラマウス中で機能的な卵子に分化し、その卵子由来の子孫にヒト2番染色体部分断片が伝達されたことを示している。

（実施例43）ヒト2番染色体部分断片を保持するマウスES細胞（TT2，XY）由来キメラマウスの子孫におけるヒト染色体保持の検出

（実施例13）で得られたキメラマウスのうちK2-18（雄、キメラ率70%）とK2-19（雌、キメラ率60%）及び同腹の非キメラ雌を混合して交配し、ES細胞由来の子孫が誕生するかを検討した。TT2細胞は（40，XY）の染色体構成を持つので雄キメラK2-18において機能的な精子に分化している可能性がある。その場合キメラマウス中のTT2細胞（野生色、優性）由来精子により受精したICR（白色：劣性）由来の卵子からは野生色の子マウスが誕生する。交配によって得られた生存可能な子マウス計110匹のうち10匹がES細胞由来の毛色である野生色を示した。これらの野生色子マウス10匹のうち7匹の尻尾より調製したゲノムDNAについてヒト染色体断片の保持をPCR法により検討した。5-1株（TT2/#2fg.，実施例12）

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で存在が確認されている2種のプライマー（Cκ、FABP1）及び、（実施例1）で示したVκ1-2 プライマーについてPCR増幅を行なった結果、7匹中2匹において3種のマーカー全ての存在が確認された。この結果は、ヒト2番染色体部分断片を保持するTT2細胞株5-1がキメラマウス中で機能的な精子に分化し、その精子由来の子孫にヒト2番染色体部分断片が伝達されたことを示している。

（実施例44）キメラマウス子孫の血清中のヒト抗体κ鎖の検出、および定量

（実施例42）のキメラマウス子孫K2-1F-1～10およびK2-4F-1～5の血清中のヒト抗体κ鎖濃度をELISA法を用いて定量した。生後約4～6週齢のマウスより採血し、血清中のヒト抗体κ鎖を（実施例20）と同様にELISA法を用いて検出した。結果を（実施例42）で得られた染色体保持の結果と共に第18表に示す。キメラマウスから生まれた子孫においてもヒト抗体κ鎖遺伝子が機能することが確認された。

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第18表　キメラマウス子孫中のヒト抗体κ鎖濃度（ELISA）

親キメラマウス	マウス個体番号	ヒト2番染色体断片	Igκ(mg/l)
K2-1F	#1	-	0.58
K2-1F	#2	+	84.1
K2-1F	#3	+	12.8
K2-1F	#4	+	15.1
K2-1F	#5	-	0.52
K2-1F	#6	-	0.58
K2-1F	#7	-	1.30
K2-1F	#8	-	0.90
K2-1F	#9	-	0.56
K2-1F	#10	+	28.8
K2-4F	#1	-	0.04以下
K2-4F	#2	+	23.3
K2-4F	#3	+	11.8
K2-4F	#4	-	0.08
K2-4F	#5	-	0.06

（実施例45）ヒト14番染色体部分断片導入キメラマウス脾臓細胞の解析

フローサイトメトリーによる解析は下記の文献に記載の方法に従った。日本生化学会編、新生化学実験講座12分子免疫学I－免疫細胞・サイトカイン－1989、東京化学同人；東京大学医科学研究所　制癌研究部編、細胞工学別冊8　新細胞工学実験プロトコール、1991、秀潤社；A. Doyle and J.B.Griffiths, “Cell & Tissue Culture: Laboratory Procedures”, published by John Wiley & Sons Ltd., 1996 。（実施例19）の生後6ヶ月齢のキメラマウス（KPG06；PG16由来、キメラ率30%）から脾臓をとりだし、塩化アンモニウム水溶液で処理した後ラッ

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ト血清を1%含むPBS中で、フルオレッセインイソチオシアネート（FITC）標識抗マウスCD45R(B220)抗体（ファーミンジェン、01124A）で細胞を染色した。洗浄後５％マウス血清を含むPBS中でビオチン標識抗ヒトIgM 抗体（ファーミンジェン、08072D）または対照としてビオチン標識抗ヒトλ鎖抗体（ファーミンジェン、08152D）と反応させた後、ストレプトアビジンフィコエリスリン（ファーミンジェン、13025D）で染色し、フローサイトメトリー（ベクトンデッキンソン、FACSort）で解析した。また対照としてヒト染色体を保持しないICRマウスも同様に解析した。第22図に結果を示す。図中横軸はヒトIgM 、縦軸はCD45R(B220）を示す。B細胞マーカーCD45R陽性（FITC）でかつヒトIgM陽性（PE）である細胞集団が4%増加しており、これによってキメラマウスではヒト抗体μ鎖を細胞表面に発現している細胞の存在が確認された。

（実施例46）ヒト抗体重鎖、κ鎖、λ鎖をそれぞれ発現するキメラマウス脾臓由来cDNAからのヒト抗体遺伝子可変領域クローニングと塩基配列決定

（実施例29）、（実施例23）、（実施例32）においてヒト抗体重鎖、κ鎖、λ鎖をそれぞれ発現することが確認されたキメラマウスK15A（1-4株由来、実施例10に示した方法で作製）、K2-8（実施例13で作製）、KPG22-2(実施例31で作製）の脾臓由来RNAより（実施例5）と同様な方法で合成したcDNAについて、下記のプライマーによりPCRを行ない、それぞれのヒト抗体遺伝子可変領域を増幅した。陰性コントロールとして非キメラマウスICRより取得した脾臓由来cDNAを用いた。参考文献の記載のないプライマーはGenbank等のデータベースより入手した塩基配列をもとに作製した。

・K15A （重鎖）

定常領域用：HIGMEX1-2：5'-CCAAGCTTCAGGAGAAAGTGATGGAGTC （配列番号53）

HIGMEX1-1：5'-CCAAGCTTAGGCAGCCAACGGCCACGCT（VH3BACKの2ndPCRに使用）（配列番号54）

可変領域用：VH1/5BACK（59℃, 35cycles, Marksら, Eur. J. Immnol., 21, 985-, 1991）, VH4BACK（59℃, 35cycles, Marksら, 前記）, VH3BACK（1stPCR:59℃, 35cycles, 2ndPCR:59℃, 35cycles, Marksら, 前記）

・K2-8（軽鎖κ）

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定常領域用：KC2H：5'-CCAAGCTTCAGAGGCAGTTCCAGATTTC （配列番号55）
可変領域用：Vk1/4BACK（55℃, 40cycles, Marksら, Eur. J. Immnol., 21, 985-, 1991）, Vk2BACK（55℃, 40cycles, Marksら, 前記）, Vk3BACK （55℃, 40cycles, Marksら, 前記）

・KPG22-2（軽鎖λ）
定常領域用：CλMIX（以下の3種のプライマーを等モル比で混合したもの）
IGL1-CR：5'-GGGAATTCGGGTAGAAGTCACTGATCAG （配列番号56）
IGL2-CR ：5'-GGGAATTCGGGTAGAAGTCACTTATGAG （配列番号57）
IGL7-CR ：5'-GGGAATTCGGGTAGAAGTCACTTACGAG （配列番号58）
可変領域用：Vλ1LEA1（55℃, 40cycles, Williamsら, Eur. J. Immunol., 23, 1456-, 1993）, Vλ2MIX（55℃, 40cycles, 前記のWilliamsらの報告にあるVλ2LEA1, Vλ2JLEAD を等モル比で混合したもの）, Vλ3MIX（55℃, 40 cycles, 前記のWilliamsらの報告にあるVλ3LEA1, Vλ3JLEAD, Vλ3BACK4を等モル比で混合したもの）

それぞれ定常領域用×可変領域用（重鎖3種、κ鎖3種、λ鎖3種）の組み合わせで94℃15秒、それぞれの可変領域プライマーに示したアニーリング温度15秒、72℃20秒、それぞれの可変領域プライマーに示したサイクル数（パーキンエルマー社、サーマルサイクラー9600使用）の条件で行なった。VH3BACKにおける2ndPCRは1stPCRの増幅産物を（HIGMEX1-1×VH3BACK）のプライマーの組み合わせで再度増幅した。全ての増幅産物は1.5%アガロース電気泳動後、臭化エチジウム染色することにより検出した。その結果、全て組み合わせにおいて、期待される長さ（重鎖：470bp付近、軽鎖κ：400bp付近、軽鎖λ：510bp付近）の増幅産物が検出された。一方、陰性コントロールでは全てにおいて同じ位置に特異的増幅産物は検出されなかった。これらの増幅産物は、アガロースゲルよりprep. A. gene（バイオラッド）により抽出した後、制限酵素処理（重鎖：HindIII-PstI、軽鎖κ：HindIII-PvuII、軽鎖λ：HindIII-EcoRI）し、pUC119（宝酒造）ベクターのHindIII, PstI 部位（重鎖）、HindIII, HincII部位（κ鎖）、HindIII, EcoRI部位（λ鎖）にクローニングした。増幅産物の挿入されたプラスミドのうち以下のもの（右側に示したクローン数）について、自動蛍光シーケンサー（Appl

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yed Bio System社）により、増幅産物の塩基配列を決定した。

・HIGMEX1-2×VH1/5BACK：10クローン、

・HIGMEX1-2×VH4BACK：8クローン、

・HIGMEX1-2(2nd PCR, HIGMEX1-1)×VH3BACK：5クローン

・KC2H×Vκ1/4BACK：6クローン、

・KC2H×Vκ2BACK：7クローン、

・KC2H×Vκ3BACK ：4 クローン、

・CλMIX×Vλ1LEA1：5クローン、

・CλMIX×Vλ2MIX：6クローン、

・CλMIX×Vλ3MIX：5クローン

得られた塩基配列をDNASIS(Hitachi Software Engneering）により解析した結果、全てがヒト由来配列であり、κ鎖、λ鎖の全て、重鎖の計23種中21種が開始コドンから定常領域に至るまで終始コドンを含まない機能的配列であった。決定された配列からお互いに同一なものを除くと重鎖17種、κ鎖11種、λ鎖12種のそれぞれ異なる可変領域配列が同定された。

（実施例47）ヒト抗体重鎖、κ鎖、λ鎖をそれぞれ発現するキメラマウス脾臓由来cDNAからのヒト抗体遺伝子可変領域塩基配列の解析

（実施例46）において決定された塩基配列（重鎖17クローン、κ鎖11クローン、λ鎖12クローン）について以下の点について解析を行った。

1．各可変領域配列において使用されている既知の生殖系列V遺伝子断片を特定

2．各可変領域配列において使用されている既知の生殖系列J遺伝子断片を特定

3．重鎖可変領域において使用されている既知の生殖系列D断片を特定

4．1、2、3の結果をもとにした重鎖可変領域におけるN領域の付加の同定

5．各可変領域塩基配列から導かれるアミノ酸配列の決定

その結果を（第19表）に示す。1、2についてはGenbankに登録されている生殖系列V及びJ断片とのホモロジー検索をDNASISにより行い特定した。VH断片は（Cookら，Nature gentics，7，162-，1994）、Vκ断片は（Kleinら，Eur. J. Immunol，23，3248-，1993）、Vλ断片は（Williamsら，前記）の表記法に従い、各V断片ファミリー名と共に表中に記した。3については（Ichiharaら，The EM

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BO J., 7, 13, 4141-, 1988）の報告に記された生殖系列D断片とのホモロジー検索をDNASISにより行い、連続して8bp以上一致することを指標として決定し表中に記した。DN*については（Greenら, Nature Genetics, 7, 13-, 1994）で報告された新しいDNファミリー断片と考えられる。4については1（V）、2（J）、3（D）の結果をもとに、いずれの生殖系列断片にも見いだされない塩基配列をN領域とみなした。その結果、D領域の特定された13種中11種にN領域が観察され、その平均の長さは8.7bpであった。5については各塩基配列をDNASISを使用して1文字表記のアミノ酸配列に変換した。表中にはCDR3領域のみ示した。表中右側に各可変領域のクローニングに使用したプライマー名及び各クローン名を記した。

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第19表

V family	V segment	CDR3	J (D)	V primer	Clone
K15A					
VH1	VH1-8	VRSSSWYEYYYYGMDV	J6 (DN1)	VH4BACK	H4-10
	VH1-18	GGITMVRGLIITDWYFDL	J2 (DXP'1)	VH1/5BACK	H1-7
	VH1-24	APYSGRFDY	J4 (DK1)	VH1/5BACK	H1-6
	VH1-46	ERYYGSGSYQDYYYYYGMDV	J6 (DXP'1)	VH1/5BACK	H1-2
	VH1-46	GGYSGYEDYYYYGMDV	J6 (DK1)	VH1/5BACK	H1-10
VH2	VH2-5	SYFDWPDFDY	J4 (DXP1)	VH4BACK	H4-14
VH3	VH3-21	EGCSGGSCLPGYYYYGMDV	J6 (DLR2)	VH1/5BACK	H1-4
	VH3-23	AHGDPYFDY	J4	VH1/5BACK	H1-3
	VH3-23	DADAFDI	J3	VH1/5BACK	H1-8
	VH3-23	SGWDY	J4 (DN1*)	VH3BACK	H3-3
	VH3-23	TGFDL	J2	VH4BACK	H4-4
	VH3-33	EGGYGSVGDYYYYGMDV	J6 (DXP'1)	VH1/5BACK	H1-9
	VH3-33	GGYSYGYDYYYYGMDV	J6 (DXP'1)	VH3BACK	H3-5
	VH3-33	GYSSGWYDY	J4 (DN1*)	VH4BACK	H4-9
VH4	VH4-34	RYSSGWYYFDY	J4 (DN1*)	VH4BACK	H4-15
	VH4-59	GRIAVASFDY	J4 (DN1*)	VH4BACK	H4-2
	VH4-59	GSGSYFHFDY	J4	VH4BACK	H4-6
K2-8					
V κ 1	O18-8	QQHDNLPFT	J3	V κ 1BACK	K1-1
	O18-8	QQYDNLPIT	J5	V κ 1BACK	K1-3
	O18-8	QQHDNLPFA	J3	V κ 2BACK	K2-2
	L1	QQYNSYPLT	J4	V κ 1BACK	K1-6
V κ 2	A17	MQGTHLLT	J4	V κ 2BACK	K2-1
	A17	MQGTHWIT	J5	V κ 2BACK	K2-5
V κ 3	A27	QQYGSSPTWT	J1	V κ 3BACK	K3-1
	A27	QQYGSSPFT	J3	V κ 3BACK	K3-4
	A27	QQYGSSPLWT	J1	V κ 3BACK	K3-5
	A27	QQYGSSPPWT	J1	V κ 3BACK	K3-6
V κ 6	A26-10	HQSSSLPQT	J1	V κ 2BACK	K2-4
KPG22-2					
V λ 1	DPL3	AAWDDSLDVV	JC3	V λ 1LEA1	L1-3
	DPL5	GTWDSSLSAGV	JC2	V λ 1LEA1	L1-4
	DPL5	GTWDSSLSAGVV	JC3	V λ 1LEA1	L1-6
	DPL5	GTWDSSLSAVV	JC2	V λ 1LEA1	L1-9
	DPL8	QSYDSSLSGVV	JC3	V λ 1LEA1	L1-8
V λ 2	DPL10	CSYAGSSTLV	JC2	V λ 2MIX	L2-4
	DPL11	SSYTSSSTVV	JC2	V λ 2MIX	L2-1
	DPL11	SSYTSSSTLV	JC2	V λ 2MIX	L2-3
	DPL11	CSYTSSSTFV	JC2	V λ 2MIX	L2-7
	DPL12	SSYAGSNNLV	JC3	V λ 2MIX	L2-5
	DPL12	SSYAGSNNFVV	JC3	V λ 2MIX	L2-6
V λ 3	DPL16	NSRDSSGNLV	JC2	V λ 3MIX	L3-1

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（実施例48）TT2(またはTT2F)ES細胞の抗体遺伝子（重鎖、軽鎖κ）ノックアウト用のターゲッティングベクターの作製

マウス抗体遺伝子（重鎖、軽鎖κ）が破壊されたTT2(またはTT2F)細胞へG418耐性遺伝子でマーキングされたヒト14番染色体断片（実施例9）およびピューロマイシン耐性遺伝子でマーキングされたヒト2番（実施例18）または22番染色体（実施例35）を導入することが可能となる。このようなヒト14番+2番またはヒト14番+22番染色体を導入したマウス抗体遺伝子（重鎖、軽鎖κ）遺伝子破壊TT2(またはTT2F)ES細胞を用いて、（重鎖＋κ鎖：実施例19、重鎖＋λ鎖：実施例36）の方法によって作成されたキメラマウスでは、主に重鎖と軽鎖がヒト由来の抗体が産生されることが期待される。以下に示す第23図～第27図における制限酵素の略称等は、以下のとおりである。

制限酵素：Kp: KpnI、B: BamHI、RI: EcoRI 、RV: EcoRV 、N: NotI 、SII: ScaII、Sca: ScaI 、Sfi: SfiI 、Sm: SmaI、X: XhoI 、SI: SalI、dKp: deletion of KpnI 、(X): ラムダベクター由来のXhoI切断部位。

点線部分：pBluescript SKII(+) プラスミドDNA

◁▷：LoxP配列

1．G418耐性遺伝子の両側にLoxP配列を入れたLoxP-pstNEOプラスミドの作製

TT2(またはTT2F)細胞の抗体遺伝子をノックアウトした後、G418耐性遺伝子を除去するためにG418耐性遺伝子（実施例1）の両端にCreリコンビネース（Sauerら，Proc. Natl. Acad. Sci. USA, 85, 5166-, 1988 ）の認識配列であるLoxP配列（Sauerら、前記）を同じ向きに入れる必要がある。pSTneoBプラスミドDNA（実施例1）より制限酵素XhoIでpstNEO遺伝子を切り出し、アガロースゲル電気泳動によりDNA断片を精製したのちT4DNAポリメラーゼ（Takara社製Blunting endキット）により両端を平滑化した。LoxP配列が入ったプラスミドDNA pBS246（Plasmid pBS246, loxP2 Cassette Vector, U.S. Patent 4959317）は、GIBCO BRL社より購入した。このプラスミドのEcoRI及びSpeI切断部位へXhoIリンカーDNAを挿入することによってXhoI認識配列に変更したものを使用した。この改変pBS246のEcoRV切断部位へ上記pstNEO DNA断片を挿入してプラスミドLoxP-pstNEOを得た（第23図）。

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2．C57BL/6由来抗体重鎖Cμ（IgM定常領域）、軽鎖Jκ-Cκ（Igκ連結領域および定常領域）を含むゲノムDNAクローンの単離

TT2(またはTT2F)細胞は、C57BL/6マウスとCBAマウスのF1マウス由来であることから、C57BL/6マウス由来のゲノムDNAクローンを用いて抗体遺伝子ノックアウト用ベクターを作製することにした。ゲノムDNAライブラリーは、Clontech社adult C57BL/6N male liver由来ラムダDNAライブラリーを使用した。スクリーニングのためにプローブとしては、以下の合成DNA配列（60 mer）を用いた。

重鎖Cμプローブ：5'-ACC TTC ATC GTC CTC TTC CTC CTG AGC CTC TTC TAC AGC ACC ACC GTC ACC CTG TTC AAG-3'(配列番号59)

軽鎖κプローブ：5'-TGA TGC TGC ACC AAC TGT ATC CAT CTT CCC ACC ATC CAG TGA GCA GTT AAC ATC TGG AGG-3'(配列番号60)

単離されたラムダクローンを解析して、重鎖Cμまたは軽鎖Jκ-Cκを含むDNA断片をpBluescript SKII(+) プラスミド（Stratagene社）へサブクローニングした（重鎖Cμ：第24図；軽鎖Jκ-Cκ：第25図）。これらのDNA断片を用いて、以下のようにTT2(またはTT2F)ES細胞中のマウス抗体遺伝子破壊のためのターゲッティングベクターを作製した。

3．マウス抗体重鎖遺伝子破壊用ベクタープラスミドの作製

2で調製したマウス抗体重鎖定常領域を含むゲノムDNAのCμをコードする領域の内で、第2～第4エクソンが含まれるDNA断片（BamHI～XhoI）を1で作製したLoxP-pstNEO遺伝子と置き換えた（第26図）。pstNEOの転写方向は、抗体遺伝子の転写方向とは逆向きとなっている。このプラスミドDNAは、大腸菌JM109を用いて増幅し、セシウムクロライド平衡遠心により精製した（細胞工学実験操作入門、1992年講談社刊）。精製したプラスミドDNAは、制限酵素SacIIにより、一箇所切断してTT2(またはTT2F) ES細胞へのトランスフェクションに使用した。形質転換体TT2(またはTT2F)ES細胞の中から抗体重鎖部分がターゲッティングベクターと相同組換えが起こったクローンを検出するための形質転換体ゲノムDNAサザンブロット用のプローブとして、Cμの上流に存在するスイッチ領域のDNA断片（約500塩基対）を用いた。このDNA断片は以下の条件で129マウスゲノムDNAをPCR増幅して得た。

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センスプライマー：5'-CTG GGG TGA GCC GGA TGT TTT G-3'(配列番号61)
アンチセンスプライマー：5'-CCA ACC CAG CTC AGC CCA GTT C-3'(配列番号62)

テンプレートDNA：EcoRI消化129マウスゲノムDNA 1μg
反応バッファー、デオキシヌクレオチドミックス、TaqDNAポリメラーゼは、Takara社製のものを使用。
反応条件：94度C,3分,1回 → 94度C, 1分；55度C,2分；72度C,2分；3回
→ 94度C, 45秒；55度C, 1分；72度C, 1分；36回

増幅されたDNAがGenbankのデータベースにあるとおり、制限酵素HindIIIで一箇所切断されえることを確認して、pBluescriptプラスミドのEcoRV切断部位へサブクローニングした。このプラスミドDNA（S8）を制限酵素BamHIとXhoIで切断して、アガロースゲル電気泳動でPCR断片（約550塩基対）を精製したものをプローブとした。ターゲッティングベクターで形質転換したTT2(またはTT2F)ES細胞のゲノムDNAを制限酵素EcoRIとXhoIで消化し、アガロースゲル電気泳動で分離後サザンブロットを行ない、上記プローブで検出する。

4．マウス抗体遺伝子軽鎖k破壊用ベクターの作製

2で調製したマウス抗体軽鎖κJ領域および定常領域を含むゲノムDNAのJ領域（J1～J5）が含まれるDNA断片（EcoRI～SacII）を1で作製したLoxP-pstNEO遺伝子と置き換えた（第27図）。pstNEOの転写方向は、抗体遺伝子の転写方向と同じ向きとなっている。このプラスミドDNAは、大腸菌JM109を用いて増幅し、セシウムクロライド平衡遠心により精製した。精製したプラスミドDNAは、制限酵素KpnIにより、一箇所切断してTT2(またはTT2F) ES細胞へのトランスフェクションに使用した。形質転換体TT2(またはTT2F)ES細胞の中から抗体重鎖部分がターゲッティングベクターと相同組換えが起こったクローンを検出するための形質転換体ゲノムDNAサザンブロット用のプローブとして、軽鎖Jκ-Cκゲノム DNA（第25図参照）の3'端のDNA断片（XhoI～EcoRI；約1.4k塩基対）を用いた。ターゲッティングベクターで形質転換したTT2(またはTT2F)ES細胞のゲノムDNAを制限酵素EcoRIとNotIで消化し、アガロースゲル電気泳動で分離後サザンブロットを行ない、上記プローブで検出する。

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（実施例49）マウスES細胞抗体重鎖遺伝子破壊株の取得

抗体重鎖遺伝子相同組換え体取得の為、（実施例48）-3で作成した抗体重鎖ターゲッティングベクターを制限酵素SacII（宝酒造）で線状化し、（相沢慎一、バイオマニュアルシリーズ8, ジーンターゲティング, 羊土社, 1995）の方法に従いマウスES細胞TT2Fへ導入した。TT2F細胞をトリプシン処理し、2.5×10^7個／mlとなるようにHBSに懸濁してから5μgDNAを加え、ジーンパルサー（バイオラッド、抵抗器ユニット接続せず）を用いてエレクトロポレーションを行なった。960μFの容量で250Vの電圧を4mm長のエレクトロポレーションセルを用いて室温で印加した。エレクトロポレーションした細胞を20mlのES培地に懸濁し、あらかじめフィーダー細胞をまいた100mm組織培養用プラスチックシャーレ（コーニング）2枚に播種した。同様に10, 15μgDNAを用いた実験も行った。1日後に300μg/mlのG418（GENETICIN, シグマ）を含む培地と置き換えた。7～9日後に生じたコロニー計176個をピックアップし、それぞれを12穴プレートでコンフルーエントになるまで増殖させ、その4/5を0.2mlの保存用培地（ES培地+10%DMSO<シグマ>）に懸濁し、-80℃にて凍結保存した。残りの1/5は12穴ゼラチンコートプレートに播種し、2日間培養して（実施例2）に示した方法でゲノムDNAを取得した。これらのG418耐性TT2F細胞ゲノムDNAを制限酵素EcoRIとXhoI（宝酒造）で消化し、アガロースゲル電気泳動で分離後サザンブロットを行ない、（実施例48）-3に示したプローブで相同組換え体を検出した。その結果176株中3株が相同組換え体であるという結果を得た。野生型TT2F細胞及び相同組換え体#131、#141のサザンブロット解析の結果を（第28図）左側3レーンに示す。野生型TT2F細胞ではEcoRI、XhoI消化により2本のバンド（a、b）が検出される。相同組換え体においては、これらのいずれかのバンドが消失し、新たに下部にバンド（c）が現われることが予想される。図中#131, #141においてaのバンドが消失し、新たにcのバンドが出現している。図中左側にはDNAの大きさを示した。すなわちこれらのクローンは抗体重鎖遺伝子の片方のアレルが相同組換えにより破壊されたものである。

（実施例50）抗体重鎖相同組換え体ES細胞からのキメラマウス作成

（実施例49）で得られた抗体重鎖相同組換え体TT2F細胞株#131を凍結ストック

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より立ち上げ、 ICRまたはMCH(ICR)（日本クレア社）雄雌マウスの交配により得られた8細胞期胚に胚あたり10～12個注入した。ES細胞用培地（実施例9）で一晩培養して胚盤胞に発生させた後、偽妊娠処理後2.5日の仮親ICRマウス（日本クレア社）の子宮に片側の子宮あたり約10個のインジェクション胚を移植した。計94個の注入胚を移植した結果、22匹の子マウスが誕生した。キメラ個体は、毛色において宿主胚（ICR）由来の白色の中にTT2F細胞由来の野生色（濃茶）が認められるかどうかにより判定される。誕生した22匹のうち毛色に明らかに野生色の部分のある、すなわち、ES細胞の貢献の認められる個体は18匹であった。うち16個体は毛色の80%以上が野生色の（ES細胞に由来する）雌キメラマウスであった。この結果より、抗体重鎖相同組換え体ES細胞株#131はキメラ形成能を保持していることが確認された。得られたキメラマウスのうち多くの個体は非常に高い貢献率を示す雌であるので、ES細胞が機能的な生殖細胞（卵子）に分化している可能性が高い。キメラマウスのうち100%の貢献率を示す雌キメラ2個体をMCH(ICR)雄マウスと交配した結果、生まれた子マウスはすべて野性色を示した。これらの子マウスは#131由来であり（実施例42参照）、2匹に1匹の割合で破壊された抗体重鎖アレルが伝達していると考えられる。

（実施例51）抗体重鎖相同組換え体からの2重破壊株の取得

片側アレルがG418耐性遺伝子の挿入により破壊されたES細胞株において、培養液中のG418濃度を上げて培養することにより得られる高濃度G418耐性株をスクリーニングすれば両側アレル共に破壊された株を取得することが可能なことが報告されている（相沢慎一ら、バイオマニュアルシリーズ8, ジーンターゲティング, 羊土社, 1995）。これに基づき、我々はTT2F抗体重鎖相同組換え体#131, #141について両側アレルの破壊株取得の為、以下の実験を行った。まず、#131, #141両株について致死G418濃度検定のため35mmシャーレ各10枚（この実施例においては栄養細胞はマイトマイシン処理をしていないG418耐性初代培養細胞を使用した。実施例9参照）に1枚あたり約100個の細胞を播種し、0, 0.5, 1, 2, 3, 5, 8, 10, 15, 20 mg/ml のG418 (GENETICIN、シグマ）をそれぞれ含むES培地中で10日間培養した。その結果3mg/mlの濃度までは明らかなコロニーが認められたが、5mg/mlではコロニー形成は認められなかった。この結果をもとに最小致死濃度を

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5mg/mlと決定し、4，5，6，7，8mg/mlの各濃度で高濃度G418耐性株の選抜を行った。#131，#141それぞれについて100mmシャーレ計10枚に1枚当たり約10^6個の細胞を播種し、上記の各濃度のG418を含むES培地（5段階、各濃度シャーレ2枚づつ）により培養した。培養開始12日後に7mg，8mg/mlのシャーレにおいて明らかなコロニー（#131：12株、#141：10株）をピックアップし、（実施例49）と同様に凍結保存、DNA取得を行った。これらの高濃度G418耐性株ゲノムDNAを制限酵素EcoRIとXhoI（宝酒造）で消化し、アガロースゲル電気泳動で分離後サザンブロットを行ない、（実施例48）-3に示したプローブで両側アレルの破壊された株を検出した。その結果#131株由来の1株（#131-3）が両側アレル破壊株であるという結果を得た。#131由来の6株についてのサザンブロット解析の結果を（第28図）に示す。野生型TT2F細胞ではEcoRI、XhoI消化により2本の野生型バンド（a、b）が検出される。片側アレル相同組換え体（#131，#141）においては、上部のバンドaが消失し、新たにバンドcが現われる（実施例49）。さらに両側アレルが破壊されることにより、もう一方の野生型バンドbが消失し、破壊型バンドcのみとなることが予想される。図中3（#131-3）のクローンにおいてこのバンドパターンが観察された。すなわちこのクローンは抗体重鎖遺伝子の両方のアレルが破壊されたものである。

（実施例52）抗体重鎖欠損ホモ接合体TT2F細胞株からのG418耐性マーカー遺伝子の除去

（実施例51）で取得された抗体重鎖両側アレル破壊株（高濃度G418耐性株#131-3）のG418耐性マーカー遺伝子を以下の手順により除去した。G418耐性マーカー遺伝子の両側に挿入したloxP配列（実施例48-1）の間で部位特異的組換えを起こすCreレコンビナーゼ遺伝子を含む発現ベクターpBS185（BRL）を（相沢慎一、バイオマニュアルシリーズ8，ジーンターゲティング，羊土社，1995、及び高津聖志ら、実験医学別冊、免疫研究の基礎技術、p255-、1995、羊土社）の方法に従い#131-3株へ導入した。#131-3細胞をトリプシン処理し、2.5×10^7個／mlとなるようにHBSに懸濁してから30μgのpBS185DNAを加え、ジーンパルサー（バイオラッド、抵抗器ユニット接続せず）を用いてエレクトロポレーションを行なった。960μFの容量で250Vの電圧を4mm長のエレクトロポレーションセル（実施例1）

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を用いて印加した。エレクトロポレーションした細胞を5mlのES培地に懸濁し、あらかじめフィーダー細胞をまいた60mm組織培養用プラスチックシャーレ（コーニング）1枚に播種した。2日後に細胞をトリプシン処理し、フィーダー細胞をまいた100mmシャーレ3枚にそれぞれシャーレ1枚当たり100、200、300細胞となるように再度播種した。ジーンパルサーの設定（抵抗器ユニット接続、抵抗値無限大）のみ変更した条件でも同様に行った。7日後に生じたコロニー計96個をピックアップし、トリプシン処理した後2つに分け、フィーダー細胞をまいた48穴プレート及びゼラチンコート処理のみを行った48穴プレート2枚にそれぞれ播種した。後者は300μ/mlのG418（GENETICIN、シグマ）を含む培地で3日間培養し、その生存率でG418耐性を判定した。その結果6クローンがG418存在下で死滅した。これらのG418感受性株を35mmシャーレでコンフルーエントになるまで増殖させ、その4/5を0.5mlの保存用培地（ES培地+10%DMSO〈シグマ〉）に懸濁し、-80℃にて凍結保存した。残りの1/5は12穴ゼラチンコートプレートに播種し、2日間培養して（実施例2）に示した方法でゲノムDNAを取得した。これらのG418感受性TT2F細胞株ゲノムDNAを制限酵素EcoRI（宝酒造）で消化し、アガロースゲル電気泳動で分離後サザンブロットを行ない、G418耐性遺伝子を含むpSTneoB由来3.2kb XhoI断片（プローブA）でG418耐性遺伝子の除去を確認した。その結果、#131-3において観察される、プローブAとハイブリダイズするバンドが、感受性株においては全く検出されなかった。これらの結果より、取得されたG418感受性株において確かにG418耐性マーカー遺伝子が除去されていることが確認された。さらに上記と同様な方法でpBS185DNAをEcoRI消化したプローブBを用いてサザン解析を行った結果、これらG418感受性株にプローブBとハイブリダイズする特異的なバンドは検出されなかったことから、Creレコンビナーゼを含むpBS185は感受性株染色体に挿入されていないと考えられる。すなわち、これらの感受性株は実施例48-4に示した抗体軽鎖ノックアウトベクター（G418耐性遺伝子の両側にloxP配列が存在する）による形質転換を行うことができる。

（実施例53）抗体重鎖欠損ES細胞株へのヒト14番染色体（抗体重鎖）の導入

（実施例52）で得られた、内在性の抗体重鎖を欠損するマウスES細胞株（TT2F由来、G418感受性）に（実施例9）で示した通りにG418耐性遺伝子によりマーキ

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ングされたヒト14番染色体（抗体重鎖遺伝子を含む）をミクロセル法により導入する。得られるG418耐性株においてPCR解析等（実施例9）によりヒト抗体重鎖遺伝子を含むヒト14番染色体（断片）の保持が確認される。

（実施例54）ヒト14番染色体（断片）を保持する抗体重鎖欠損ES細胞株へのヒト2番染色体断片あるいは22番染色体の導入

（実施例53）で得られた、ヒト14番染色体断片を保持する抗体重鎖欠損マウスES細胞株（G418耐性）に（実施例18、35）で示した通りにピューロマイシン耐性遺伝子によりマーキングされたヒト2番染色体断片（抗体軽鎖κ遺伝子を含む）あるいはヒト22番染色体（抗体軽鎖λ遺伝子を含む）をミクロセル法により導入する。得られるピューロマイシン、G418二重薬剤耐性株においてPCR解析等（実施例18、35）によりヒト14番染色体（断片）及び2番染色体断片あるいは22番染色体（断片）の保持が確認される。

（実施例55）ヒト抗体重鎖遺伝子を含む14番染色体（断片）を保持する内在性抗体重鎖欠損マウスES細胞からのキメラマウス作成

（実施例53）で取得されるヒト抗体重鎖遺伝子を含む14番染色体（断片）を保持する内在性抗体重鎖遺伝子欠損マウスES細胞株からのキメラマウス作成は（実施例10）で示した方法により行う。ここで得られるキメラマウスにおいては、ES細胞株由来のB細胞で産生されるヒト抗体重鎖が（実施例14）で示した方法により検出される。また、このES細胞由来のB細胞において機能的な抗体重鎖遺伝子は導入染色体上のヒト由来のもののみであるので、ES細胞株由来B細胞の多くはヒト抗体重鎖を産生する。

（実施例56）ヒト14番＋2番、14番＋22番染色体（断片）を保持する内在性抗体重鎖欠損マウスES細胞からのキメラマウス作成

（実施例54）で取得されるヒト14番＋2番、14番＋22番染色体（断片）を保持する内在性抗体重鎖遺伝子欠損マウスES細胞株からのキメラマウス作成は（実施例19、36）等で示した方法により行う。ここで得られるキメラマウスにおいては、ES細胞株由来のB細胞でヒト抗体重鎖及び軽鎖κあるいは軽鎖λが（実施例14、23、32）で示した方法により検出される。また、（実施例55）と同様にこのES細胞由来のB細胞において機能的な抗体重鎖遺伝子は導入染色体上のヒト由来のも

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ののみであるので、ES細胞由来B細胞の多くはヒト抗体重鎖を産生する。さらに、（実施例37、38）で示したように重鎖、軽鎖が共にヒト由来である完全なヒト抗体分子が検出される。

（実施例57）ヒト14番＋2番、14番＋22番染色体（断片）を保持する内在性抗体重鎖欠損マウスES細胞由来キメラマウスからのヒト抗体産生ハイブリドーマ取得

（実施例15、25、34）と同様に（実施例56）で作成されるキメラマウスに目的とする抗原で免疫し、脾臓を取り出し、ミエローマ細胞と細胞融合し、ハイブリドーマを作成する。1～3週間培養し培養上清をELISA法で解析する。ELISA法は（実施例14、15、21、24、25、33、34、37、38）に示した方法で行ない、ヒト抗体陽性及びヒト抗体陽性かつ免疫した抗原特異的クローンを得る。

（実施例58）抗体重鎖欠損ホモ接合体マウスES細胞からの抗体軽鎖遺伝子破壊株の取得

（実施例52）で取得した抗体重鎖欠損ホモ接合体TT2F細胞株（G418感受性）においてさらに抗体軽鎖遺伝子を破壊した相同組換え体を以下の手順で取得する。（実施例48-4）で作成した抗体軽鎖ターゲッティングベクターを制限酵素KpnI（宝酒造）で線状化し、（相沢慎一、バイオマニュアルシリーズ8,ジーンターゲティング，羊土社，1995）の方法に従い上記TT2F細胞株（G418感受性）へ導入する。7～9日後に生じたコロニーをピックアップし、（実施例49）に示した方法で凍結保存、ゲノムDNAを取得する。G418耐性株ゲノムDNAを制限酵素EcoRIとNotI（宝酒造）で消化し、アガロースゲル電気泳動で分離後サザンブロット解析を行ない、（実施例48-4）に示したプローブで相同組換え体を検出する。

（実施例59）抗体軽鎖相同組換え体からの2重破壊株の取得

（実施例58）で得られたTT2F抗体軽鎖相同組換え体（かつ抗体重鎖欠損ホモ接合体）について軽鎖両側アレルの破壊株を以下の手順により取得する。（実施例51）と同様な方法で高濃度G418耐性株を取得し、凍結保存、DNA取得を行なう。高濃度G418耐性株ゲノムDNAを制限酵素EcoRIとNotI（宝酒造）で消化し、アガロースゲル電気泳動で分離後サザンブロットを行ない、（実施例48-4）に示したプローブで両側アレルの破壊された株を検出する。

（実施例60）抗体軽鎖欠損ホモ接合体（かつ抗体重鎖欠損ホモ接合体）TT2F細胞

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株からのG418耐性マーカー遺伝子の除去

（実施例59）で取得された抗体軽鎖両側アレル破壊株（高濃度G418耐性株）のG418耐性マーカー遺伝子を（実施例52）で示した手順により除去する。G418耐性マーカー遺伝子の両側に挿入したloxP配列（実施例48-1）の間で部位特異的組換えを起こすCreレコンビナーゼ遺伝子を含む発現ベクターpBS185（BRL）を（実施例52）の方法に従い上記の株へ導入する。（実施例52）と同様に得られるG418感受性株を35mmシャーレでコンフルーエントになるまで増殖させ、その4/5を0.5mlの保存用培地（ES培地+10%DMSO<シグマ>）に懸濁し、-80℃にて凍結保存する。残りの1/5は12穴ゼラチンコートプレートに播種し、2日間培養して（実施例2）に示した方法でゲノムDNAを取得する。これらのG418感受性TT2F細胞ゲノムDNAを制限酵素EcoRI（宝酒造）で消化し、アガロースゲル電気泳動で分離後サザンブロットを行ない、G418耐性遺伝子を含むpSTneoB由来3.2kbXhoI断片をプローブとし、G418耐性遺伝子の除去を確認する。

（実施例61）抗体重鎖、軽鎖欠損ES細胞株へのヒト14番染色体（抗体重鎖）の導入

（実施例60）で得られた、内在性の抗体重鎖及び軽鎖の両者を欠損するマウスES細胞株（TT2F由来、G418感受性）に（実施例9）で示した通りにG418耐性遺伝子によりマーキングされたヒト14番染色体（ヒト抗体重鎖遺伝子を含む）をミクロセル法により導入する。得られるG418耐性株においてPCR解析等（実施例9）によりヒト抗体重鎖遺伝子を含むヒト14番染色体（断片）の保持が確認される。

（実施例62）抗体重鎖、軽鎖欠損かつヒト14番染色体（抗体重鎖）を保持するES細胞株へのヒト2番染色体（軽鎖κ）の導入

（実施例61）で得られた、内在性抗体重鎖及び軽鎖欠損かつヒト14番染色体を保持するマウスES細胞株（TT2F由来、G418耐性）に（実施例18）で示した通りにピューロマイシン耐性遺伝子でマーキングされたヒト2番染色体（ヒト抗体軽鎖κ遺伝子を含む）部分断片をミクロセル法により導入する。得られるピューロマイシン、G418二重薬剤耐性株においてPCR解析等（実施例18）によりヒト14番染色体（断片）及び2番染色体断片の保持が確認される。

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（実施例63）抗体重鎖、軽鎖欠損かつヒト14番染色体（抗体重鎖）を保持するES細胞株へのヒト22番染色体（軽鎖λ）の導入

（実施例61）で得られた、内在性抗体重鎖及び軽鎖欠損かつヒト14番染色体を保持するマウスES細胞株（TT2F由来、G418耐性）に（実施例35）で示した通りにピューロマイシン耐性遺伝子でマーキングされたヒト22番染色体（ヒト抗体軽鎖λ遺伝子を含む）をミクロセル法により導入する。得られるピューロマイシン、G418二重耐性株においてPCR解析等（実施例35）によりヒト14番染色体（断片）及び22番染色体（断片）の保持が確認される。

（実施例64）ヒト2番（抗体軽鎖κ）、14番（抗体重鎖）、22番（抗体λ鎖）染色体あるいはその部分断片を同時に保持する内在性抗体重鎖、軽鎖欠損マウスES細胞の取得

3種のヒト染色体を同時に保持するマウスES細胞を得るために、ヒト2番あるいは22番染色体をブラストサイジン耐性（Izumiら、Exp. Cell. Res., 197, 229, 1991）、ハイグロマイシン耐性（Windら、Cell, 82, 321-, 1995）等のマーカー遺伝子の挿入によりマーキングする。これは（実施例16、26）に示した方法により行う。（実施例62）で得られた、内在性抗体重鎖及び軽鎖欠損でありかつヒト14番染色体（断片）及び2番染色体部分断片を保持するマウスES細胞株（TT2F由来、G418耐性、ピューロマイシン耐性）に（実施例9）で示した方法と同様にブラストサイジン耐性あるいはハイグロマイシン耐性等でマーキングされたヒト22番染色体（ヒト抗体軽鎖λ遺伝子を含む）を導入する。ES細胞培養用のフィーダー細胞はそれぞれの選択マーカーに適したものを使用する。ハイグロマイシン耐性マーカーを使用する場合は、そのマーカーを保持し、発現するトランスジェニックマウス系統より得た初代培養繊維芽細胞（Johnsonら、Nucleic Acids Research, vol. 23, No. 7, 1273-, 1995）を使用する。得られるG418、ピューロマイシン、ハイグロマイシン（あるいはブラストサイジン）三重耐性株は上記3種のヒト染色体（断片）を保持していることがPCR解析等（実施例9、18、35）により確認される。（実施例63）で得られた内在性抗体重鎖及び軽鎖欠損でありかつヒト14番染色体（断片）及び22番染色体部分断片を保持するマウスES細胞株（TT2F由来、G418耐性、ピューロマイシン耐性）へのハイグロマイシンあるいは

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ブラストサイジン耐性遺伝子でマーキングされたヒト2番染色体断片の導入も同様に行う。

（実施例65）ヒト抗体遺伝子（重鎖＋軽鎖）を含む染色体（断片）を保持する内在性抗体重鎖、軽鎖遺伝子欠損マウスES細胞からのキメラマウス作成

（実施例61、62、63、64）で取得されるヒト抗体遺伝子を含む染色体（断片）を保持する内在性抗体重鎖、軽鎖遺伝子欠損マウスES細胞株からのキメラマウス作成は（実施例10）等で示した方法により行う。ここで得られるキメラマウスにおいては、宿主胚由来のB細胞で産生されるマウス抗体とES細胞株由来のB細胞で主に産生されるヒト抗体が（実施例14、23、32）で示した方法により検出される。また、このES細胞由来のB細胞において機能的な抗体重鎖及び軽鎖κ遺伝子は導入染色体上のヒト由来のもののみであるので、ES細胞由来のB細胞の多くはヒト抗体重鎖及び軽鎖κを産生する（ Lonbergら, Nature, 368, 856-, 1994）。さらに、（実施例37、38）で示した方法により重鎖、軽鎖が共にヒト由来である完全なヒト抗体分子が検出される。

（実施例66）ヒト抗体遺伝子（重鎖＋軽鎖）を含む染色体（断片）を保持する内在性抗体重鎖、軽鎖遺伝子欠損マウスES細胞由来キメラマウスからのヒト抗体産生ハイブリドーマ取得

（実施例25）と同様に（実施例65）で得られるキメラマウスに目的とする抗原で免疫し、脾臓を取り出し、ミエローマ細胞と細胞融合し、ハイブリドーマを作成する。1～3週間培養し培養上清をELISA法で解析する。ELISA法は（実施例14、15、21、22、23、24、25、33、34、37、38）に示した方法で行ない、ヒト抗体陽性及びヒト抗体陽性かつ免疫した抗原特異的クローンを得る。

（実施例67）重鎖遺伝子欠損宿主胚とのキメラマウス作成

（実施例49）で作成した内在性抗体重鎖片側アレル欠損TT2F細胞株由来キメラマウスの子孫のうち野性色を示すものについてサザン解析あるいはＰＣＲ（実施例49）等により欠損アレルを保持する個体を選抜する（期待される確率は1/2である）。これらの抗体重鎖欠損ヘテロ接合体の雌雄個体の交配により生まれる子マウスについてサザン解析（実施例49参照）、リンパ球表面でのμ鎖発現（Kitamuraら, Nature, 350, 423-, 1991）等の解析を行い、両側アレルが欠損し、自身

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