HUMANIZED IMMUNOGLOBULIN REACTIVE WITH B7 MOLECULES AND METHODS OF TREATMENT THEREWITH

RELATED APPLICATION(S)

This application is a Continuation-In-Part of Application No. 09/339,596, filed June 24, 1999, which is a Continuation-In-Part of 09/249,011, filed February 12,1999, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Antigen specific T-cell activation and the initiation of an immune response depend initially on the interaction of the T-cell receptor (TCR) complex with the peptide/major histocompatibility complex (MHC) present on antigen presenting cells (APC). B7 molecules, B7-1 and B7-2, are molecules which are present on APCs. A "co-stimulatory" signal, provided by the interaction of B7-1 and B7-2 on the APC with their ligands CD28 and CTLA4 on T-cells, is required to complete T-cell activation and the subsequent regulation of an immune response. A need exists to regulate the B7-1 and B7-2 pathway, referred to as the B7:CD28/CTLA4 pathway. A further need exists to develop treatments for diseases that are affected by this pathway.

SUMMARY OF THE INVENTION

The invention relates to humanized immunoglobulins having binding specificity for B7 molecules. In particular, the invention includes a humanized immunoglobulin having binding specificity for B7-2 or B7-1, wherein the immunoglobulin comprises an antigen binding region of non-human origin (e.g. rodent) and at least a portion of human origin (e.g. a human constant region such as an IgG constant region and/or a human framework region). In one embodiment, the human constant region of either the humanized anti-B7-2 or humanized anti-B7-1 immunoglobulin can also contain a mutation that reduces the effector function of the humanized immunoglobulin. The humanized B7-2 immunoglobulin described

herein can compete with murine 3D1 for binding to B7-2. Similarly, the humanized B7-1 immunoglobulin described herein can compete with murine 1F1 for binding to B7-1. In particular embodiments, the antigen binding region of the humanized B7-2 immunoglobulin is derived from the 3D1 monoclonal antibody, and the antigen binding region of the humanized B7-1 immunoglobulin is derived from the 1F1 monoclonal antibody.

The humanized immunoglobulins of the present invention can comprise a constant region of human origin and an antigen binding region, wherein the antigen binding region of non-human origin comprises one or more complementarity determining regions (CDRs) of rodent origin (e.g., derived from 3D1 monoclonal antibody) that binds to B7-2, or one or more CDRs of rodent origin (e.g., derived from 1F1 monoclonal antibody) that binds to B7-1, and the portion of an immunoglobulin of human origin is derived from a human framework region (FR).

The antigen binding region of the humanized B7-2 antibody can further comprise a light chain and a heavy chain, wherein the light and heavy chain each have three CDRs derived from the 3D1 antibody. The FR of the light chain for the humanized B7-2 antibody can be derived, for example, from the light chain of the human H2F antibody and the heavy chain can be derived, for example, from the heavy chain of the human I2R antibody. In a particular embodiment, the invention is a humanized immunoglobulin having binding specificity for B7-2 that is derived from the cell line deposited with the American Type Culture Collection (A.T.C.C.), Accession No. CRL-12524.

The antigen binding region of the humanized B7-1 antibody can comprise a light chain and a heavy chain, wherein the light and heavy chain each have three CDRs derived from the 1F1 antibody. The FR of the humanized B7-1 light and heavy chain can be derived, for example, from the light and heavy chains of the human III-2R antibody. In a particular embodiment, the invention is a humanized immunoglobulin having binding specificity for B7-1 that is derived from the cell line deposited with the American Type Culture Collection (A.T.C.C.), Accession No. PTA-263.

The invention also embodies a humanized immunoglobulin having a binding specificity for B7-1 or B7-2 comprising a heavy chain and/or a light chain. In one embodiment, the humanized immunoglobulin has binding specificity for B7-2 and the light chain comprises at least one CDR (e.g., CDR1, CDR2 and CDR3) derived from an antibody of non-human origin which binds B7-2 and a FR derived from a light chain of human origin (e.g., H2F antibody); and the heavy chain comprises at least one CDR (e.g., CDR1, CDR2 and CDR3) derived from an antibody of non-human origin which binds B7-2 and a FR region derived from a heavy chain of human origin (e.g., the human I2R antibody). In another embodiment, the humanized immunoglobulin has binding specificity for the B7-1 and the light and/or heavy chain comprise at least one CDR (e.g., CDR1, CDR2 and CDR3) derived from an antibody of non-human origin which binds B7-1 and a FR derived from a light and/or heavy chain of human origin (e.g., III-2R). The immunoglobulins can further comprise CDR1, CDR2 and CDR3 for the light or heavy chain having the amino acid sequence set forth herein or an amino acid substantially the same as the amino acid sequence such that the antibody specifically binds to B7-2 or B7-1. The present invention also relates to humanized immunoglobulin light chains and to humanized immunoglobulin heavy chains. The invention further relates to isolated nucleic acids comprising a sequence which encodes the humanized immunoglobulins of the present invention (e.g., a single chain antibody), and the invention also relates to isolated nucleic acids that comprise a sequence which encodes the B7-2 or B7-1 humanized immunoglobulin light chain or heavy chain.

One embodiment of the invention is a humanized immunoglobulin light chain having binding specificity for B7-2 comprising CDR1, CDR2 and/or CDR3 of the light chain of murine 3D1 antibody, and a human light chain FR (e.g., H2F antibody). Similarly, the invention also comprises a humanized B7-1 immunoglobulin light chain having binding specificity for B7-1 comprising CDR1, CDR2 and/or CDR3 of the light chain of the murine 1F1 antibody, and a human light chain FR (e.g., III-2R antibody). Another embodiment is a humanized B7-2 or B7-1 immunoglobulin light chain that comprises a variable region shown in Figure

2B (SEQ ID NO: 8) or the variable region shown in Figure 7B (SEQ ID NO: 28). The invention also relates to an isolated nucleic acid sequence that encodes a humanized variable light chain specific for B7-2 or B7-1 that comprises a nucleic acid sequence shown in Figure 2B (SEQ ID NO: 7) or Figure 7B (SEQ ID NO.: 27), respectively, a nucleic acid sequence that encodes the amino acid sequence shown in Figure 2B (SEQ ID NO: 8) or Figure 7B (SEQ ID NO: 28), respectively, a nucleic acid sequence which hybridizes thereto under stringent hybridization conditions, or a nucleic acid sequence which is the complement thereof.

Another embodiment of the invention is a humanized immunoglobulin heavy chain that is specific for B7-2 and comprises CDR1, CDR2 and/or CDR3 of the heavy chain of the 3D1 antibody, and a human heavy chain FR (e.g., I2R antibody). Similarly, the invention also relates to a B7-1 humanized immunoglobulin heavy chain that is specific for B7-1 and comprises CDR1, CDR2, and/or CDR3 of the heavy chain of the 1F1 antibody, and a human heavy chain FR (e.g., III-2R antibody). The invention pertains to a humanized immunoglobulin heavy chain that comprises a variable region shown in Figure 2A (SEQ ID NO: 6) or Figure 7A (SEQ ID NO: 26). The invention also pertains to an isolated nucleic acid sequence that encodes a humanized variable heavy chain specific for B7-2 that comprises a nucleic acid sequence shown in Figure 2A (SEQ ID NO: 5), an isolated nucleic acid sequence that encodes the amino acid sequence shown in Figure 2A (SEQ ID NO: 6), a nucleic acid sequence which hybridizes thereto under stringent hybridization conditions, or a nucleic acid sequence which is the complement thereof. The invention relates to an isolated nucleic acid sequence that encodes a humanized variable heavy chain specific for B7-1 that comprises a nucleic acid sequence shown in Figure 7A (SEQ ID NO: 25), an isolated nucleic acid sequence that encodes the amino acid sequence shown in Figure 7A (SEQ ID NO: 26), a nucleic acid sequence which hybridizes thereto under stringent hybridization conditions, or a nucleic acid sequence which is the complement thereof.

In particular, an embodiment of the invention is a humanized immunoglobulin which specifically binds to B7-2 and comprises a humanized light

chain comprising three light chain CDRs from the mouse 3D1 antibody, and a light chain variable region framework sequence from a human immunoglobulin light chain, and a humanized heavy chain comprising three heavy chain CDRs from the mouse 3D1 antibody, and a heavy chain variable region framework sequence from a human immunoglobulin heavy chain. The mouse 3D1 antibody can further have a mature light chain variable domain, such as the mature light chain variable domain shown in Figure 1B (SEQ ID NO.: 4) and a mature heavy chain variable domain such as the mature heavy chain variable region shown in Figure 1A (SEQ ID NO.: 2).

Another embodiment of the invention is a humanized immunoglobulin which specifically binds to B7-1 and comprises a humanized light chain comprising three light chain CDRs from the mouse 1F1 antibody, and a light chain variable region framework sequence from a human immunoglobulin light chain, and a humanized heavy chain comprising three heavy chain CDRs from the mouse 1F1 antibody, and a heavy chain variable region framework sequence from a human immunoglobulin heavy chain. Similarly, the mouse 1F1 antibody can further have a mature light chain variable domain, such as the mature light chain variable domain shown in Figure 6B (SEQ ID NO.: 24) and a mature heavy chain variable domain such as the mature heavy chain variable region shown in Figure 6A (SEQ ID NO.: 22).

The invention includes an expression vector that comprises a fused gene which encodes humanized B7-1 and/or B7-2 immunoglobulin light and/or heavy chains. The gene comprises a nucleotide sequence encoding a CDR derived from a light and/or heavy chain of a non-human antibody having binding specificity for B7-2 and/or B7-1 (e.g., murine 3D1 or 1F1 antibody, respectively) and a FR derived from a light and/or heavy chain of human origin.

The present invention also relates to a host cell comprising a nucleic acid of the present invention, including one or more constructs comprising nucleic acid of the present invention. In one embodiment, the invention encompasses a host cell comprising a first B7-2 recombinant nucleic acid that encodes a humanized B7-2 immunoglobulin light chain and a second B7-2 recombinant nucleic acid that

encodes a humanized B7-2 immunoglobulin heavy chain. The first B7-2 nucleic acid comprises a nucleotide sequence encoding at least one CDR derived from the light chain of murine 3D1 antibody and a FR derived from a light chain of human origin. The second B7-2 nucleic acid comprises a nucleotide sequence encoding at least one CDR derived from the heavy chain of murine 3D1 antibody and a FR derived from a heavy chain of human origin. In another embodiment, the invention encompasses a host cell comprising a first B7-1 recombinant nucleic acid that encodes a humanized B7-1 immunoglobulin light chain and a second B7-1 recombinant nucleic acid that encodes a humanized B7-1 immunoglobulin heavy chain. The first B7-1 nucleic acid comprises a nucleotide sequence encoding at least one CDR derived from the light chain of murine 1F1 antibody and a FR derived from a light chain of human origin. The second B7-1 nucleic acid comprises a nucleotide sequence encoding at least one CDR derived from the heavy chain of murine 1F1 antibody and a FR derived from a heavy chain of human origin. The invention further relates to a host cell comprising a vector or a nucleic acid that encodes the humanized B7-1 and/or B7-2 immunoglobulins, as described herein.

The invention further pertains to methods of preparing humanized immunoglobulins that comprise maintaining a host cell that encodes a humanized immunoglobulin that is specific for B7-2 or B7-1, as described herein, under conditions appropriate for expression of a humanized immunoglobulin, wherein a humanized immunoglobulin chain (one or more) are expressed and a humanized immunoglobulin is produced. The method further comprises the step of isolating the humanized B7-1 or B7-2 immunoglobulin.

The invention encompasses methods of inhibiting the interaction of a first cell bearing a B7-2 receptor with a second cell bearing B7-2 comprising contacting the second cell with an effective amount of a humanized B7-2 immunoglobulin, as described herein. The invention also encompasses methods of inhibiting the interaction of a first cell bearing a B7-1 receptor with a second cell bearing B7-1 comprising contacting the second cell with an effective amount of a humanized B7-1 immunoglobulin, as described herein. Accordingly, the invention relates to

inhibiting both a B7-1 and B7-2 receptor with B7-1 and B7-2 ligands, comprising contacting the cells having the B7-1 and B7-2 receptors with an amount of humanized anti-B7-1 and B7-2 immunoglobulins. Thus, the invention pertains to various methods of treatment. The invention includes a method for modulating an immune response of an individual (e.g., patient), or treating an individual having a transplanted organ, tissue, cell or the like comprising administering an effective amount of a humanized B7-1 and/or B7-2 immunoglobulin, as described herein, with or without a carrier (e.g., pharmaceutical carrier), wherein the immune response is modulated. The invention pertains to treating acute and/or chronic transplant rejection, for example, for a prolonged periods of time (e.g., days, months, or years). The invention also pertain to methods of treating a disease associated with modulation of the B7-2 and/or B7-1 molecules (e.g., autoimmune diseases, infectious diseases, inflammatory disorders, systemic lupus erythematosus, diabetes mellitus, asthma, insulitis, arthritis, inflammatory bowel disease, inflammatory dermatitis, and multiple sclerosis), comprising administering to an individual (e.g., patient) an effective amount (e.g., a therapeutically effective amount) of the B7-2 and/or B7-1 humanized immunoglobulins, as described herein, with or without a carrier. Accordingly, the invention encompasses a pharmaceutical composition comprising the B7-1 and/or B7-2 humanized immunoglobulins, as described herein.

The invention also embodies methods of making a humanized immunoglobulin specific to B7-2 from a murine antibody specific to B7-2, and/or methods of making a humanized immunoglobulin specific to B7-1 from a murine antibody specific to B7-1. The methods comprise determining or ascertaining the CDRs of an antibody of non-human origin (e.g., murine origin) which has binding specificity for B7-2 or B7-1; obtaining a human antibody having a framework region amino acid sequence suitable for grafting of the CDRs, and grafting the CDRs of an antibody of non-human origin into the FR of the human antibody.

The invention also relates to methods for determining the presence or absence of B7-2 and/or B7-1 in a sample. The methods comprise obtaining the sample to be tested, contacting the sample with the humanized antibody specific to

B7-2 and/or B7-1, or a fragment thereof, sufficiently to allow formation of a complex between B7-2 and/or B7-1 and the anti-B7-2 and/or anti-B7-1 antibody, respectively, and detecting the presence or absence of the complex formation. The presence of the complex indicates the presence of B7-2 and/or B7-1 in the sample.

The invention relates to methods for treating an individual having a disease comprising administering an amount (e.g., therapeutically effective amount) of a humanized immunoglobulin specific to B7-1 and/or an amount (e.g., therapeutically effective amount) of a humanized immunoglobulin specific to B7-2. The diseases, as described herein, include, for example, autoimmune diseases, infectious diseases, asthma, inflammatory disorders, systemic lupus erythematosus, diabetes mellitus, insulitis, arthritis, inflammatory bowel disease, inflammatory dermatitis, and multiple sclerosis. This method also pertains to modulating the immune response of an individual having a transplanted organ, tissue, cell or the like comprising administering an effective amount of a humanized immunoglobulin that binds to B7-1 and/or a humanized immunoglobulin that binds to B7-2. This method further includes administering a drug that is used to modulate the immune response of an individual having a transplanted organ, tissue, cell or the like. The drug can be, for example, methotrexate, rapamycin, cyclosporin, steroids, anti-CD40 pathway inhibitors (e.g., anti-CD40 antibodies, anti-CD40 ligand antibodies and small molecule inhibitors of the CD40 pathway), transplant salvage pathway inhibitors (e.g., mycophenolate mofetil (MMF)), IL-2 receptor antagonists (e.g., Zeonpax® from Hoffmann-la Roche Inc., and Simulet from Novartis, Inc.) and analogs thereof. These drugs can be administered prior to, with or after administration of the humanized immunoglobulins.

The invention also pertains to methods for transplanting cells (e.g., bone marrow, blood cells, blood components and other cells) to an individual in need thereof comprising obtaining cells (e.g., bone marrow, or blood cells or components) from a donor, and contacting the cells with an immunoglobulin specific to B7-1 and/or an immunoglobulin specific to B7-2, and recipient cells, thereby obtaining a mixture. The immunoglobulins and the recipient cells are maintained for a period of

time sufficient for tolerance induction. The mixture (referred to as a bone marrow composition or blood cell composition) is then introduced into the individual. The recipient cells can be lymphocytes (e.g. lymphocytes that express class 1 antigens (MHCI) or peripheral blood lymphocyte (PBL)). Instead of using recipient cells, the method also comprise utilizing tissue, organs or cells that express MHC Class I antigens, B7-1 and/or B7-2 molecules. The cells can be engineered to express recipient molecules. The cells from the donor can be bone marrow cells, or cells/components from blood (e.g., stem cells or immature cells). The B7 immunoglobulins are in contact with the donor bone marrow and the recipient cells for a period of time that is long enough to induce tolerance induction (e.g., about 1 to 96 hours, and, preferably about 36-48 hours). An individual in need of such a transplant is one who has a disease that is benefitted by or treatable with a bone marrow transplant. Such diseases, for example, are proliferative diseases (e.g. leukemia, lymphoma and cancer), anemia (e.g. sickle-cell anemia, thalassemia, and aplastic anemia), inborn errors of metabolism, congenital immunodeficiency diseases, and myeloid dysplasia syndrome (MDS). The method further includes administering to the individual a drug that is used to modulate the immune response (e.g., methotrexate; rapamycin; cyclosporin; steroids; anti-CD40 pathway inhibitors such as anti-CD40 antibodies, anti-CD40 ligand antibodies and small molecule inhibitors of the CD40 pathway; transplant salvage pathway inhibitors such as mycophenolate mofetil (MMF), IL-2 receptor antagonists such as Zeonpax® from Hoffmann-la Roche Inc., and Simulet from Novartis, Inc.; or analogs thereof).

In particular, the invention includes methods for transplanting bone marrow to an individual having a disease (e.g., proliferative diseases such as leukemia, lymphoma, cancer; anemia such as sickle-cell anemia, thalassemia, and aplastic anemia; inborn errors of metabolism; congenital immunodeficiency diseases; and myeloid dysplasia syndrome) that is treated with a bone marrow transplant comprising obtaining bone marrow from a donor, and contacting the bone marrow with immunoglobulins specific to B7-1 and/or an immunoglobulin specific to B7-2, and recipient cells (e.g., lymphocyte). The bone marrow, immunoglobulin(s) and

recipient cells are in contact for a period of time sufficient for tolerance induction (e.g., about 1-96 hours, preferably about 36-48 hours). The method then comprises re-introducing the treated bone marrow to the individual. The method further includes administering to the individual a drug that is used to modulate the immune response (e.g., methotrexate; rapamycin; cyclosporin; steroids; anti-CD40 pathway inhibitors such as anti-CD40 antibodies, anti-CD40 ligand antibodies and small molecule inhibitors of the CD40 pathway; transplant salvage pathway inhibitors such as mycophenolate mofetil (MMF), IL-2 receptor antagonists such as Zeonpax® from Hoffmann-la Roche Inc., and Simulet from Novartis, Inc.; or analogs thereof).

The invention also includes methods of treating a transplant recipient or preventing transplant rejection in a transplant recipient by administering to the recipient an effective amount of an immunoglobulin specific to B7-1 and/or an effective amount of an immunoglobulin specific to B7-2. The immunoglobulin specific to B7-1 is administered in an amount between about 1 mg/kg and 100 mg/kg, and the immunoglobulin specific to B7-2 is administered in an amount between about 1 mg/kg and about 100 mg/kg. The immunoglobulins specific to B7-1 and B7-2 can be administered on the day the recipient receives the transplantation (e.g., in an amount between about 1 mg/kg and about 25 mg/kg), and can also be administered periodically (e.g., daily, weekly or monthly) after the recipient receives the transplantation (e.g., in an amount between about 1 mg/kg and about 5 mg/kg). The method further includes administering a composition that is used in transplant rejection therapy, such as calcineurin inhibitors (e.g., cyclosporin A or FK506), steroids (e.g., methyl prednisone or prednisone), or immunosuppressive agents that arrest the growth of immune cells (e.g., rapamycin), anti-CD40 pathway inhibitors (e.g., anti-CD40 antibodies, anti-CD40 ligand antibodies and small molecule inhibitors of the CD40 pathway) transplant salvage pathway inhibitors (e.g., mycophenolate mofetil (MMF)), IL-2 receptor antagonists (e.g., Zeonpax® from Hoffmann-la Roche Inc., and Simulet from Novartis, Inc.), or analogs thereof. Also, the present invention relates to a method of transplanting cells, tissue or organs to an individual in need thereof, by transplanting the cells,

tissue or organs; and administering an effective amount of an immunoglobulin specific to B7-1 and an effective amount of an immunoglobulin specific to B7-2 to the individual, as described above.

The invention also includes methods of decreasing an antibody response to an antigen in a mammal by administering to the individual an effective amount of a humanized immunoglobulin specific to B7-1 and/or a humanized immunoglobulin specific to B7-2, in the presence of the antigen. The method further includes administering the antigen (e.g., tetanus toxoid, Factor VIII, Factor IX, insulin, growth hormone, or a gene delivery vector) to the individual. The antigen can be administered in a polypeptide form or in a nucleic acid form (e.g., gene therapy with delivery through adeno-associated viruses (AAV), retroviruses, naked DNA vectors, etc.).

Advantages of the invention include the ability to regulate or modulate the B7 co-stimulatory pathway. Manipulation of this co-stimulatory pathway with humanized anti-B7-2 and/or anti-B7-1 antibodies provide methods of treatments for various diseases. The humanized anti-B7-2 and anti-B7-1 antibodies maintain about the same specificity for respective B7 molecule as the corresponding murine antibody, but with a reduced immunogenicity in humans and an extended half-life, as compared with the murine counterpart. Accordingly, the invention can advantageously be used to treat immune-related diseases/disorders, or diseases in which the B7-2 and/or B7-1 molecules play an important role. Particularly, the invention relates to methods for treating autoimmune diseases, and methods for modulating the immune response for individuals with transplanted organs, tissue or cells.

BRIEF DESCRIPTION OF THE FIGURES

The foregoing and other embodiments, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying figures.

Figure 1A is a sequence listing of the heavy chain variable region nucleic acid and amino acid sequences (SEQ ID NOS: 1 and 2, respectively) of the murine 3D1 antibody, wherein the amino acid sequences of the CDRs (CDR1, CDR2 and CDR3) are underlined, and the first amino acid of the mature, heavy chain is double underlined.

Figure 1B is a sequence listing of the light chain variable region nucleic acid and amino acid sequences (SEQ ID NOS: 3 and 4, respectively) of the murine 3D1 antibody wherein, the nucleic and amino acid sequences of the CDRs (CDR1, CDR2 and CDR3) are underlined, and the first amino acid of the mature light chain is double underlined.

Figure 2A is a sequence listing of the heavy chain variable region nucleic acid and amino acid sequences (SEQ ID NOs: 5 and 6, respectively) of the humanized 3D1 antibody, wherein the nucleic and amino acid sequences of the CDRs (CDR1, CDR2 and CDR3), and underlined and the first amino acid of the mature heavy chain is double underlined.

Figure 2B contains the light chain variable region nucleic acid and amino acid sequences (SEQ ID NOs: 7 and 8, respectively) of the humanized 3D1 antibody, wherein the nucleic and amino acid sequences of CDR1, CDR2 and CDR3. The CDRs are underlined, and the first amino acid of the mature light chain is double underlined.

Figure 3 is a graph depicting the results of a competitive binding assay. The graph depicts the results of a competitive binding assay of murine or humanized anti-human B7-2 mAbs to CHO cells expressing rhB7-2 (CHO/hB7-2) on their surface. Increasing concentrations of unlabeled competitor antibodies were incubated with CHO/hB7-2 cells in the presence of radiolabeled tracer murine anti-human B7-2 mAb and the ratio of bound/free antibody was determined.

Figure 4 is a graph depicting the results of a direct binding assay of murine or humanized anti-human B7-2 mAbs to CHO/hB7-2 cells. Increasing concentrations of radiolabeled antibodies were incubated with CHO or CHO/hB7-2

cells and the amount of specific antibody bound to the CHO/hB7-2 cells was determined.

Figure 5 is a graph depicting the results of a T cell proliferation assay. Increasing concentrations of murine or humanized anti-human B7-2 mAbs were added to $CD28^+$ human T cells stimulated with PMA and CHO/hB7-2 cells and the inhibition of T cell proliferation by these mAbs was determined.

Figure 6A is a sequence listing of the heavy chain variable region nucleic acid and amino acid sequences (SEQ ID NOS: 21 and 22, respectively) of the murine 1F1 antibody, wherein the amino acid sequences of the CDRs (CDR1, CDR2 and CDR3) are underlined, and the first amino acid of the mature heavy chain is double underlined.

Figure 6B is a sequence listing of the light chain variable region nucleic acid and amino acid sequences (SEQ ID NOS: 23 and 24, respectively) of the murine 1F1 antibody wherein, the nucleic and amino acid sequences of the CDRs (CDR1, CDR2 and CDR3) are underlined, and the first amino acid of the mature light chain is double underlined.

Figure 7A is a sequence listing of the heavy chain variable region nucleic acid and amino acid sequences (SEQ ID NOs: 25 and 26, respectively) of the humanized 1F1 antibody (hu1F1), wherein the nucleic and amino acid sequences of the CDRs (CDR1, CDR2 and CDR3) are underlined, and the first amino acid of the mature heavy chain is double underlined.

Figure 7B contains the light chain variable region nucleic acid and amino acid sequences (SEQ ID NOs: 27 and 28, respectively) of the humanized 1F1 (hu1F1) antibody, wherein the nucleic and amino acid sequences of CDR1, CDR2 and CDR3. The CDRs are underlined, and the first amino acid of the mature light chain is double underlined.

Figure 8 is a graph depicting the results of a competitive binding assay. The graph depicts the results of a competitive binding assay of murine or humanized anti-human B7-1 mAbs to CHO transfected with rhB7-1 (CHO/hB7-1). Increasing concentrations of unlabeled competitor antibodies were incubated with CHO/hB7-1

cells in the presence of radiolabeled tracer humanized 1F1, and the ratio of bound/free antibody was determined.

Figure 9A is a graph showing the Scatchard analysis of the binding of mouse 1F1 antibodies to CHO cells transfected with rhB7-1. Radiolabeled mouse 1F1 antibodies were incubated with CHO cells transfected with rhB7-1, and the ratio of bound/free radioactivity was determined.

Figure 9B is a graph showing the Scatchard analysis of the binding of humanized 1F1 antibodies to CHO cells transfected with rhB7-1. Radiolabeled humanized 1F1 antibodies were incubated with CHO cells transfected with rhB7-1, and the ratio of bound/free radioactivity was determined.

Figure 10 is a graph depicting the results of a competitive binding assay of murine or humanized anti-human B7-1 mAbs to CHO expressing rhB7-1 (CHO/hB7-1) on their surface. Increasing concentrations of unlabeled competitor antibodies were incubated with CHO/hB7-1 cells in the presence of radiolabeled tracer murine anti-human B7-1 mAb and the ratio of bound/free antibody was determined.

Figure 11 is a graph depicting the results of a direct binding assay of murine or humanized anti-human B7-1 mAbs to CHO/hB7-1 cells. Increasing concentrations of radiolabeled antibodies were incubated with CHO or CHO/hB7-1 cells and the amount of specific antibody bound to the CHO/hB7-1 cells was determined.

Figure 12 is a graph depicting the results of a T cell proliferation assay. Increasing concentrations of murine or humanized anti-human B7-1 mAbs were added to $CD28^+$ human T cells stimulated with PMA and CHO/hB7-1 cells and the inhibition of T cell proliferation by these mAbs was determined.

Figure 13 is a graph depicting the results of a one way mixed lymphocyte reaction (MLR) assay. Fixed concentrations of murine or humanized anti-human B7-2 (IgG2.M3 isotype) or human CTLA4Ig were added to a mixture of human responder and stimulator PBLs and the proliferation of the responder PBLs was determined on days 3, 4, and 5 by the addition of radiolabeled thymidine.

Figure 14 is a graph depicting the results of a one way secondary MLR assay using PBLs from a primary MLR as responders and PBLs from the same or a different individual as in the primary MLR as stimulators. The humanized anti-human B7-1 mAb was added to the primary MLR only. Proliferation of the responder PBLs in the secondary MLR was determined on days 3, 4, and 5 by the addition of radiolabeled thymidine.

Figure 15 is a graph depicting the results of a one way secondary MLR assay using PBLs from a primary MLR as responders and PBLs from the same or a different individual as in the primary MLR as stimulators. The humanized anti-human B7-2 mAb (IgG2.M3 isotype) was added to the primary MLR only. Proliferation of the responder PBLs in the secondary MLR was determined on days 3, 4, and 5 by the addition of radiolabeled thymidine.

Figure 16 is a graph depicting the results of a one way secondary MLR assay using PBLs from a primary MLR as responders and PBLs from the same or a different individual as in the primary MLR as stimulators. The humanized anti-human B7-1 and B7-2 mAbs (IgG2.M3 isotype) were added to the primary MLR only. Proliferation of the responder PBLs in the secondary MLR was determined on days 3, 4, and 5 by the addition of radiolabeled thymidine.

Figure 17 is a graph showing the results from a one way primary MRL assay using PBLs as responders and irradiated "B" PBL stimulators. The responder and stimulator were treated with the humanized anti-B7-2 antibody, humanized anti-B7-1 mAbs, combined humanized anti-B7-1 and humanized anti-B7-2 mAbs, 10 μg/ ml CTLA4 Ig, 20 μg/ ml CTLA4 Ig, or control Ig. The culture proliferation was measured on days 3, 4, and 5 by the addition of radiolabeled thymidine.

Figure 18 is a graph showing the results from a one way primary MRL assay using PBLs as responders and irradiated "C" PBL stimulators. The responder and stimulator were treated with the humanized anti-B7-2 antibody, humanized anti-B7-1 mAbs, combined humanized anti-B7-1 and humanized anti-B7-2 mAbs, 10 μg/ ml CTLA4 Ig, 20 μg/ ml CTLA4 Ig, or control Ig. The culture proliferation was measured on days 3, 4, and 5 by the addition of radiolabeled thymidine.

Figure 19 is a graph showing the results from a one way secondary MRL assay using responders from the "B" stimulator primary MLR (See Figure 17) and fresh "B" stimulators. The responders and stimulators were treated with the humanized anti-B7-2 mAb, humanized anti-B7-1 mAb, combined humanized anti-B7-1 and humanized anti-B7-2 mAbs, 10 μg/ ml CTLA4 Ig, 20 μg/ ml CTLA4 Ig, or control Ig in the primary MLR only. There were no additions to the secondary MLR. The culture proliferation was measured on days 3, 4, 5 and 6 by the addition of radiolabeled thymidine.

Figure 20 is a graph showing the results from a one way secondary MRL assay using responders from the "B" stimulator primary MLR (See Figure 17) and fresh "C" stimulators. The responder and stimulator were treated with the humanized anti-B7-2 mAb, humanized anti-B7-1 mAb, combined humanized anti-B7-1 and humanized anti-B7-2 mAbs, 10 μg/ ml CTLA4 Ig, 20 μg/ ml CTLA4 Ig, or control Ig in the primary MLR only. There were no additions to the secondary MLR. The culture proliferation was measured on days 3, 4, 5 and 6 by the addition of radiolabeled thymidine.

Figure 21 is a graph showing the results from a one way secondary MRL assay using responders from the "B" stimulator primary MLR (See Figure 17) and fresh "B" or "C" stimulators. The responder and stimulator were treated with the humanized anti-B7-2 mAb, humanized anti-B7-1 mAb, combined humanized anti-B7-1 and humanized anti-B7-2 mAbs, 10 μg/ ml CTLA4 Ig, 20 μg/ ml CTLA4 Ig, or control Ig in the primary MLR only. There were no additions to the secondary MLR. The culture proliferation was measured on days 3, 4, 5 and 6 by the addition of radiolabeled thymidine. Figure 21 is a compilation of Figures 19 and 20.

Figure 22 is a graph depicting the anti-tetanus response (log titer) in non-human primates immunized with tetanus toxoid. Cynomolgus monkeys were immunized with purified tetanus toxoid and treated with humanized anti-B7-1 and humanized anti-B7-2 antibodies. Serum anti-tetanus antibody titers (IgM & IgG) were measured weekly over a 26 week period.

Figure 23 is a graph depicting the anti-tetanus response (log titer) in non-human primates immunized with tetanus toxoid. Cynomolgus monkeys were immunized with tetanus toxoid on Day 0 and treated with a single IV dose of humanized anti-B7-1 and humanized anti-B7-2 antibodies in combination or vehicle on Day 0. At Week 14 animals were immunized with tetanus toxoid a second time without additional humanized anti-B7-1 or humanized anti-B7-2 antibody treatment. Serum anti-tetanus antibody titers (IgM & IgG) were measured weekly over an 18 week period.

Figure 24 is a graph depicting the anti-tetanus response (log titer) in non-human primates immunized with tetanus toxoid. Cynomolgus monkeys were immunized with tetanus toxoid on Day 0 and treated with a single IV dose of humanized anti-B7-1 antibody alone or vehicle on Day 0. At Week 14 animals were immunized with tetanus toxoid a second time without additional humanized anti-B7-1 antibody treatment. Serum anti-tetanus antibody titers (IgM & IgG) were measured weekly over an 18 week period.

Figure 25 is a graph depicting the anti-tetanus response (log titer) in non-human primates immunized with tetanus toxoid. Cynomolgus monkeys were immunized with tetanus toxoid on Day 0 and treated with a single IV dose of humanized anti-B7-2 antibody alone or vehicle on Day 0. At Week 14 animals were immunized with tetanus toxoid a second time without additional humanized anti-B7-2 antibody treatment. Serum anti-tetanus antibody titers (IgM & IgG) were measured weekly over an 18 week period.

Figure 26 is a bar graph showing the area under the anti-tetanus antibody titer curve (log titer) for each treatment Group (Group designations: Group 1: vehicle control; Groups 2-4: 10, 1, or 0.1 mg/kg of h1F1 alone; Groups 5-7: 10, 1, or 0.1 mg/kg of h3D1 alone; Groups 8-11: 10, 1, 0.1, or 0.01 mg/kg of h1F1 and h3D1 in combination). Cynomolgus monkeys were immunized with tetanus toxoid 0 and treated with a single IV dose of humanized anti-B7-1 and humanized anti-B7-2 antibodies in combination, humanized anti-B7-1 antibody or humanized anti-B7-2 antibody alone, or vehicle (n=3 per group). Area Under the Curve (AUC) values

were calculated from time 0 to 14 weeks. All tetanus titers were normalized to a baseline of zero before AUCs of the tetanus titer curves were calculated. These AUC values were weighted by the fraction of the number of animals in each group that produced detectable antibody titers to account for the number of responding animals in each group.

Figure 27 is a graph showing the serum concentration of anti-B7-1 and anti-B7-2 (IgG2.M3 isotype) mAbs at various times after administration of an I.V. dose of 10mg/kg.

Figure 28 is a graph depicting the percent survival over about 1 year of Rhesus Monkeys that received a renal allotransplantation. The monkeys were treated with humanized anti-B7-1 and humanized anti-B7-2 antibodies in combination, humanized anti-B7-1 antibody or humanized anti-B7-2 antibody alone, or a vehicle. The humanized antibodies were given at an initial dose of 20 mg/kg followed by 5 mg/kg and then weekly doses of 5 mg/kg for between 60-80 days.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to humanized immunoglobulins having binding specificity for B7-2 or B7-1, comprising an antigen binding region of non-human origin and at least a portion of an immunoglobulin of human origin. Preferably, the humanized immunoglobulins can bind B7-2 or B7-1 with an affinity of at least about $10^7 M^{-1}$, preferably at least about $10^8 M^{-1}$, and more preferably at least about $10^9 M^{-1}$. In one embodiment, the humanized immunoglobulins include an antigen binding region of non-human origin which binds B7-2 or B7-1 and a constant region derived from a human constant region. The human constant region can have non-human residues in the framework region (FR). In another embodiment, the humanized immunoglobulins which binds B7-2 or B7-1 comprise a complementarity determining region (one or more) of non-human origin and a variable framework region (one or more) of human origin, and optionally, a constant region of human origin. Optionally, the FR region of the immunoglobulins can comprise residues of non-human origin. For example, the humanized immunoglobulins can comprise a

heavy chain and a light chain, wherein the light chain comprises a complementarity determining region derived from an antibody of non-human origin which binds B7-2 and a framework region derived from a light chain of human origin, and the heavy chain comprises a complementarity determining region derived from an antibody of non-human origin which binds B7-2 and a framework region derived from a heavy chain of human origin. In another example, the humanized immunoglobulins can comprise a heavy chain and a light chain, wherein the light chain comprises a complementarity determining region derived from an antibody of non-human origin which binds B7-1 and a framework region derived from a light chain of human origin, and the heavy chain comprises a complementarity determining region derived from an antibody of non-human origin which binds B7-1 and a framework region derived from a heavy chain of human origin. Also, the invention, individually or in a functional combination, embodies the light chain, the heavy chain, the variable region, the variable light chain and the variable heavy chain.

The invention relates to a humanized B7-2 antibody that has substantially the same binding specificity as the murine B7-2 antibody from which the humanized antibody is made, but with reduced immunogenicity in primates (e.g., humans). Similarly, the invention also relates to a humanized B7-1 antibody that has substantially the same binding specificity as the murine B7-1 antibody, respectively, from which the humanized antibody is made, but with reduced immunogenicity in primates (e.g., humans). The humanized B7-2 or B7-1 antibody can have about a lesser, substantially the same, or greater binding affinity as the murine counterpart. See Figures 3, 4, 8, 9A and 9B.

Naturally occurring immunoglobulins have a common core structure in which two identical light chains (about 24 kD) and two identical heavy chains (about 55 or 70 kD) form a tetramer. The amino-terminal portion of each chain is known as the variable (V) region, also referred to as the "antigen binding" region, and can be distinguished from the more conserved constant (C) regions of the remainder of each chain. Within the variable region of the light chain is a C-terminal portion known as the J region. Within the variable region of the heavy

chain, there is a D region in addition to the J region. Most of the amino acid sequence variation in immunoglobulins is confined to three separate locations in the V regions known as hypervariable regions or complementarity determining regions (CDRs) which are directly involved in antigen binding. The variable region is the portion of the antibody that binds to the antigen. The constant region allows for various functions such as the ability to bind to Fc receptors on phagocytic cells, placental cells, mast cells, etc. The light and heavy chains each have a variable region and a constant region. Accordingly, the invention relates to humanized immunoglobulins having binding specificity to B7-2 or B7-1. The humanized B7-1 or B7-2 immunoglobulin comprises a light chain and a heavy chain in which two light chains and two heavy chains form the tetramer.

The variable region further constitutes two types of regions, a framework region (FR) and a complementarity determining region (CDR). CDRs are hypervariable regions that contain most of the amino acid sequence variation in between immunoglobulins. Proceeding from the amino-terminus, these regions are designated CDR1, CDR2 and CDR3, respectively. See Figures 1A-1B, 2A-2B, 6A-6B and 7A-7B. The CDRs are connected by more conserved FRs. Proceeding from the amino-terminus, these regions are designated FR1, FR2, FR3, and FR4, respectively. The locations of CDR and FR regions and a numbering system have been defined by Kabat *et al.* (Kabat, E.A. *et al., Sequences of Proteins of Immunological Interest,* Fifth Edition, U.S. Department of Health and Human Services, U.S. Government Printing Office (1991); Kabat, E.A. *Structural Concepts in Immunology and Immunochemistry,* Second Edition, Holt, Rinehart and Winston, New York (1976); Kabat, E.A. *Sequences of Immunoglobulin Chains: Tabulation and Analysis of Amino Acid Sequences of Precursors, V-regions, C-regions, J-Chain and β2-Microglobulins,* U.S. Department of Health, Education and Welfare, Public Health Service, (1979); Kabat, E.A. *Structural Concepts in Immunology and Immunochemistry,* Holt, Rinehart and Winston, New York (1968); Kabat, E.A. *Experimental Immunochemistry,* Second Edition, Springfield, Thomas (1967). During the process of humanizing an immunoglobulin, one or more of the CDRs

from an antibody having specificity for B7-2 or B7-1 from a non-human species is grafted into the FRs of a human antibody. In addition, certain non-human framework substitutes can be made according to the methods described herein. The resulting humanized antibody has CDRs from a non-human species such as a mouse and FRs from a human antibody, whereby the humanized antibody maintains its antigenic specificity and affinity to B7-1 or B7-2 .

The invention also relates to a B7-1 or B7-2 humanized immunoglobulin light chain, or a B7-1 or B7-2 humanized immunoglobulin heavy chain. In one embodiment, the invention relates to a humanized B7-2 light chain comprising one or more light chain CDRs (e.g., CDR1 (SEQ ID NO: 16), CDR2 (SEQ ID NO: 18) and/or CDR3 (SEQ ID NO: 20)) of non-human origin and a human light chain framework region (See Figure 2B). In another embodiment, the invention relates to a humanized B7-2 immunoglobulin heavy chain comprising one or more heavy chain CDRs (e.g., CDR1 (SEQ ID NO: 10), CDR2 (SEQ ID NO: 12), and/or CDR3 (SEQ ID NO: 14)) of non-human origin and a human heavy chain framework region (See Figure 2A). The CDRs can be derived from a non-human immunoglobulin such as murine heavy (e.g., SEQ ID NO: 1, Figure 1A) and light (e.g., SEQ ID NO: 3, Figure 1B) variable region chains of the 3D1 antibody which are specific to B7-2.

In another embodiment, the invention relates to a humanized B7-1 light chain comprising one or more light chain CDRs (e.g., CDR1 (SEQ ID NO: 36), CDR2 (SEQ ID NO: 38) and/or CDR3 (SEQ ID NO: 40)) of non-human origin and a human light chain framework region (See Figure 7B). The invention also pertains to a B7-1 humanized immunoglobulin heavy chain comprising one or more heavy chain CDRs (e.g., CDR1 (SEQ ID NO: 30), CDR2 (SEQ ID NO: 32), and/or CDR3 (SEQ ID NO: 34)) of non-human origin and a human heavy chain framework region (See Figure 7A). The CDRs can be derived from a non-human immunoglobulin such as murine heavy (e.g., SEQ ID NO: 21, Figure 6A) and light (e.g., SEQ ID NO: 23, Figure 6B) variable region chains of the 1F1 antibody which are specific to B7-1.

The invention also embodies the humanized anti-B7-2 antibody expressed by a cell line deposited with the A.T.C.C., 10801 University Boulevard, Manassas, VA 02110-2209, on May 5, 1998, A.T.C.C. No: CRL-12524. The cell line which expresses the humanized anti-B7-2 antibody, deposited with the A.T.C.C., is designated as a recombinant CHO cell line (PA-CHO-DUKX-1538) expressing the humanized anti-human B7-2 (CD86) monoclonal antibody (#HF2-3D1) of the IgG2.M3 isotype.

The invention also embodies the humanized anti-B7-1 antibody expressed by a cell line deposited with the A.T.C.C., 10801 University Boulevard, Manassas, VA 02110-2209, on June 22, 1999, under A.T.C.C. No: PTA-263. The cell line which expresses the humanized anti-B7-1 antibody, deposited with the A.T.C.C., is designated as a recombinant CHO cell line (PA-CHO-DUKX-1538) expressing the humanized anti-human B7-1 (CD80) monoclonal antibody (#1F1).

Human immunoglobulins can be divided into classes and subclasses, depending on the isotype of the heavy chain. The classes include IgG, IgM, IgA, IgD and IgE, in which the heavy chains are of the gamma (γ), mu (μ), alpha (α), delta (δ) or epsilon (ϵ) type, respectively. Subclasses include IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2, in which the heavy chains are of the γ1, γ2, γ3, γ4, α1 and α2 type, respectively. Human immunoglobulin molecules of a selected class or subclass may contain either a kappa (κ) or lambda (λ) light chain. See e.g., *Cellular and Molecular Immunology*, Wonsiewicz, M.J., Ed., Chapter 45, pp. 41-50, W. B. Saunders Co, Philadelphia, PA (1991); Nisonoff, A., *Introduction to Molecular Immunology*, 2nd Ed., Chapter 4, pp. 45-65, Sinauer Associates, Inc., Sunderland, MA (1984).

The terms "HF2.3D1" and "3D1" refer to a murine immunoglobulin specific to B7-2. The terms "humanized HF2.3D1," "humanized 3D1", "hu3D1," "h3D1," "B7-2 humanized immunoglobulin" or "humanized B7-2 immunoglobulin" refer to a humanized immunoglobulin specific to human B7-2 (e.g., mouse anti-human B7-2 antibody). The terms "1F1" or "mouse 1F1" refer to a murine immunoglobulin that is specific to B7-1. The terms "humanized 1F1", "hu1F1," "h1F1," "B7-1

humanized immunoglobulin" or "humanized B7-1 immunoglobulin" refer to a humanized immunoglobulin specific to human B7-1 (e.g., mouse anti-human B7-1 antibody). The term "B7 molecules" refer to the B7-1 and B7-2 molecules. The term "B7 antibodies" refer to the anti-human B7-1 and anti-human B7-2 antibodies.

The terms "immunoglobulin" or "antibody" include whole antibodies and biologically functional fragments thereof. Such biologically functional fragments retain at least one antigen binding function of a corresponding full-length antibody and preferably, retain the ability to inhibit the interaction of B7-2 or B7-1 with one or more of its receptors (e.g., CD28, CTLA4). In a preferred embodiment, biologically functional fragments can inhibit binding of B7-2 and/or B7-1 for manipulation of the co-stimulatory pathway. Examples of biologically functional antibody fragments which can be used include fragments capable of binding to B7-2 or B7-1, such as single chain antibodies, Fv, Fab, Fab' and $F(ab')_2$ fragments. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For instance, papain or pepsin cleavage can be used to generate Fab or $F(ab')_2$ fragments, respectively. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding the heavy chain of a $F(ab')_2$ fragment can be designed to include DNA sequences encoding the CH_1 domain and hinge region of the heavy chain. The invention includes single chain antibodies (e.g., a single chain FV) that contain both portions of the heavy and light chains.

The term "humanized immunoglobulin" as used herein refers to an immunoglobulin comprising portions of immunoglobulins of different origin, wherein at least one portion is of human origin. For example, the humanized antibody can comprise portions derived from an immunoglobulin of non-human origin with the requisite specificity, such as a mouse, and from immunoglobulin sequences of human origin (e.g., chimeric immunoglobulin). These portions can be joined together chemically by conventional techniques (e.g., synthetic) or prepared as a contiguous polypeptide using genetic engineering techniques (e.g., DNA

encoding the protein portions of the chimeric antibody can be expressed to produce a contiguous polypeptide chain). Another example of a humanized immunoglobulin of the invention is an immunoglobulin containing one or more immunoglobulin chains comprising a CDR derived from an antibody of non-human origin and a framework region derived from a light and/or heavy chain of human origin (e.g., CDR-grafted antibodies with or without framework changes). Chimeric or CDR-grafted single chain antibodies are also encompassed by the term humanized immunoglobulin. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Padlan, E.A. *et al.*, European Patent Application No. 0,519,596 A1. See also, Ladner *et al.*, U.S. Patent No. 4,946,778; Huston, U.S. Patent No. 5,476,786; and Bird, R.E. *et al.*, *Science, 242:* 423-426 (1988), regarding single chain antibodies.

As embodied in the exemplified antibody of the present invention, the term "humanized immunoglobulin" also refers to an immunoglobulin comprising a human framework, at least one CDR from a non-human antibody, and in which any constant region present is substantially identical to a human immunoglobulin constant region, e.g., at least about 60-90%, preferably at least 95% identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDR's, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. In some instances, the humanized immunoglobulin, in addition to CDRs from a non-human antibody, would include additional non-human residues in the human framework region.

The design of humanized immunoglobulins can be carried out as follows. When an amino acid falls under the following categories, the framework amino acid of a human immunoglobulin to be used (acceptor immunoglobulin) is replaced by a framework amino acid from a CDR-providing non-human immunoglobulin (donor immunoglobulin):

(a) the amino acid in the human framework region of the acceptor immunoglobulin is unusual for human immunoglobulin at that position, whereas the corresponding amino acid in the donor immunoglobulin is typical for human immunoglobulin in that position:

(b) the position of the amino acid is immediately adjacent to one of the CDR's; or

(c) the amino acid is capable of interacting with the CDR's in a tertiary structure immunoglobulin model (see, Queen *et al.*, op. cit., and Co *et al.*, Proc. Natl. Acad. Sci. USA 88, 2869 (1991)).

For a detailed description of the production of humanized immunoglobulins, See Queen *et al.*, op. cit. and Co *et al*, op. cit. and U.S. Patents 5,585,089; 5,693,762, 5,693,761, and 5,530,101.

Usually, the CDR regions in humanized antibodies are substantially identical, and more usually, identical to the corresponding CDR regions in the mouse antibody from which they were derived. Although not usually desirable, it is sometimes possible to make one or more conservative amino acid substitutions of CDR residues without appreciably affecting the binding affinity of the resulting humanized immunoglobulin. Occasionally, substitutions of CDR regions can enhance binding affinity.

Other than for the specific amino acid substitutions discussed above, the framework regions of humanized immunoglobulins are usually substantially identical, and more usually, identical to the framework regions of the human antibodies from which they were derived. Of course, many of the amino acids in the framework region make little or no direct contribution to the specificity or affinity of an antibody. Thus, many individual conservative substitutions of framework residues can be tolerated without appreciable change of the specificity or affinity of the resulting humanized immunoglobulin.

The antigen binding region of the humanized B7-2 immunoglobulin (the non-human portion) can be derived from an immunoglobulin of non-human origin,

referred to as a donor immunoglobulin, having specificity for B7-2 (e.g., the 3D1 antibody) or B7-1 (e.g., the 1F1 antibody). For example, a suitable antigen binding region for the humanized B7-2 antibody can be derived from the HF2.3D1 monoclonal antibody, a murine anti-human B7-2 antibody. U.S. Serial No. 08/101,624, filed on July 26, 1993, 08/109,393, filed August 19, 1993 and 08/147,773, filed November 3, 1993, entitled, "B7-2:CTLA4/CD28 Counter Receptor". See also, Freeman, *et al.*, WO 95/03408, " B7-2: CTLA4/CD 28 Counter Receptor, published on February 2, 1995. A suitable antigen binding region for the humanized B7-1 antibody can be derived from the murine 1F1 monoclonal antibody, a murine-anti-human B7-1 antibody. Other sources include B7-2 or B7-1 specific antibodies obtained from non-human sources, such as rodent (e.g., mouse and rat), rabbit, pig, goat or non-human primate (e.g., monkey) or camelid animals (e.g., camels and llamas).

Additionally, other polyclonal or monoclonal antibodies, such as antibodies which bind to the same or similar epitope as the murine HF2.3D1 or 1F1 antibodies, can be made (e.g., Kohler *et al.*, *Nature, 256*:495-497 (1975); Harlow *et al.*, 1988, Antibodies: A Laboratory Manual, (Cold Spring Harbor, NY); and Current Protocols in Molecular Biology, Vol. 2 (Supplement 27, Summer '94), Ausubel *et al.*, Eds. (John Wiley & Sons: New York, NY), Chapter 11 (1991)). For example, antibodies can be raised against an appropriate immunogen in a suitable mammal such as a mouse, rat, rabbit, sheep, or camelid. Cells bearing B7-2 or B7-1, membrane fractions containing B7-2 or B7-1 immunogenic fragments of B7-2 or B7-1, and a B7-2 or B7-1 peptide conjugated to a suitable carrier are examples of suitable immunogens (e.g., DNA or peptide immunogens). Antibody-producing cells (e.g., a lymphocyte) can be isolated, for example, from the lymph nodes or spleen of an immunized animal. The cells can then be fused to a suitable immortalized cell (e.g., a myeloma cell line), thereby forming a hybridoma. Fused cells can be isolated employing selective culturing techniques. Cells which produce antibodies with the desired specificity can be selected by a suitable assay, such as an ELISA. Immunoglobulins of non-human origin having binding specificity for B7-2 or B7-1

can also be obtained from antibody libraries, such as a phage library comprising non-human Fab molecules. Humanized immunoglobulins can be made using other techniques.

In one embodiment, the antigen binding region of the humanized immunoglobulins comprise a CDR of non-human origin. In this embodiment, humanized immunoglobulins having binding specificity for B7-2 or B7-1 comprise at least one CDR of non-human origin. For example, CDRs can be derived from the light and heavy chain variable regions of immunoglobulins of non-human origin, such that a humanized B7-2 immunoglobulin includes substantially the heavy chain CDR1 (e.g., SEQ ID NO: 10), CDR2 (e.g., SEQ ID NO: 12) and/or CDR3 (e.g., SEQ ID NO: 14) amino acid sequences, and/or light chain CDR1 (e.g., SEQ ID NO: 16), CDR2 (e.g., SEQ ID NO: 18) and/or CDR3 (e.g., SEQ ID NO: 20) amino acid sequences, from one or more immunoglobulins of non-human origin, and the resulting humanized immunoglobulin has binding specificity for B7-2. CDRs can also be derived from light and heavy chain variable regions of immunoglobulins of non-human origin that are specific for B7-1. The humanized B7-1 antibody comprises substantially the heavy chain CDR1 (SEQ ID NO: 30), CDR2 (SEQ ID NO: 32) and/or CDR3 (e.g., SEQ ID NO: 34) amino acid sequences, and/or light chain CDR1 (SEQ ID NO: 36), CDR2 (SEQ ID NO: 38) and/or CDR3 (SEQ ID NO: 40) amino acid sequences, from one or more immunoglobulins of non-human origin, and the resulting humanized immunoglobulin has binding specificity for B7-1. All three CDRs of a selected chain can be substantially the same as the CDRs of the corresponding chain of a donor, and preferably, all three CDRs of the light and heavy chains are substantially the same as the CDRs of the corresponding donor chain. The nucleic acid sequences of the B7-2 heavy chain CDR1 (e.g., SEQ ID NO: 9), CDR2 (e.g., SEQ ID NO: 11) and CDR3 (e.g., SEQ ID NO: 13) and/or B7-2 light chain CDR1 (e.g., SEQ ID NO: 15), CDR2 (e.g., SEQ ID NO: 17), and CDR3 (e.g., SEQ ID NO: 19) can also be used in grafting the CDRs into the human framework. Additionally, the nucleic acid sequences of the B7-1 heavy chain CDR1 (SEQ ID NO: 29), CDR2 (SEQ ID NO: 31) and CDR3 (SEQ ID NO: 33) and/or B7-

1 light chain CDR1 (SEQ ID NO: 35), CDR2 (SEQ ID NO: 37) and CDR3 (SEQ ID NO:39) can be used in grafting the CDRs into the human framework.

In another embodiment, the invention pertains to humanized immunoglobulins having a binding specificity for either B7-2 or B7-1 comprising a heavy chain and a light chain. The light chain can comprise a CDR derived from an antibody of non-human origin which binds B7-2 or B7-1 and a FR derived from a light chain of human origin. For example, the light chain can comprise CDR1, CDR2 and/or CDR3 which have the amino acid sequence set forth below or an amino acid sequence substantially the same as the amino acid sequence such that the antibody specifically binds to B7-2: CDR1 KSSQSLLNSRTRENYLA (SEQ ID NO: 16), CDR2 WASTRES (SEQ ID NO: 18), and CDR3 TQSYNLYT (SEQ ID NO: 20). The heavy chain can comprise a CDR derived from an antibody of non-human origin which binds B7-2 and a FR derived from a heavy chain of human origin. For example, the B7-2 heavy chain can comprise CDR1, CDR2 and CDR3 which have the amino acid sequence set forth below or an amino acid sequence substantially the same as said amino acid sequence such that the antibody specifically binds to the B7-2: heavy chain: CDR1 DYAIQ (SEQ ID NO: 10), CDR2 VINIYYDNTNYNQKFKG (SEQ ID NO: 12), CDR3 AAWYMDY (SEQ ID NO: 14).

The light chain that is specific to B7-1 can comprise CDR1, CDR2 and/or CDR3 that have the amino acid sequence set forth below or an amino acid sequence substantially the same as the amino acid sequence such that the antibody specifically binds to B7-1: CDR1 SVSSSISSSNLH (SEQ ID NO: 30), CDR2 GTSNLAS (SEQ ID NO: 32) and CDR3 QQWSSYPLT (SEQ ID NO: 34). The heavy chain can comprise a CDR derived from an antibody of non-human origin which binds B7-1 and a FR derived from a heavy chain of human origin. The heavy chain that is specific to B7-1 can comprise CDR1, CDR2 and/or CDR3 that have the amino acid sequence set forth below or an amino acid sequence substantially the same as the amino acid sequence such that the antibody specifically binds to B7-1: CDR1

DYYMH (SEQ ID NO: 36), CDR2 WIDPENGNTLYDPKFQG (SEQ ID NO: 38), and CDR3 EGLFFAY (SEQ ID NO: 40).

An embodiment of the invention is a humanized immunoglobulin which specifically binds to B7-2 and comprises a humanized light chain comprising three light chain CDRs from the mouse 3D1 antibody and a light chain variable region framework sequence from a human immunoglobulin light chain. The invention further comprises a B7-2 humanized heavy chain that comprises three heavy chain CDRs from the mouse 3D1 antibody and a heavy chain variable region framework sequence from a human immunoglobulin heavy chain. The mouse 3D1 antibody can further have a mature light chain variable domain as shown in Figure 1B (SEQ ID NO.: 4) and a mature heavy chain variable domain as shown in Figure 1A (SEQ ID NO.: 2).

In another embodiment of the invention is a humanized immunoglobulin which specifically binds to B7-1 and comprises a humanized light chain comprising three light chain CDRs from the mouse 1F1 antibody, and a light chain variable region framework sequence from a human immunoglobulin light chain. The invention further comprises a B7-1 humanized heavy chain that comprises three heavy chain CDRs from the mouse 1F1 antibody, respectively, and a heavy chain variable region framework sequence from a human immunoglobulin heavy chain. The mouse 1F1 antibody can have a mature light chain variable domain as shown in Figure 6B (SEQ ID NO: 24) and a mature heavy chain variable domain, as shown in Figure 6A (SEQ ID NO: 22).

The portion of the humanized immunoglobulin or immunoglobulin chain which is of human origin (the human portion) can be derived from any suitable human immunoglobulin or immunoglobulin chain. For example, a human constant region or portion thereof, if present, can be derived from the κ or λ light chains, and/or the γ (*e.g.*, $\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$), μ, α (*e.g.*, $\alpha 1$, $\alpha 2$), δ or ϵ heavy chains of human antibodies, including allelic variants. A particular constant region, such as IgG2 or IgG4, variants or portions thereof can be selected to tailor effector function. For example, a mutated constant region, also referred to as a "variant," can be

incorporated into a fusion protein to minimize binding to Fc receptors and/or ability to fix complement (see *e.g.*, Winter *et al.*, U.S. Patent No. 5,648,260 and 5,624,821; GB 2,209,757 B; Morrison *et al.*, WO 89/07142; Morgan *et al.*, WO 94/29351, December 22, 1994). In addition, a mutated IgG2 Fc domain can be created that reduces the mitogenic response, as compared to natural Fc regions (see e.g., Tso *et al.*, U.S. Patent No. 5,834,597, the teachings of which are incorporated by reference herein in their entirety). See Example 3 for mutations performed to the humanized anti-B7-2 antibody and Example 10 for mutations performed to the humanized anti-B7-1 antibody.

If present, human FRs are preferably derived from a human antibody variable region having sequence similarity to the analogous or equivalent region of the antigen binding region donor. Other sources of FRs for portions of human origin of a humanized immunoglobulin include human variable consensus sequences (See, Kettleborough, C.A. *et al.*, *Protein Engineering* 4:773-783 (1991); Queen *et al.*, U.S. patent Nos: 5,585,089, 5,693,762 and 5,693,761). For example, the sequence of the antibody or variable region used to obtain the non-human portion can be compared to human sequences as described in Kabat, E.A., *et al., Sequences of Proteins of Immunological Interest,* Fifth Edition, U.S. Department of Health and Human Services, U.S. Government Printing Office (1991). In a preferred embodiment, the FRs of the humanized immunoglobulin chains are derived from a human variable region having at least about 60% overall sequence identity, and preferably at least about 80% overall sequence identity, with the variable region of the non-human donor (e.g., murine HF2.3D1 or 1F1 antibody). For example, the overall sequence identity between the mouse HF2.3D1 and human H2F light chain variable framework regions is 82.5%, and the overall sequence identity between the mouse HF2.3D1 and human I2R heavy chain variable framework regions is 62.5%. For the B7-1 antibody, the overall sequence identity between the murine 1F1 and humanized III-2R light chain variable frame-work region is 69%, and the overall sequence identity between the murine III-2R heavy chain variable frame-work region is 79%.

The phrase "substantially identical," in context of two nucleic acids or polypeptides (e.g., DNAs encoding a humanized immunoglobulin or the amino acid sequence of the humanized immunoglobulin) refers to two or more sequences or subsequences that have at least about 80%, most preferably 90-95% or higher nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using the following sequence comparison method and/or by visual inspection. Such "substantially identical" sequences are typically considered to be homologous. Preferably, the "substantial identity" exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues, or over the full length of the two sequences to be compared. As described below, any two antibody sequences can only be aligned in one way, by using the numbering scheme in Kabat. Therefore, for antibodies, percent identity has a unique and well-defined meaning.

Amino acids from the variable regions of the mature heavy and light chains of immunoglobulins are designated Hx and Lx respectively, where x is a number designating the position of an amino acid according to the scheme of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987 and 1991). Kabat lists many amino acid sequences for antibodies for each subgroup, and lists the most commonly occurring amino acid for each residue position in that subgroup. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence. Kabat's scheme is extendible to other antibodies not included in the compendium by aligning the antibody in question with one of the consensus sequences in Kabat. The use of the Kabat numbering system readily identifies amino acids at equivalent positions in different antibodies. For example, an amino acid at the L50 position of a human antibody occupies the equivalent position to an amino acid position L50 of a mouse antibody.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-

terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. The variable regions of each light/heavy chain pair form the antibody binding site. Thus, an intact antibody has two binding sites.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See generally, *Fundamental Immunology,* Paul, W., ed., 3rd ed. Raven Press, N.Y., 1993, Ch. 9).

From N-terminal to C-terminal, both light and heavy chain variable regions comprise alternating framework and (CDRs)" FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each region is in accordance with the definitions of Kabat (1987) and (1991), *supra* and/or Chothia & Lesk, *J. Mol. Biol.* 196:901-917 (1987); Chothia *et al., Nature* 342:878-883 (1989).

In one embodiment, the humanized immunoglobulins comprise at least one of the FRs derived from one or more chains of an antibody of human origin. Thus, the FR can include a FR1, FR2, FR3 and/or FR4 derived from one or more antibodies of human origin. Preferably, the human portion of a selected humanized chain includes FR1, FR2, FR3 and/or FR4 derived from a variable region of human origin (e.g., from a human immunoglobulin chain, from a human consensus sequence). In a preferred embodiment, the FRs for the B7-2 light chain variable region are from the H2F human antibody and the FRs for the B7-2 heavy chain variable region are from the I2R human antibody. The FRs for B7-1 heavy and light chain variable regions are from the III-2R antibody.

The immunoglobulin portions of non-human and human origin for use in the invention have sequences that are identical to immunoglobulins or immunoglobulin portions from which they are derived, or to variants thereof. Such variants include

mutants differing by the addition, deletion, or substitution of one or more residues. As indicated above, the CDRs which are of non-human origin are substantially the same as in the non-human donor, and preferably are identical to the CDRs of the non-human donor. As described herein, changes in the FR, such as those which substitute a residue of the FR of human origin with a residue from the corresponding position of the donor can be made. One or more mutations in the FR can be made, including deletions, insertions and substitutions of one or more amino acids. Several such substitutions are described in the design of a humanized HF2.3D1 antibody in Example 2 and for the humanized 1F1 in Example 9. For a selected humanized antibody or chain, framework mutations can be designed as described herein. Preferably, the B7-2 and B7-1 humanized immunoglobulins can bind B7-2 and B7-1, respectively, with an affinity similar to or better than that of the non-human donor. Variants can be produced by a variety of suitable methods, including mutagenesis of non-human donor or acceptor human chains.

The humanized immunoglobulins of the invention have binding specificity for human B7-2 or B7-1, and include humanized immunoglobulins (including fragments) which can bind determinants of B7-2 or B7-1. In a preferred embodiment, the humanized immunoglobulin of the present invention has at least one functional characteristic of murine HF2.3D1 or 1F1 antibody, such as binding function (e.g., having specificity for B7-2 or B7-1, having the same or similar epitopic specificity), and/or inhibitory function (e.g., the ability to inhibit the binding of a cell bearing CD28 or CTLA4 to the B7-2 or B7-1 ligand). Thus, preferred humanized immunoglobulins can have the binding specificity of the murine HF2.3D1 or 1F1 antibody, the epitopic specificity of the murine HF2.3D1 or 1F1 antibody (e.g., can compete with murine HF2.3D1 or 1F1, a chimeric HF2.3D1 or 1F1 antibody, or humanized HF2.3D1 or 1F1 for binding to B7-2 or B7-1, respectively) and/or inhibitory function.

The binding function of a humanized immunoglobulin having binding specificity for B7-2 or B7-1 can be detected by standard immunological methods, for example, using assays which monitor formation of a complex between

humanized immunoglobulin and B7-2 or B7-1 (e.g., a membrane fraction comprising B7-2 or B7-1, or human lymphocyte cell line or recombinant host cell comprising nucleic acid which expresses B7-2 or B7-1).

Binding and/or adhesion assays or other suitable methods can also be used in procedures for the identification and/or isolation of humanized immunoglobulins (e.g., from a library) with the requisite specificity (e.g., an assay which monitors adhesion between a cell bearing a B7-2 or B7-1 receptor and B7 molecule, or other suitable methods).

The immunoglobulin portions of non-human and human origin for use in the invention include light chains, heavy chains and portions of light and heavy chains. These immunoglobulin portions can be obtained or derived from immunoglobulins (e.g., by de novo synthesis of a portion), or nucleic acids encoding an immunoglobulin or chain thereof having the desired property (e.g., binds B7-2 or B7-1, sequence similarity) can be produced and expressed. Humanized immunoglobulins comprising the desired portions (e.g., antigen binding region, CDR, FR, C region) of human and non-human origin can be produced using synthetic and/or recombinant nucleic acids to prepare genes (e.g., cDNA) encoding the desired humanized chain. To prepare a portion of a chain, one or more stop codons can be introduced at the desired position. For example, nucleic acid sequences coding for newly designed humanized variable regions can be constructed using PCR mutagenesis methods to alter existing DNA sequences (see e.g., Kamman, M., *et al.*, *Nucl. Acids Res. 17*:5404 (1989)). PCR primers coding for the new CDRs can be hybridized to a DNA template of a previously humanized variable region which is based on the same, or a very similar, human variable region (Sato, K., *et al.*, *Cancer Research 53*:851-856 (1993)). If a similar DNA sequence is not available for use as a template, a nucleic acid comprising a sequence encoding a variable region sequence can be constructed from synthetic oligonucleotides (see e.g., Kolbinger, F., *Protein Engineering 8*:971-980 (1993)). A sequence encoding a signal peptide can also be incorporated into the nucleic acid (e.g., on synthesis, upon insertion into a vector). If the natural signal peptide sequence is unavailable, a

signal peptide sequence from another antibody can be used (see, e.g., Kettleborough, C.A., *Protein Engineering 4*:773-783 (1991)). Using these methods, methods described herein or other suitable methods, variants can be readily produced. In one embodiment, cloned variable regions can be mutagenized, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber *et al.*, U.S. 5,514,548; Hoogengoom *et al.*, WO 93/06213, published April 1, 1993)).

Nucleic Acids and Constructs Comprising Same:

The invention also relates to isolated and/or recombinant (including, e.g., essentially pure) nucleic acids comprising sequences which encode a humanized B7-1 or B7-2 immunoglobulin, or humanized B7-1 or B7-2 immunoglobulin light or heavy chain of the present invention.

Nucleic acids referred to herein as "isolated" are nucleic acids which have been separated away from the nucleic acids of the genomic DNA or cellular RNA of their source of origin (e.g., as it exists in cells or in a mixture of nucleic acids such as a library), and include nucleic acids obtained by methods described herein or other suitable methods, including essentially pure nucleic acids, nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods, and recombinant nucleic acids which are isolated (see e.g., Daugherty, B.L. *et al., Nucleic Acids Res., 19(9):* 2471-2476 (1991); Lewis, A.P. and J.S. Crowe, *Gene, 101*: 297-302 (1991)).

Nucleic acids referred to herein as "recombinant" are nucleic acids which have been produced by recombinant DNA methodology, including those nucleic acids that are generated by procedures which rely upon a method of artificial recombination, such as the polymerase chain reaction (PCR) and/or cloning into a vector (e.g., plasmid) using restriction enzymes. "Recombinant" nucleic acids are also those that result from recombination events that occur through the natural mechanisms of cells, but are selected for after the introduction to the cells of nucleic acids designed to allow and make probable a desired recombination event.

The invention also relates, more specifically, to isolated and/or recombinant nucleic acids comprising a nucleotide sequence which encodes a humanized HF2.3D1 or 1F1 immunoglobulin, also referred to as "humanized 3D1" or "humanized 1F1," respectively, (e.g., a humanized immunoglobulin of the invention in which the non-human portion is derived from the murine HF2.3D1 or 1F1 monoclonal antibody), or chain thereof. In one embodiment, the light chain comprises three complementarity determining regions derived from the light chain of the HF2.3D1 or 1F1 antibody, and the heavy chain comprises three complementarity determining regions derived from the heavy chain of the HF2.3D1 or 1F1 antibody. Such nucleic acids include, for example, (a) a nucleic acid comprising a sequence which encodes a polypeptide comprising the amino acid sequence of the heavy chain variable region of a humanized HF2.3D1 or 1F1 immunoglobulin (e.g., SEQ ID NO: 5, See Figure 2A or SEQ ID NO: 25, See Figure 7A), (b) a nucleic acid comprising a sequence which encodes a polypeptide comprising the amino acid sequence of the light chain variable region of a humanized HF2.3D1 or 1F1 immunoglobulin (e.g., SEQ ID NO: 7, See Figure 2B or SEQ ID NO: 27, See Figure 7B), (c) a nucleic acid comprising a sequence which encodes at least a functional portion of the light or heavy chain variable region of a humanized HF2.3D1 or 1F1 immunoglobulin (e.g., a portion sufficient for antigen binding of a humanized immunoglobulin which comprises the chain). Due to the degeneracy of the genetic code, a variety of nucleic acids can be made which encode a selected polypeptide. In one embodiment, the nucleic acid comprises the nucleotide sequence of the variable region as set forth or substantially as set forth in Figure 2A and/or Figure 2B, or Figure 7A and/or Figure 7B, including double or single-stranded polynucleotides. Isolated and/or recombinant nucleic acids meeting these criteria can comprise nucleic acids encoding sequences identical to sequences of humanized HF2.3D1 or humanized 1F1 antibody or variants thereof, as discussed above.

Nucleic acids of the invention can be used in the production of humanized immunoglobulins having binding specificity for B7-2 or B7-1. For example, a

nucleic acid (e.g., DNA) encoding a humanized immunoglobulin of the invention can be incorporated into a suitable construct (e.g., a vector) for further manipulation of sequences or for production of the encoded polypeptide in suitable host cells.

Method of Producing Humanized Immunoglobulins Having Specificity for B7-2 and/or B7-1:

Another aspect of the invention relates to a method of preparing a humanized immunoglobulin which has binding specificity for B7-2 or B7-1. The humanized immunoglobulin can be obtained, for example, by the expression of one or more recombinant nucleic acids encoding a humanized immunoglobulin having binding specificity for B7-2 or B7-1 in a suitable host cell.

Constructs or expression vectors suitable for the expression of a humanized immunoglobulin having binding specificity for B7-2 and/or B7-1 are also provided. The constructs can be introduced into a suitable host cell, and cells which express a humanized immunoglobulin of the invention, can be produced and maintained in culture. Suitable host cells can be procaryotic, including bacterial cells such as *E. coli, B. subtilis* and or other suitable bacteria, or eucaryotic, such as fungal or yeast cells (e.g., *Pichia pastoris, Aspergillus species, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Neurospora crassa*), or other lower eucaryotic cells, and cells of higher eucaryotes such as those from insects (e.g., Sf9 insect cells (WO 94/26087, O'Connor, published November 24, 1994)). Suitable host cells can also come from plants, transgenic animals, or mammals (e.g., COS cells, NSO cells, SP2/0, Chinese hamster ovary cells (CHO), HuT 78 cells, 293 cells). (See, e.g., Ausubel, F.M. *et al.,* eds. *Current Protocols in Molecular Biology,* Greene Publishing Associates and John Wiley & Sons Inc., (1993)).

Host cells which produce a humanized immunoglobulin having binding specificity for B7-2 and/or B7-1 can be produced as follows. For example, a nucleic acid encoding all or part of the coding sequence for the desired humanized immunoglobulin can be inserted into a nucleic acid vector, e.g., a DNA vector, such as a plasmid, virus or other suitable expression unit. A variety of vectors are

available, including vectors which are maintained in single copy or multiple copy, or which become integrated into the host cell chromosome.

Suitable expression vectors can contain a number of components, including, but not limited to one or more of the following: an origin of replication; a selectable marker gene; one or more expression control elements, such as a transcriptional control element (e.g., a promoter, an enhancer, terminator), and/or one or more translation signals; a signal sequence or leader sequence for membrane targeting or secretion. In a construct, a signal sequence can be provided by the vector or other source. For example, the transcriptional and/or translational signals of an immunoglobulin can be used to direct expression.

A promoter can be provided for expression in a suitable host cell. Promoters can be constitutive or inducible. For example, a promoter can be operably linked to a nucleic acid encoding a humanized immunoglobulin or immunoglobulin chain, such that it directs expression of the encoded polypeptide. A variety of suitable promoters for procaryotic (e.g., lac, tac, T3, and T7 promoters for *E. coli*) and eucaryotic (e.g., yeast alcohol dehydrogenase (ADH) and SV40, CMV) hosts are available.

In addition, the expression vectors typically comprise a selectable marker for selection of host cells carrying the vector, and, in the case of replicable expression vector, an origin of replication. Genes encoding products which confer antibiotic or drug resistance are common selectable markers and can be used in procaryotic (e.g., β-lactamase gene (ampicillin resistance) and *Tet* gene (tetracycline resistance)) and eucaryotic cells (e.g., neomycin (G418 or geneticin), gpt (mycophenolic acid), ampicillin, and hygromycin resistance genes). Dihydrofolate reductase marker genes permit selection with methotrexate in a variety of hosts. Genes encoding the gene product of auxotrophic markers of the host (e.g., *LEU2, URA3* and *HIS3*) are often used as selectable markers in yeast. Use of viral (e.g., baculovirus) or phage vectors, and vectors which are capable of integrating into the genome of the host cell, such as retroviral vectors, are also contemplated. The invention also relates to cells carrying these expression vectors.

For example, a nucleic acid (e.g., one or more nucleic acids) encoding the heavy and light chains of a humanized immunoglobulin having binding specificity for B7-2 or B7-1, or a construct (e.g., one or more constructs) comprising such nucleic acid(s), can be introduced into a suitable host cell by a method appropriate to the host cell selected (e.g., transformation, transfection, electroporation, infection), such that the nucleic acid(s) are operably linked to one or more expression control elements (e.g., in a vector, in a construct created by processes in the cell, integrated into the host cell genome). Host cells can be maintained under conditions suitable for expression (e.g., in the presence of inducer, suitable media supplemented with appropriate salts, growth factors, antibiotic, nutritional supplements, etc.), whereby the encoded polypeptide(s) are produced. If desired, the encoded protein (e.g., humanized HF2.3D1 or 1F1 antibody) can be isolated from, for example, host cells, medium or milk. This process encompasses expression in a host cell of a transgenic animal (see e.g., WO 92/03918, GenPharm International, published March 19, 1992).

Fusion proteins can be produced in which a humanized immunoglobulin or immunoglobulin chain is linked to a non-immunoglobulin moiety (e.g., a moiety which does not occur in immunoglobulins as found in nature) in an N-terminal location, C-terminal location or internal to the fusion protein. For example, some embodiments can be produced by the insertion of a nucleic acid encoding immunoglobulin sequences into a suitable expression vector, such as a pET vector (e.g., pET-15b, Novagen), a phage vector (e.g., pCANTAB 5 E, Pharmacia), or other vector (e.g., pRIT2T Protein A fusion vector, Pharmacia). The resulting construct can be introduced into a suitable host cell for expression. Upon expression, some fusion proteins can be isolated or purified from a cell lysate by means of a suitable affinity matrix (see e.g., *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., Vol. 2, Suppl. 26, pp. 16.4.1-16.7.8 (1991)).

Therapeutic Methods and Compositions:

Two types of T-cells exist: helper T cells and cytotoxic T cells. The helper T cells can recognize an antigen that is coupled with a major histocompatibility complex (MHC). Antigen presenting cells internalize an antigen and re-express the antigen with the MHC molecule. Upon recognition of the antigen, secretion of cytokines occur. Cytokine secretion activates B-lymphocytes, cytotoxic T cells, phagocytes and other cells. However, cytokine secretion and cellular proliferation require more than recognition of the antigen. Complete T-cell activation requires a second signal referred to as the "co-stimulatory signal." These co-stimulatory signals serve to initiate, maintain, and regulate the activation cascade. An important co-stimulatory pathway is called the B7:CD28/CTLA4 pathway.

The B7:CD28/CTLA4 pathway involves two co-stimulatory ligands, B7-1 (CD80) and B7-2 (CD86). The B7-1 and B7-2 ligands which are present on the antigen presenting cell each bind to two receptors on T-cells called CD28 and CTLA4.

The expression of B7 polypeptides, B7-1 (CD80) and B7-2 (CD86), is tightly regulated. (Linsley, PS *et al.*, Immunity 1:793-801 (1994). Unstimulated antigen-presenting cells generally do not express B7-1 and B7-2, except in dendritic cells. After activation, dendritic cells, epidermal Langerhans' cells, B cells, and macrophages up-regulate the expression of B7-2 and B7-1. Additionally, B7-2 can be expressed on granulocytes and on T-cell molecules, and B7-1 is expressed in fibroblasts and T-cell molecules. (Reiser, *et al., New England J. of Med., 335:*18, 1369-1377, 1371 (1996).

In most immune responses, B7-2 is induced earlier than B7-1 and rises to higher levels. B7-2 also affects the production of interleukin-4 (IL-4) and the generation of type 2 helper cells. B7 molecules (B7-1 and B7-2) are also responsible for costimulating CD8 T cells in the absence of CD4 T cells which can be helpful in generating vaccines against melanoma. B7 molecules can costimulate natural killer cells and γ/δ T cells. Hence, modulation of B7 molecules is helpful in anti-tumor and anti-microbial immunity.

The B7:CD28/CTLA4 pathway participates in various disease states including the pathogenesis of infectious diseases, asthma, autoimmune diseases, inflammatory disorders, the rejection of grafted organs and graft versus host disease. This pathway also participates in prophylaxis and mechanisms that stimulate the immune system. Transfection with genes encoding costimulators, such as B7, are applicable for anti-tumor and anti-viral vaccines. Also, the B7 molecules participate in autoimmune diseases such as systemic lupus erythematosus, diabetes mellitus, asthma, insulitis, arthritis, inflammatory bowel disease, inflammatory dermatitis (psoriasis vulgaris and atopic dermatitis), and multiple sclerosis. (Reiser, *et al., New England J. of Med., 335 (18):* 1369 (1996). Accordingly, the invention encompasses methods for treating the disease, as described herein, comprising administering immunoglobulin(s) that binds to B7-1 and/or B7-2. The immunoglobulin should be administered in therapeutically effective amounts and, optionally, in a carrier.

Treating an individual having a disease refers to minimizing or alleviating one or more symptoms associated with the disease. Treating an individual with a transplant rejection means minimizing or alleviating one or more symptoms associated with transplant rejection (e.g., fever, loss of kidney function, distended kidneys, T-cell/APC cell attack the rejection). Preventing a disease in an individual refers to preventing the occurrence of one or more symptoms of the disease. Preventing a transplant rejection means reducing one or more of the immune responses associated with such a transplant rejection.

Therefore, modulating or influencing the B7 molecules role can be useful in treating individuals with the diseases, described herein. B7 modulation is also useful in treating individuals with immune-related or autoimmune diseases and disorders in which B7-2 and/or B7-1 participates. The modulation of B7-2 or B7-1 can also be used for diseases related to or affected by IL-4 and/or the generation of type 2 helper cells. These disorders/diseases can be treated using an antibody specific to B7-2 and/or B7-1. Preferably, the antibody is a humanized antibody specific to B7-2 or B7-1. Treatment of these diseases can be facilitated with co-

administration of an anti-B7-2 antibody, an anti-B7-1 antibody, including chimeric and humanized versions thereof, and/or antibodies to the corresponding receptors, CD28 and CTLA4.

In addition to the diseases described herein, the immunoglobulins that bind B7-1 and/or B7-2 can be administered to a person having transplanted tissue, organ or cells. Inhibiting the B7 pathway prevents or reduces the rejection of the transplanted tissue, organ or cell. The invention pertains to treating acute and/or chronic transplant rejection for a prolonged period of time (e.g., days, months, years). Acute transplant rejection generally occurs within the first few weeks of the transplant, whereas chronic rejection occurs after the first few weeks. The amounts of the anti-B7-1 and anti-B7-2 antibodies administered are described herein.

In particular, the invention pertains to methods of treating a transplant recipient or preventing transplant rejection. This method involves administering the humanized anti-B7-1 and humanized anti-B7-2 antibodies in sufficient amounts to prevent rejection of the transplant. The transplant can be cells, tissue, or an organ. The antibodies can be administered before and/or after the transplant, or at the time of the transplant. The antibodies are administered in doses (e.g., between about 1 mg/kg and about 100 mg/kg). In particular, the antibodies are administered in higher doses (e.g., between about 1mg/kg and about 25 mg/kg) on the day of the transplant, and then at lower doses periodically after the transplant (e.g., weekly at a dose between about 1 mg/kg and about 5 mg/kg). Remarkably, such administration results in a significant decrease and even complete prevention of an immune response directed toward the transplant rejection. See Examples 22 and 23.

Methods of treatment also involve co-administration of a humanized anti-B7-2 antibody and/or humanized anti-B7-1 antibody with other known standard of care drugs (e.g., drugs that are used to modulate the immune response of an individual having a transplanted organ, tissue, cell or the like). Such drugs include, for example, methotrexate, immunosuppressive agents that arrest the growth of immune cells or inhibit cell cycle progression (e.g., rapamycin), steroids (e.g., prednisone or derivative thereof), calcineurin inhibitors (e.g., cyclosporin or

FK506), anti-CD40 pathway inhibitors (e.g., anti-CD40 antibodies, anti-CD40 ligand antibodies and small molecule inhibitors of the CD40 pathway), transplant salvage pathway inhibitors (e.g., mycophenolate mofetil (MMF)), IL-2 receptor antagonists (e.g., Zeonpax® from Hoffmann-la Roche Inc., and Simulet from Novartis, Inc.) and analogs thereof, or transplant rejection drugs developed in the future. The data described herein show that Cyclosporin A, prednisone and rapamycin work particularly well in preventing transplant rejection when any one of these compounds are administered with humanized anti-B7-1 and humanized anti-B7-2 antibodies. The amounts administered of these compounds vary. The amounts administered depend their serum concentrations in the individual. A higher serum concentration warrants a lower dosage, and lower serum concentration warrants a higher dosage. For example, Cyclosporin A can be administered in an amount between about 150 ng/ml and about 100 mg/ml (e.g., 200-300 ng/ml), prednisone can be administered between about 0.2 mg/kg and about 2.0 mg/ml, methylprednisone can be administered between about 0.2 mg/kg and 2.0 mg/kg, and rapamycin can be administered between about 0.5 mg/kg and about 2.0 mg/kg, when administered with the humanized anti-B7-1 and humanized anti-B7-2 antibodies.

The invention includes *ex vivo* methods for transplanting cells (e.g., blood cells or components, or bone marrow) to an individual in need thereof. An individual in need thereof is one, for example, having a disease that is treated with such a transplant (e.g., proliferative diseases such as leukemia, lymphoma, cancer; anemia such as sickle-cell anemia, thalassemia, and aplastic anemia; inborn errors of metabolism; congenital immunodeficiency diseases; and myeloid dysplasia syndrome). The method comprises obtaining cells from a donor. Generally, donor bone marrow contains both immature and mature lymphocytes. The blood cells from a donor can be stem cells or immature blood cells in addition to bone marrow cells. The cells of the donor preferably comes from, but is not limited to a person who has similar characteristics as the patient/recipient (e.g., the donor's bone marrow is a match to the patient's bone marrow). The characteristics that are

analyzed to determine whether a donor is a match to the patient are MHC class 1 and 2 (e.g., HLA-A, HLA-B, and/or HLA-DR). The method involves contacting the cells (e.g., bone marrow or other blood components) with an immunoglobulin specific to B7-1 and/or an immunoglobulin specific to B7-2 and recipient cells (e.g., lymphocyte from the patient) to obtain a mixture, referred to as "treated cells". The amount of antibody utilized depends on the number of cells present. A greater amount of cells requires more antibody, and a lesser amount of cells requires less antibody. The experiments described herein utilized 10 mg/ml of each antibody which is in excess of 10-100 fold. The amount of the anti-B7-1 and/or anti-B7-2 antibodies used in this embodiment should be sufficient to induce anergy, e.g., about .01 to about 10 mg/ml. The donor cells, immunoglobulin(s) and recipient cells are in contact for a period of time sufficient for tolerance induction (e.g., about 1-96 hours, preferably about 36-48 hours). Tolerance induction (e.g., anergy) refers to the lack of responsiveness to an antigen that has been induced with a treatment with B7-1 and/or B7-2 antibodies, such that the T-cell can no longer adequately or fully respond to that antigen. Example 18. The recipient cells (e.g., Peripheral Blood Lymphocytes (PBL), or lymphocytes that express class I antigens (MHC-I)) are irradiated to prevent cells from dividing. A substitute for recipient cells can be tissue, organs or engineered cells that express MHC class I antigens, and B7-1 and/or B7-2 molecules. The method then includes introducing the mixture (e.g., the treated cells) or treated bone marrow to the patient. This method of treatment is aimed at preventing graft vs. host disease. For example, cells in the treated bone marrow become tolerant to recipient alloantigen thereby reducing or eliminating graft vs. host disease, and improving engraphment of donor marrow (e.g., stem cells). Accordingly, the methods of the present invention include treating, preventing or aiding in the prevention of graft vs. host disease. The anti-B7-1 and anti-B7-2 antibodies reduce rejection by donor bone marrow or donor cells of the recipient. However, the methods are able to reduce rejection without significantly compromising the patient's ability to detect and develop an immune response to other foreign cells and antigens. Hence, these methods allow the transplantation to

be recipient specific, and allow for the rejection of foreign antigens without compromising the transplant. See Exemplification Section.

The invention also relates to methods for decreasing an antibody response to an antigen in an individual by administering the humanized anti-B7-1 and/or humanized anti-B7-2 antibodies to the individual. The antibodies can be administered in the presence of the antigen. The antigen can be growth factors, clotting factors, cytokines, chemokines, gene therapy vehicles or hormones. In particular, the antigen can be, for example, tetanus toxoid, Factor VIII, Factor IX, insulin, growth hormone, or a gene delivery vector. The antigens can be administered in a polypeptide form, or in a nucleic acid form (e.g., gene delivery through adeno-associated viruses, retroviruses, naked DNA vectors, etc.). Suppression of the antibody response to these antigens is helpful in the treatment of a number of diseases, such as in hemophiliacs who develop antibody responses to their administered Factor VIII or Factor IX, thereby leading to blood clotting problems. The data described herein shows that administering effective amounts of the humanized anti-B7-1 and/or humanized anti-B7-2 antibodies in conjunction with the model antigen, tetanus toxoid, inhibit an antibody response to the tetanus toxoid. The antibodies can be administered together with the antigen, or close enough in time (e.g., shortly before or shortly thereafter) to confer the desired effect, e.g., suppression of the antibody response. The humanized anti-B7-1 and humanized anti-B7-2 antibodies are administered within about 3 weeks (the ½ life of the antibodies) of the antigen, e.g., between about 14 days before and about 2 days after administration of the antigen. The humanized anti-B7-1 and humanized anti-B7-2 antibodies are administered between about .01 mg/kg and about 10 mg/kg.

The invention pertains to a pharmaceutical composition comprising a humanized anti-B7-1 and/or humanized anti-B7-2 antibody, with or without a carrier. A preferred embodiment is to administer the B7-1 and/or B7-2 immunoglobulins in injectable or capsule form. In particular, the injectable form can be intravenous or subcutaneous injections. The terms "pharmaceutically acceptable carrier" or a "carrier" refer to any generally acceptable excipient or drug

delivery composition that is relatively inert and non-toxic. Exemplary carriers include calcium carbonate, sucrose, dextrose, mannose, albumin, starch, cellulose, silica gel, polyethylene glycol (PEG), dried skim milk, rice flour, magnesium stearate, and the like. Suitable formulations and additional carriers are described in Remington's Pharmaceutical Sciences, (17th Ed., Mack Pub. Co., Easton, PA).

Suitable carriers (e.g., pharmaceutical carriers) also include, but are not limited to sterile water, salt solutions (such as Ringer's solution), alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrolidone, etc. Such preparations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, and/or aromatic substances and the like which do not deleteriously react with the immunoglobulin. They can also be combined where desired with other active substances, e.g., enzyme inhibitors, to reduce metabolic degradation. A carrier (e.g., a pharmaceutically acceptable carrier) is preferred, but not necessary to administer the immunoglobulins.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. In particular, carriers for parenteral administration include aqueous solutions of dextrose, saline, pure water, ethanol, glycerol, propylene glycol, peanut oil, sesame oil, polyoxyethylene-polyoxypropylene block polymers, and the like. Ampules, vials and syringes are convenient unit dosages.

Immunoglobulins of the invention can be administered intravenously, parenterally, intramuscular, subcutaneously, orally, nasally, by inhalation, by implant, by injection, or by suppository. The composition can be administered in a single dose or in more than one dose over a period of time to confer the desired effect.

The actual effective amounts of immunoglobulin can vary according to the specific immunoglobulin being utilized, the particular composition formulated, the mode of administration, and the age, weight, condition of the patient, and severity of the disorder or disease, for example. As used herein, an effective amount of the B7-2 and/or B7-2 immunoglobulins is an amount which modulates or inhibits the B7/CD28/CTLA4 pathway. Dosages for a particular patient are described herein and can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

The administration of the humanized B7-1 antibody, humanized B7-2 antibody and/or other drugs can occur simultaneously or sequentially in time. These compounds or compositions can be administered before, after or at the same time. Thus, the term "co-administration" is used herein to mean that the humanized B7-1 and/or B7-2 antibodies and/or other compositions are administered at times to treat the diseases described herein or induce tolerization (e.g., methotrexate; rapamycin; cyclosporin; steroids; anti-CD40 pathway inhibitors such as anti-CD40 antibodies, anti-CD40 ligand antibodies and small molecule inhibitors of the CD40 pathway; transplant salvage pathway inhibitors such as mycophenolate mofetil (MMF); IL-2 receptor antagonists such as Zeonpax® from Hoffmann-la Roche Inc. and Simulet from Novartis, Inc. and analogs thereof). The methods of the present invention are not limited to the sequence in which the antibodies or compositions are administered, so long as they are administered close enough in time to produce the desired effect.

The invention also pertains to methods for determining the presence, absence or level of B7-2 or B7-1 using a humanized anti-B7-2 or B7-1 antibody, respectively. The presence or absence of B7-2 or B7-1 can be detected in an assay (e.g., ELISA, radioimmunoassay (RIA), FACS or Immunohistochemistry). The assay can be a direct detection or an indirect detection (e.g. a competitive assay).

For example, to determine the presence or absence of B7-2 or B7-1 using an ELISA assay in a suitable sample, the method comprises combining a suitable

sample with a composition comprising a humanized or murine anti-B7-2 or B7-1 antibody as detector (e.g., biotinylated anti-B7-2 or B7-1 mAb and HRP-streptavidin, or HRP-conjugated anti-B7-2 or B7-1 mAb) and a solid support (e.g., a microtiter plate), having an anti-B7-2 or B7-1 capture antibody bound (directly or indirectly) thereto. The detector antibody can bind to a different B7-2 or B7-1 epitope from that recognized by the capture antibody, under conditions suitable for the formation of a complex between the anti-B7-2 or B7-1 antibodies and B7-2 or B7-1, respectively. The method further comprises determining the formation of complex in the sample.

The presence of B7-2 or B7-1 can also be determined in a radioimmunoassay or a fluorescent assay. For example, the presence of B7-2 or B7-1 can be assessed by an immunobinding assay comprising obtaining a sample, contacting the sample with a composition comprising an anti-B7-2 or B7-1 antibody (e.g., a humanized or murine anti-B7-2 or B7-1 antibody comprising a radioactive or fluorescent label; or a humanized or murine anti-B7-2 or B7-1 antibody comprising a binding site for a second antibody which comprises a radioactive or fluorescent label), preferably in an amount in excess of that required to bind the B7-2 or B7-1, under conditions suitable for the formation of labeled complexes. The method further comprises determining (detecting or measuring) the formation of complex in the samples. Similarly, the present, absence or level of B7-1 or B7-2 can be determined using Fluorescent-Activated Cell Sorting (FACS) analysis, or histochemical analysis of tissues, using the humanized anti-B7-1 or B7-2 antibody of the present invention.

EXEMPLIFICATION

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

Example 1: Cloning and Sequencing of Mouse 3D1 Variable Region cDNAs:

Mouse 3D1 (also referred to as HF2.3D1) heavy and light chain variable region cDNAs were cloned from mRNA isolated from hybridoma cells using

anchored PCR (Co *et al., J. Immunol. 148:* 1149 (1992)). The 5' primers used annealed to the poly-dG tails added to the cDNA, and the 3' primers annealed to the constant regions. The amplified gene fragments were then inserted into the plasmid pUC18. Nucleotide sequences were determined from several independent clones for both V_L and V_H cDNA. For the heavy chain, a single, unique sequence was identified, typical of a mouse heavy chain variable region. For the light chain, two unique sequences, both homologous to murine light chain variable region sequences, were identified. However, one sequence was not functional because of a missing nucleotide that caused a frame shift at the V-J junction, and was identified as the non-productive allele. The other sequence was typical of a functional mouse kappa chain variable region. The variable region cDNA sequences of the heavy chain and the functional light chain and the translated amino acid sequences are shown in Figures 1A-1B. The mouse V_L sequence belongs to Kabat's mouse kappa chain subgroup I. The mouse V_H belongs to Kabat's heavy chain subgroup II(A).

Example 2: Design of humanized 3D1 variable regions:

To retain the binding affinity of the mouse antibody in the humanized antibody, the general procedures of Queen *et al.* were followed (Queen *et al., Proc. Natl. Acad. Sci. USA 86:* 10029 (1989), U.S. Patent Nos. 5,585,089 and 5,693,762, the teachings of which are incorporated herein in their entirety). The choice of framework residues can be critical in retaining high binding affinity. In principle, a framework sequence from any human antibody can serve as the template for CDR grafting; however, it has been demonstrated that straight CDR replacement into such a framework can lead to significant loss of binding affinity to the antigen (Tempest *et al., Biotechnology* 9: 266 (1992); Shalaby *et al., J. Exp. Med. 17*: 217 (1992)). The more homologous a human antibody is to the original murine antibody, the less likely the human framework will introduce distortions into the mouse CDRs that could reduce affinity. Based on a sequence homology, I2R was selected to provide the framework for the humanized 3D1 heavy chain and H2F for the humanized 3D1

light chain variable region. Manheimer-Lory, A. *et al., J. Exp. Med.* 174(6):1639-52 (1991). Other highly homologous human antibody chains would also be suitable to provide the humanized antibody framework, especially kappa light chains from human subgroup 4 and heavy chains from human subgroup 1 as defined by Kabat.

Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembling of the two chains. The I2R antibody shows a high homology to the 3D1 heavy and light chains and thus, was chosen to provide the framework for the initial humanized antibody model. The 3D1 light chain variable region, however, shows a significantly higher homology to the H2F framework compared to any others, including I2R. Therefore, H2F was chosen instead to provide the framework for the humanized 3D1 light chain variable region, while I2R was selected to provide the framework for the heavy chain variable region.

The computer programs ABMOD and ENCODE (Levitt *et al., J. Mol. Biol. 168:* 595 (1983)) were used to construct a molecular model of the 3D1 variable domain, which was used to locate the amino acids in the 3D1 framework that are close enough to the CDRs to potentially interact with them. To design the humanized 3D1 heavy and light chain variable regions, the CDRs from the mouse 3D1 heavy chain were grafted into the framework regions of the human I2R heavy chain and the CDRs from the mouse 3D1 light chain grafted into the framework regions of the human H2F light chain. At framework positions where the computer model suggested significant contact with the CDRs, the amino acids from the mouse antibody were substituted for the original human framework amino acids. For humanized 3D1, this was done at residues 27, 30, 48, 67, 68, 70 and 72 of the heavy chain and at residue 22 of the light chain. Furthermore, framework residues that occurred only rarely at their positions in the database of human antibodies were replaced by a human consensus amino acid at those positions. For humanized 3D1 this was done at residue 113 of the heavy chain and at residue 3 of the light chain.

The sequence of the humanized 3D1 antibody heavy chain and light chain variable regions is shown in Figures 2A-2B. However, many of the potential CDR-contact residues are amenable to substitutions of other amino acids that may still allow the antibody to retain substantial affinity to the antigen. Table 1 lists a number of positions in the framework where alternative amino acids may be suitable (LC = light chain, HC = heavy chain). The position specified in the table is the number of amino acids from the first amino acid of the mature chain, which is indicated by a double underline (Figures 2A-2B). For example, position LC-22 is the twenty second amino acid beginning from the doubled underlined Aspartic Acid, D, (or the forty second amino acid from the start codon).

Table 1. Amino Acids Substitutes and/or Alternatives

Position	Humanized 3D1	Alternatives
LC-22	S	N
HC-27	Y	G
HC-30	T	S
HC-48	I	M
HC-67	K	R
HC-68	A	V
HC-70	M	I
HC-72	V	A

Likewise, many of the framework residues not in contact with the CDRs in the humanized 3D1 heavy and light chains can accommodate substitutions of amino acids from the corresponding positions of I2R and H2F frameworks, from other human antibodies, from the mouse 3D1 antibody, or from other mouse antibodies, without significant loss of the affinity or non-immunogenicity of the humanized antibody. Table 2 lists a number of additional positions in the framework where alternative amino acids may be suitable.

Table 2. Framework Region Amino Acid Substitutes and/or Alternatives

Position	Humanized 3D1	Alternatives
LC-3	V	Q
HC-113	T	I

Selection of various alternative amino acids may be used to produce versions of humanized 3D1 that have varying combinations of affinity, specificity, non-immunogenicity, ease of manufacture, and other desirable properties. Thus, the examples in the above tables are offered by way of illustration, not of limitation.

Example 3: Construction of Humanized 3D1:

Once the humanized variable region amino acid sequences had been designed, as described above, genes were constructed to encode them, including signal peptides, splice donor signals and appropriate restriction sites (Figure 2A-2B). The light and heavy chain variable region genes were constructed and amplified using eight overlapping synthetic oligonucleotides ranging in length from approximately 65 to 80 bases (see He *et al.*, *J. Immunol. 160*: 1029 (1998)). The oligos were annealed pairwise and extended with the Klenow fragment of DNA polymerase I, yielding four double-stranded fragments. The resulting fragments were denatured, annealed, and extended with Klenow, yielding two fragments. These fragments were denatured, annealed pairwise, and extended once again, yielding a full-length gene. The resulting product was amplified by polymerase chain reaction (PCR) using Taq polymerase, gel-purified, digested with XbaI, gel-purified again, and subcloned into the XbaI site of the pVk for the expression of light chain and pVg4 or pVg2.M3 for the expression of heavy chains. The pVk vector for kappa light chain expression has been previously described (See Co *et al.*, *J. Immunol. 148*:1149 (1992)). The pVg4 vector for the γ 4 heavy chain expression was constructed by replacing the XbaI - BamHI fragment of pVg1 containing the γ1 constant region gene (See Co *et al.*, *J. Immunol. 148*: 1149 (1992)) with an approximately 2000 bp fragment of the human g4 constant region gene (Ellison and

Hood, *Proc. Natl. Acad. Sci. USA* 79: 1984 (1982)) that extended from the HindIII site preceding the C_H1 exon of the γ4 gene to 270 bp after the NsiI site following the C_H4 exon of the gene. The pVg2.M3 vector for the γ 2 heavy chain expression was described in Cole, *et al., J. Immunol. 159*: 3613 (1997). The pVg2.M3 is mutated from the human wildtype IgG2 by replacing the amino acids Val and Gly at positions 234 and 237 with Ala. This variant has a reduced interaction with its Fc receptors and thus has minimal antibody effector activity.

The structure of the final plasmids was verified by nucleotide sequencing and restriction mapping. All DNA manipulations were performed by standard methods well-known to those skilled in the art.

Two humanized 3D1, an IgG4 and an IgG2.M3, were generated for comparative studies. To construct a cell line producing humanized 3D1 (IgG4 or IgG2.M3), a light chain and the respective heavy chain plasmids were transfected into the mouse myeloma cell line Sp2/0-Ag14 (ATCC CRL 1581). Plasmids were also transfected into CHO cells using known methods in the art. Before transfection, the heavy and light chain-containing plasmids were linearized using restriction endonucleases. The kappa chain and the γ2 chain were linearized using FspI; the γ4 chain was linearized using BstZ17I. Approximately 20 μg of the light chain and a heavy chain plasmid was transfected into 1 x 10^7 cells in PBS. Transfection was by electroporation using a Gene Pulser apparatus (BioRad) at 360 V and 25 μFD capacitance according to the manufacturer's instructions. The cells from each transfection were plated in four 96-well tissue culture plates, and after two days, selection medium (DMEM, 10% FCS, 1xHT supplement (Sigma), 0.25 mg/mL xanthine, 1 μg/mL mycophenolic acid) was applied.

After approximately two weeks, the clones that appeared were screened for antibody production by ELISA. Antibody from a high-producing clone was prepared by growing the cells to confluency in regular medium (DMEM with 10% FCS), then replacing the medium with a serum-free medium (Hybridoma SMF; Gibco) and culturing until maximum antibody titers were achieved in the culture. The culture supernatant was run through a protein A-Sepharose column

(Pharmacia); antibody was eluted with 0.1 M Glycine, 100 mM NaCl, pH 3, neutralized and subsequently exchanged into phosphate-buffered saline (PBS). The purity of the antibody was verified by analyzing it on an acrylamide gel, and its concentration was determined by an OD_{280} reading, assuming 1.0 mg of antibody protein has an OD_{280} reading of 1.4.

Example 4: Affinity of Humanized Anti-B7-2 Antibody:

Competitive Binding Assay:

The relative affinities, of the murine and humanized 3D1 antibodies for the B7-2 antigen were determined by competitive binding assays. Three-fold serial dilutions of unlabeled humanized or murine 3D1 antibodies were mixed with a fixed amount of radio-iodinated murine 3D1 antibody (40,000-50,000 cpm per test in PBS containing 2% fetal calf serum).

1 x 10^5 CHO cells expressing cell surface rhB7-2 (CHO/hB7-2) were added subsequently and the mixture (in a total volume of 200 µL) was incubated for 2 hr at 4° C with gentle shaking. The cell-antibody suspension was then transferred to Sarstedt Micro Tubes (part #72.702) containing 100 µL of 80% dibutyl phthalate-20% olive oil. After centrifugation in a microfuge, the Sarstedt tubes were plunged into dry ice for several minutes. Cell-bound ^{125}I was determined by clipping tips of each tube (containing cell pellets) into scintillation vials and counting in a gamma counter. Bound and free counts were determined and the ratio plotted against the concentrations of the cold competitor antibodies according to the method of Berzofsky and Berkower (J. A. Berzofsky and I. J. Berkower, in Fundamental Immunology 9ed. W.E. Paul), Raven Press (New York), 595 (1984)).

Cell Line:

Recombinant Chinese Hamster Ovary (CHO) cell lines expressing hB7-2 on their membrane surfaces were cloned from cells transfected with B7-2 cDNA sequence and G418 resistance. Expression of hB7-2 on the CHO cell surface over

many passages under selective pressure has been confirmed using murine anti-B7 antibodies and FACS analysis.

Preparation of ^{125}I labeled anti-hB7 mAb and characterization:

Anti-hB7 antibodies were labeled with ^{125}I by reaction with ^{125}I-Bolton-Hunter reagent according to manufacturers instructions (Amersham Corp., Arlington Hts, IL). Protein was separated from free reagent with a NAP-25 column. An HPLC size-exclusion column was used to confirm that the antibodies remained intact and were not aggregated, and to measure protein concentration against standards prepared from non-labeled antibody. Labeling typically resulted in 4 to 8 microcuries per microgram of protein, or approximately 30 to 60% of the antibody molecules labeled.

Results:

The competitive binding graph is shown in Figure 3. Each data point represents the average of triplicate determinations. Results showed that both humanized IgG4 and humanized IgG2.M3 anti-human B7-2 antibodies have a similar high binding affinity as the murine anti-human B7-2 antibody (approximately 1 x 10^9 M^{-1}), indicating no loss of affinity for B7-2 in the humanization of 3D1. Both murine and humanized anti-B7-2 recognize cell surface expressed hB7-2 with high affinity.

Example 5. Direct binding of Humanized Anti-B7 mAbs to CHO/hB7 Cells:

Cell binding assay:

Binding assays were begun by plating cells onto 96-well tissue culture plates at 10,000 CHO/hB7-2 cells per well. Two days later, adherent cells were gently washed with assay buffer containing nonfat dry milk protein (for blocking nonspecific binding) and sodium azide (to prevent internalization of antibodies by cells). For direct binding assays, ^{125}I-labeled anti-B7 antibodies (^{125}I -murine anti-human B7-2; 826 cpm/fmol; humanized anti-human B7-2, 883 cpm/fmol) were

serially diluted in assay buffer and incubated on cells overnight, allowing antibodies to bind to cell-surface B7 and come to equilibrium. Unbound antibody was gently washed from cells, and bound ^{125}I-labeled antibody was detected using an ^{125}I scintillant and photodetector system. Non-specific binding to CHO cells was determined for each dilution in the same manner, but on cells expressing the B7-1 molecule that is not recognized by the antibody being tested.

Results:

The direct binding graph is shown in Figure 4. The data, means of triplicate wells with nonspecific binding subtracted, were fit to a hyperbolic saturation curve using Graphpad PrismJ software. K_D of the antibodies determined as the concentration corresponding to half-maximal binding indicated that the murine and humanized anti-B7-2 mAbs had similar and high binding affinities (~10^{-9}m) for B7-2. Both murine and humanized anti-B7-2 antibodies recognize cell surface expressed hB7-2 with high affinity.

Example 6: Binding of Humanized Anti-B7 mAbs to Protein Ligands:

Affinity determination by BIACORE®:

The BIACORE® biosensor (BIACORE®; Uppsalla, Sweden) was used to determine binding kinetics of murine and humanized anti-B7-2 human antibodies to human B7-2Ig. Human B7-2Ig (hB7-2Ig) was immobilized onto the dextran matrix of a BIACORE® sensor chip. Humanized and murine anti-human B7-2 were tested at 200, 100, 50, and 20 nM. Each dilution was tested 4 times per run and a total of three separate runs performed. Anti-human B7-2 antibody binding was measured in real time by Surface Plasmon Resonance (SPR) and global analysis was performed using the bivalent binding model in BIA evaluation software (version 3.1). For each sample, the association (k_a), dissociation (k_d), and equilibrium dissociation constant (K_D) were determined.

Preparation of hB7-2 Ig:

A soluble form of hB7-2Ig was recovered from culture medium of CHO cells engineered to secrete this protein. Recombinant hB7-2Ig was derived by fusing the DNA coding sequences corresponding to the extracellular domain of B7-2 gene to the hinge-CH2-CH3 domains of the human IgG1 heavy chain. Recombinant hB7-2Ig was purified from the culture medium by protein A.

Results:

Table 3 reports the mean values obtained for both murine and humanized anti-human B7-2 mAbs. The binding constants for the murine and humanized anti-B7-2 mAbs determined by SPR shows that the murine and humanized forms of the anti-B7-2 mAbs are similar and that the murine anti-B7-2 mAb has a slightly higher binding constant for the immobilized hB7-2 Ig than does the humanized anti-B7-2. The approximately 2.8 fold higher affinity calculated for the murine anti-B7-2 mAb may represent a real, but slight difference between the murine and humanized anti-B7-2 mAbs introduced during the humanization process. Another possibility may be due to technical variation in the preparation, processing and analysis of these antibodies. As shown in Examples 4, 5, and 7, a difference was not observed in humanized hB7-2 binding affinity in cell based assays.

Table 3: Affinity of anti-B7-2 mAbs as determined by BIACORE®

mAb	Mean K_D
murine Anti-B7-2	1.8×10^{-9} M
humanized Anti-B7-2	5.1×10^{-9} M

Example 7. Inhibition of T cell costimulation by humanized anti-B7-2.

$CD28^+$T cell/CHO-B7 proliferation assay

$CD28^+$ T cells, isolated as described herein, were washed once and resuspended in RPMI complete medium, supplemented with 2 ng/mL PMA (Calbiochem), to a cell density of $5x10^5$ cells/mL. The $CD28^+$ T cells (100 µL, $5x10^4$ cells) were added to the antibody/CHO/hB7-2 mixture (see below), incubated for 3

days at 37°C, 5% CO_2, and T cell proliferation was measured by pulsing for the last 6 hours of culture with 1 uCi of [^{3}H]-thymidine (NEN, Boston, MA). The cells were harvested on a filter and the incorporated radioactivity was measured in a scintillation counter.

Materials:

$CD28^+$ human T cells were isolated by negative selection with immunoabsorption from human peripheral blood lymphocytes, as described (June *et al., Mol. Cell. Biol. 7*:4472-4481 (1987)). Buffy coats were obtained by leukophoresis of healthy human donors and peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation. Monocytes were depleted from the PBL by plastic absorption. $CD28^+$ T cells were isolated from the non-adherent cells by negative selection using antibodies to CD11, CD20, CD16 and CD14, (this set of antibodies will coat all B cells, monocytes, large granular lymphocytes, and $CD28^-$ T cells) and magnetic bead separation using goat anti-mouse immunoglobulin-coated magnetic particles.

CHO/hB7-2 cells were detached from the tissue culture plates by incubation in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) with 0.5 mM EDTA, washed, and fixed with freshly prepared paraformaldehyde.

Various concentrations of anti-B7-2 antibody (in duplicate) were preincubated for 1 hour at 37°C, 5% CO_2 with $1x10^4$ CHO/hB7-2 cells in 100 µL RPMI complete medium (RPMI 1640 medium, 10% fetal bovine serum (FBS),100 U/mL penicillin, 100 µg/mL streptomycin) in a microtiter plate (flat-bottom, 96-well, Costar, Cambridge, MA).

Results:

Figure 5 shows the results of the inhibition of human $CD28^+$T cell proliferation by the murine and humanized anti-hB7-2 mAbs. Both antibodies exhibit dose dependent inhibition of B7-2 driven T cell proliferation with similar IC_{50} (Inhibitory concentration 50%; amount of antibody required to inhibit the

maximal T cell proliferation by 50%) values of 72 pm (murine anti-hB7-2) and 50 pm (humanized anti-hB7-2) indicating that both antibodies were similar and very effective in inhibiting the B7-2 T cell stimulatory signal. This demonstrates that the high affinity anti-B7-2 mAbs can block B7-2 functionality by inhibiting (e.g., preventing) the activation and/or proliferation of human T cells. These mAbs are expected to exhibit similar capability in *in vivo* use to inhibit T cell response.

Example 8: Cloning and sequencing of mouse 1F1 variable region cDNAs

Mouse 1F1 heavy and light chain variable region cDNAs were cloned from mRNA isolated from hybridoma cells using anchored PCR (Co *et al., J. Immunol.* 148: 1149 (1992)). The 5' primers used annealed to the poly-dG tails added to the cDNA, and the 3' primers annealed to the constant regions. The amplified gene fragments were then inserted into the plasmid pUC19. Nucleotide sequences were determined from several independent clones for both V_H and V_L cDNA. For the heavy chain, a single, unique sequence was identified, typical of a mouse heavy chain variable region. For the light chain, two unique sequences, both homologous to mouse light chain variable regions, were identified. However, one sequence was not functional because of a missing nucleotide that caused a frame shift at the V-J junction, and was identified as the non-productive allele. The other sequence was typical of a functional mouse kappa chain variable region. The variable region cDNA sequences of the heavy chain and the functional light chain, and the translated amino acid sequences are shown in Figures 6A and 6B, respectively. The mouse V_H belongs to Kabat's heavy chain subgroup II(C). The mouse V_K sequence belongs to Kabat's mouse kappa chain subgroup IV.

Example 9: Design of humanized 1F1 variable regions

To retain the binding affinity of the mouse antibody in the humanized antibody, the general procedures of Queen *et al.* were followed (Queen *et al., Proc. Natl. Acad. Sci. USA* 86: 10029 (1989) and U.S. Patent Nos. 5,585,089 and 5,693,762). The choice of framework residues can be critical in retaining high binding affinity. In principle, a framework sequence from any human antibody can serve as the template for CDR grafting; however, it has been demonstrated that straight CDR replacement into such a framework can lead to significant loss of binding affinity to the antigen (Tempest *et al., Biotechnology* 9: 266 (1992); Shalaby *et al., J. Exp. Med.* 17: 217 (1992)). The more homologous a human antibody is to the original mouse antibody, the less likely that the human framework will introduce distortions into the mouse CDRs that could reduce affinity. Based on a sequence

homology search against the Kabat antibody sequence database (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th ed., U.S. Department of Health and Human Services (1991)),III-2R (Manheimer-Lory *et al.*, *J. Exp. Med.* 176: 309 (1992)) was selected to provide the framework for both the humanized 1F1 heavy chain variable region and for the humanized 1F1 light chain variable region. Other highly homologous human antibody chains would also be suitable to provide the humanized antibody framework, especially heavy chains from human subgroup 1 and kappa light chains from human subgroup 1 as defined by Kabat.

Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembly of the two chains. The III-2R antibody shows a high homology to the 1F1 heavy and light chains and thus was chosen to provide the framework for the humanized antibody. The humanized 1F1 heavy chain variable domain has 69 residues out of 87 framework residues that are identical to those of the mouse 1F1 heavy chain framework, or 79% sequence identity. The humanized 1F1 light chain variable domain has 55 residues out of 80 framework residues that are identical to those of the mouse 1F1 light chain framework, or 69% sequence identity.

The computer programs ABMOD and ENCAD (Levitt *et al.*, *J. Mol. Biol.* 168: 595 (1983)) were used to construct a molecular model of the 1F1 variable domain, which was used to locate the amino acids in the 1F1 framework that are close enough to the CDRs to potentially interact with them. To design the humanized 1F1 heavy and light chain variable regions, the CDRs from the mouse 1F1 heavy chain were grafted into the framework regions of the human III-2R heavy chain and the CDRs from the mouse 1F1 light chain were grafted into the framework regions of the human III-2R light chain. At framework positions where the computer model suggested significant contact with the CDRs, the amino acids from the mouse antibody were substituted for the original human framework amino acids. For humanized 1F1, this was done at residues 1, 24, 27, 28, 29, 30, 48, 67, and 68 of the heavy chain and at residues 47 and 72 of the light chain. Furthermore,

framework residues that occurred only rarely at their positions in the database of human antibodies were replaced by human consensus amino acids at those positions. For humanized 1F1 this was done at residues 16, 74, and 113 of the heavy chain and at residue 44 of the light chain. Overall, the humanized 1F1 heavy chain variable domain has 88 residues that are identical to the human III-2R heavy chain variable domain, and the humanized 1F1 light chain variable domain has 88 residues that are identical to the III-2R light chain variable domain.

The sequences of the humanized 1F1 antibody heavy chain and light chain variable regions are shown in Figures 7A and 7B. However, many of the potential CDR-contact residues are amenable to substitutions of other amino acids that still allow the antibody to retain substantial affinity to the antigen. Table 4 lists a number of positions in the framework where alternative amino acids may be suitable (LC = light chain, HC = heavy chain).

Table 4.

Position	Humanized 1F1	Alternatives
HC-1	E	Q
HC-24	P	A
HC-27	F	G,Y
HC-28	N	T
HC-29	I	F
HC-30	K	S,T
HC-48	I	M
HC-67	K	R
HC-68	A	V
LC-72	Y	F

Likewise, many of the framework residues not in contact with the CDRs in the humanized 1F1 heavy and light chains can accommodate substitutions of amino acids from the corresponding positions of the III-2R framework, from other human antibodies, from the mouse 1F1 antibody, or from other mouse antibodies, without significant loss of the affinity or non-immunogenicity of the humanized antibody.

Table 5 lists a number of additional positions in the framework where alternative amino acids may be suitable.

Table 5

Position	Humanized 1F1	Alternatives
HC-16	A	S
HC-74	T	K
HC-113	T	I
LC-44	A	S,V

Selection of various alternative amino acids may be used to produce versions of humanized 1F1 that have varying combinations of affinity, specificity, non-immunogenicity, ease of manufacture, and other desirable properties. Thus, the examples in the above tables are offered by way of illustration, not of limitation.

Example 10: Construction of humanized 1F1

Once the humanized variable region amino acid sequences had been designed as described above, genes were constructed to encode them, including signal peptides, splice donor signals and appropriate restriction sites (Figures 7A and 7B). The heavy and light chain variable region genes were constructed and amplified using eight overlapping synthetic oligonucleotides ranging in length from approximately 65 to 80 bases (see He *et al.*, *J. Immunol.* 160: 1029 (1998)). The oligos were annealed pairwise and extended with the Klenow fragment of DNA polymerase I, yielding four double-stranded fragments. The resulting fragments were denatured, annealed pairwise, and extended with Klenow, yielding two fragments. These fragments were denatured, annealed pairwise, and extended once again, yielding a full-length gene. The resulting product was amplified by polymerase chain reaction (PCR) using Taq polymerase, gel-purified, digested with XbaI, gel-purified again, and subcloned into the XbaI site of pVg2.M3 for the expression of heavy chain, and pVk for the expression of light chain. The pVg2.M3 vector for human γ 2 heavy chain expression has been previously described (Cole *et*

al., J. Immunol. 159: 3613 (1997)). The human γ2 constant region in the pVg2.M3 plasmid is mutated from the wildtype human γ2 constant region by replacing the amino acids Val and Gly at positions 234 and 237 with Ala. This variant has a reduced interaction with its Fc receptors and thus has minimal antibody effector activity. The pVk vector for human kappa light chain expression has been previously described (see Co *et al., J. Immunol.* 148: 1149 (1992)).

The structures of the final plasmids were verified by nucleotide sequencing and restriction mapping. All DNA manipulations were performed by standard methods well-known to those skilled in the art.

An IgG2.M3 form of the humanized 1F1 antibody was generated for binding studies. To construct a cell line producing humanized 1F1, the heavy and light chain plasmids were transfected into mouse myeloma cell line Sp2/0-Ag14 (ATCC CRL 1581). Before transfection, the heavy and light chain plasmids were linearized using restriction endonucleases. The γ2 heavy chain plasmid and the kappa light chain plasmid were linearized using FspI. Approximately 40 μg of the heavy chain plasmid and 20 μg of the light chain plasmid were transfected into 1 x 10^7 cells in PBS. Transfection was by electroporation using a Gene Pulser apparatus (BioRad) at 360 V and 25 μFD capacitance according to the manufacturer's instructions. The cells from each transfection were plated in four 96-well tissue culture plates, and after two days selection medium (DMEM, 10% FCS, 1 x HT supplement (Sigma), 0.25 mg/mL xanthine, 1 μg/mL mycophenolic acid) was applied.

After approximately two weeks, the clones that appeared were screened for antibody production by ELISA. Antibody from a high-producing clone was prepared by growing the cells to confluency in regular medium (DMEM with 10% FCS), then replacing the medium with a serum-free medium (Hybridoma SFM; GIBCO) and culturing until maximum antibody titers were achieved in the culture. The culture supernatant was run through a protein A-Sepharose column (Pharmacia); antibody was eluted with 0.1 M Glycine, 100 mM NaCl, pH 3, neutralized, and subsequently exchanged into phosphate-buffered saline (PBS). The purity of the antibody was verified by analyzing it on an acrylamide gel, and its

concentration was determined by an OD_{280} reading, assuming 1.0 mg of antibody protein has an OD_{280} reading of 1.4.

An IgG4 form of the humanized 1F1 antibody was also generated and purified following the methods described above.

In order to permit high level expression in CHO cells, the complete humanized human 1F1 (h1F1) and human 3D1 (h3D1) light chain and heavy chain genes were each independently subcloned into the selectable, amplifiable expression vector pED (Kaufman R.J., *et al.*, *Nucl Acids Res.*, 19:4485-4490 (1991)). The pED-derived expression plasmids were sequenced to confirm that they encoded the appropriate h1F1 and h3D1 light and heavy chains. The penultimate amino acid of the lgG2m3 CH3 domain was found to be serine in contrast to the glycine residue reported at this position for all published lgG2 and lgG1 sequences. This serine was replaced with the more common glycine for the pED expression constructs. The h1F1 light chain and heavy chain expression plasmids (pED.1F1v2KA and pED.1F1v2G2m3gly) were linearized and cotransfected into the CHO PA-DUKX.153.8 cell line, which has been pre-adapted for growth in serum-free suspension culture (Sinacore M.S. *et al.*, *Biotechnol. Bioeng.* 52:518-528 (1996)). Cell lines expressing h3D1 were generated in the same manner by cotransfection with linearized plasmids pED.3D1KA and pED.3D1G2m3gly. In each case, stable integration of light chain and heavy chain genes into the CHO cell genome, followed by methotrexate selection and amplification, resulted in recombinant h1F1 or h3D1 cell lines. The CHO cell lines expressing anti-B7-1 or anti-B7-2 were cultured in serum-free growth medium and the secreted antibodies purified from the conditioned culture supernatant by chromatography on protein A sepharose. Bound antibody was eluted in acid buffer followed by neutralization to pH 7.0 with. The purified antibody was buffer exchanged into PBS and sterile filtered.

Example 11: Properties of humanized 1F1

The affinity of the mouse and humanized 1F1 antibodies for the B7-1 antigen was determined by competitive binding with radio-iodinated humanized 1F1 antibody. Three-fold serial dilutions of unlabeled mouse or humanized 1F1 antibodies were mixed with a fixed amount of radio-iodinated humanized 1F1 antibody (40,000 - 50,000 cpm per test) in PBS containing 2% fetal calf serum, 0.1% sodium azide. 3 x 10^4 CHO cells expressing cell surface rhB7-1 were added subsequently and the mixture (in a total volume of 200 µL) was incubated for 2 hr at 4° C with gentle shaking. The cell-antibody suspension was then transferred to Sarstedt Micro Tubes (part #72.702) containing 100 µL of 80% dibutyl phthalate-20% olive oil. After centrifugation in a microfuge, the Sarstedt tubes were plunged into dry ice for several minutes. Cell-bound ^{125}I was determined by clipping the tips of each tube (containing cell pellets) into scintillation vials and counting in a gamma counter. Bound and free counts were determined and the ratio plotted against the concentrations of the cold competitor antibodies according to the method of Berzofsky and Berkower (J. A. Berzofsky and I. J. Berkower, in *Fundamental Immunology*, W.E. Paul, Raven Press, New York, pp. 595-644 (1984)).

The competitive binding graphs are shown in Figure 8. Each data point represents the average of triplicate determinations. The results showed that the IgG2.M3 antibody has a similar binding affinity to that of the mouse antibody (approximately 1 x 10^9 M^{-1}), indicating no loss of affinity in the humanization of 1F1.

The affinity of the mouse and humanized 1F1 antibodies for the B7-1 antigen was confirmed by Scatchard analysis of binding of radiolabeled antibodies. Two-fold serial dilutions of radiolabeled mouse or humanized 1F1 antibodies were incubated with 5 x 10^4 CHO cells expressing cell surface rhB7-1 in PBS containing 2% FCS, 0.1% sodium azide in a total volume of 200 µL. The mixture was incubated for 6 hr at 4°C with gentle shaking. The cell-antibody suspension was then transferred to Sarstedt Micro Tubes (part #72.702) containing 100 µL of 80% dibutyl phthalate-20% olive oil. After centrifugation in a microfuge, the Sarstedt tubes were plunged into dry ice for several minutes. Cell-bound ^{125}I was determined

by clipping the tips of each tube (containing cell pellets) into scintillation vials and counting in a gamma counter. Bound and free counts were determined and the ratio plotted against the concentration of bound antibody following the method of Scatchard (Scatchard, *Ann. N. Y. Acad. Sci.* 51: 660 (1949)). A least squares method was used to fit a line to the data, and the apparent Ka was determined from the slope of the line.

The Scatchard plots are shown in Figures 9A and 9B. Each data point represents the average of duplicate determinations. The results showed that the IgG2.M3 antibody has a similar binding affinity to that of the mouse antibody (approximately 1 x 10^9 M^{-1}), confirming no loss of affinity in the humanization of 1F1.

Example 12: Affinity of Humanized anti-B7-1 Monoclonal Antibody

Competitive Binding Assay:

The relative affinities of the murine and humanized anti-B7-1 (1F1) antibodies for the B7-1 antigen were determined by competitive binding assays. Three-fold serial dilutions of the unlabeled murine or humanized anti-B7-1 mAbs were mixed with a fixed amount of radio-labeled murine anti-B7-1 mAb (^{125}I-anti-B7-1, 2800 cpm/fmol; 40,000-50,000 cpm/test in PBS containing 2% fetal calf serum). 1 x 10^5 CHO/hB7-1 cells expressing hB7-1 on their surface were added subsequently and the mixture (total volume = 200 µL) incubated for 2 hrs at 4°C with gentle shaking. The cell antibody mixture was then transferred to Sarstedt Micro tubes (Part # 72.702) containing 100 µL of 80% dibutyl phthlate-20% olive oil. After centrifugation in a microfuge, the Sarstedt tubes were plunged into dry ice for several minutes. Cell bound ^{125}I-labeled mAb was determined by clipping the cell pellet containing tips of each tube into scintillation vials and counting in a gamma counter. Bound and free counts were determined. The bound counts were plotted against the concentration of the cold competitor mAbs.

CHO/hB7-1 cell line:

A recombinant Chinese Hamster Ovary (CHO) cell line expressing hB7-1 on its surface was cloned form CHO cells transfected with the hB7-1 cDNA sequence and G418 resistance marker. Stable expression of the hB7-1 on the CHO cell surface over many passages under selective pressure has been confirmed using the murine anti-B7-1 mAb and FACS analysis.

Preparation of ^{125}I-labeled anti-B7-1 mAbs:

Murine and Humanized anti-B7-1 mAbs were labeled with ^{125}Iodine by reaction with ^{125}I-Bolton Hunter reagent according to the manufacturers' instructions (Amersham Corp, Arlington Heights, IL). Protein was separated from free reagent with a NAP-25 column. HPLC size-exclusion chromatography was used to verify antibody integrity and aggregation state post labeling and to determine protein concentration. Labeling typically resulted in 4 to 8 microcuries ^{125}I/μg of mAb with an estimated 30 to 60% of the antibody molecules labeled. The murine and humanized anti-B7-1 mAbs had a specific activity of 2800 cpm/fmol and 950 cpm/fmol, respectively.

Results:

The graphical representation of the competitive binding data is shown in Figure 10. Each data point represents the average of triplicate determinations. Results show that both the humanized anti-B7-1 mAb and the murine anti-B7-1 mAb from which it was derived have a similar high affinity for B7-1 (approximately 1×10^{-9} M) indicating no loss of affinity for the humanized anti-B7-1 mAb. Both the murine and humanized anti-B7-1 compete similarly and effectively with labeled murine anti-B7-1 mAb for binding to cell surface expressed B7-1

Example 13: Direct Binding of Humanized anti-B7-1 mAb to B7-1

Cell Binding assay:

Binding assays were begun by plating CHO/hB7-1 cells onto 96 well tissue culture plates at 10,000 cells/well in complete medium and the plates incubated at

37°C for two days. Adherent cells were washed gently with assay buffer (PBS containing nonfat dry milk and sodium azide). Murine and humanized anti-B7-1 mAbs labeled with ^{125}I were serially diluted in assay buffer and incubated with the cells overnight at 4°C to allow biding to reach equilibrium. Unbound labeled antibody was removed from the cells by a series of gentle washings with assay buffer and the bound 125I-labeled antibody was detected using a ^{125}I scintillant and detector system. Non-specific binding was determined for each dilution of labeled antibody in an identical manner as described above except that the target CHO cells expressed hB7-2 which is not bound by either the murine or humanized anti-B7-1 mAbs.

Results:

The graphical depiction of the results of the direct binding experiment is shown in Figure 11. The mean value of triplicate determinations minus the non-specific binding was calculated and fit to a hyperbolic saturation binding curve using Graphpad Prism software. Binding constants (K_D), determined as the concentration of antibody corresponding to half-maximal saturation of binding sites, for both the murine and humanized antibodies indicated that both antibodies had similar and high affinities for B7-1 (approximately 10^{-9}M). Both murine and humanized anti-B7-1 mAbs recognize cell surface expressed human B7-1

Example 14: Binding of Murine and Humanized anti-B7-1 mAbs to Protein Ligands:

Affinity determination by BIACORE®:

The BIACORE® biosensor (BIACORE®, Uppsalla, Sweden) was used to determine binding kinetics of murine and humanized anti-B7-1 monoclonal antibodies to human B7-1Ig (hB7-1Ig) protein. Human B7-1Ig was immobilized onto the dextran matrix of a BIACORE® sensor chip. Humanized and murine anti-B7-1 mAbs were tested at 200, 100, 50, and 20nM on the immobilized hB7-1Ig. Each mAb dilution was tested 4 times per run and a total of three separate runs were performed. Anti-human B7-1Ig binding was measured in real time by Surface Plasmon Resonance (SPR) and global analysis was performed using the bivalent binding model in BIA evaluation software (Version 3.1). For each sample, the association (k_a), dissociation (k_d) and equilibrium dissociation constant (K_D) were determined.

Table 6 reports the mean values determined for both the murine and humanized anti-B7-1 mAbs. The binding constants for the murine and humanized anti-B7-1mAbs determined by SPR shows that the murine and humanized forms of the anti-B7-1 mAbs are similar and that the murine anti-B7-1 mAb had a slightly higher binding affinity for the hB7-1Ig protein than did the humanized anti-B7-1. The 5 fold higher binding affinity found for the murine anti-B7-1 mAb may

represent a real but slight difference between the murine and humanized anti-B7-1 mAbs introduced during the humanization process. Alternatively, technical variations in the preparation, processing, and analysis of the individual mAbs may explain these minor differences. As shown in Examples 12, 14, and 19, no difference was observed between the murine and humanized anti-B7-1 mAbs in cell binding or functional assays.

Table 6. Affinity of anti-B7-1 mAbs as determined by BIACORE®

mAb	Mean K_D	Std deviation
Murine anti-B7-1	5.6×10^{-10} M	1.9×10^{-10} M
Humanized anti-B7-1	2.8×10^{-9} M	1.2×10^{-9} M

Preparation of hB7-1Ig:

A soluble form of hB7-1Ig was recovered from culture medium of CHO cells engineered to secrete this protein. Recombinant hB7-1Ig was derived by fusing the DNA sequences encoding the extracellular domain of hB7-1 gene to the hinge-CH2-CH3 domains of human IgG1 heavy chain. Recombinant protein was purified from culture medium by protein A chromatography.

Example 15. Inhibition of T cell costimulation by humanized B7-1 mAb.

$CD28^+$ T cell/CHO-B7 proliferation assay

$CD28^+$ T cells, isolated as described herein, were washed once and resuspended in RPMU complete medium supplemented with 2 ng/mL PMA (Calbiochem) to a cell density of 5×10^5 cells/mL. The $CD28^+$ T cells (100 µL; 5×10^4 cells) were added to the antibody/CHO/hB7-1 cell mixture (see below), incubated for 3 days at 37°C, 5% CO_2, and the T cell proliferation measured by pulsing for the last 6 hours of culture with 1 uCi of [^{3}H]-thymidine (NEN, Boston, MA). The cells were harvested on a filter and the incorporated radioactivity was measured by scintillation counting.

Materials:

$CD28^+$ human T cells were isolated by negative selection with immunoabsorption from human peripheral blood lymphocytes as described (June *et al., Mol. Cell Biol.* 7:4472-4481 (1987)). Briefly, buffy coats were obtained by leukophoresis of healthy human donors and the peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation. Monocytes were depleted from the PBLs by adsorption onto plastic. $CD28^+$ T cells were isolated from the non-adherent cells be negative selection using antibodies to CD11, CD20, CD16, and CD14 (this set of antibodies will coat B cells, monocytes, large granular lymphocytes, and $CD28^-$ T cells) and magnetic bead separation using goat anti-mouse immunoglobulin-coated magnetic beads.

CHO/hB7-1 cells were detached from the tissue culture plates by incubation with phosphate -buffered saline lacking Ca^{2+} or Mg^{2+} with 0.5 mM EDTA. The cells were washed and then fixed with freshly prepared paraformaldehyde.

Various concentrations of the anti-B7-1 antibody (in duplicate) were preincubated with 1 x 10^4 CHO/hB7-1 cells for 1 hour at 37°C in 100 µL RPMI complete medium (RPMI 1640, 10 % FBS, 100U/mL penicillin, 100 µg/mL streptomycin) in a microtiter plate (flat bottomed, 96 well, Costar, Cambridge MA)

Results:

Figure 12 shows the results of the inhibition of human $CD28^+$ T cell proliferation by the murine and humanized forms of the anti-B7-1 mAbs. Both monoclonal antibodies exhibit dose dependent inhibition of B7-1 driven proliferation of human T cells with similar IC50 (Inhibitory concentration 50%, amount of antibody required to inhibit maximal T cell proliferation by 50%) values of 110 pM (humanized anti-B7-1) and 220 pM (murine anti-B7-1) indicating that both antibodies were similar and very effective in inhibiting the B7-1 T cell co-stimulatory signal. This demonstrates that the high affinity anti-B7-1 mAbs can block B7-1 functionally by inhibiting or preventing the activation and/or proliferation of human T cells. These mAbs are expected to exhibit similar capability in *in vivo* use to inhibit T cell responses.

Example 16. Inhibition of mixed lymphocyte reactions by anti-B7-1 and anti-B7-2 mAbs

Mixed lymphocyte reactions (MLR): Human normal peripheral blood lymphocytes (PBL) (responders) were cultured with irradiated (2,500 cGy) normal donor PBL (stimulators) in RPMI 1640 containing 5% heat-inactivated human AB serum at 37°C in 5% CO2 at a final concentration of 10^6 cells/mL. Where indicated, murine anti-hB7-1 or murine anti-hB7-2 antibodies were added alone (10 μg/mL), in combination (10 μg/mL each), and in comparison with CTLA4Ig (10 or 20 μg/mL). Cells were cultured in triplicate in microtiter plates in a final volume of 200 μL and proliferation was assessed by [^{3}H]-thymidine incorporation for the last 16 hours of culture. Secondary MLR was performed using the cells derived from the primary MLRs as responders. These cells were washed, cultured overnight, and restimulated as above using the same or different, third party stimulator PBLs. No inhibitors were added to the secondary MLRs.

Results:

The determinations shown in Figure 13 were made by performing primary one-way MLRs in the absence or presence of B7 inhibitors (anti-B7, CTLA4Ig). The proliferation was measured after 3,4, or 5 days of culture.

In the primary MLR, the additional anti-B7-1 mAb alone had no inhibitory effect indicating a minor role for B7-1 alone in driving proliferation of responder T cells. Anti-B7-2 alone inhibited T cell proliferation on all days tested at a level comparable to human CTL4Ig (hCTL4Ig), a recombinant protein known to bind to both B7-1 and B7-2. The combination of anti-B7-1 and anti-B7-2 was the most effective inhibitor of T cell proliferation that completely inhibited this response on all days tested. The superior ability of the combined anti-B7-1 and anti-B7-2 to inhibit T cell proliferation, as compared to hCTL4Ig, reflects the higher affinity of the anti-B7 mAbs for B7-1 and B7-2 as compared to hCTL4Ig. The combined anti-B7-1 and anti-B7-2 mAbs were better inhibitors of T cell proliferation than anti-B7-

2 alone, demonstrating the need to block both stimulatory receptors to completely inhibit T cell responses. These results show that complete blockade of the B7-1 and B7-2 costimulators more completely abrogates alloresponsiveness in the MLR. Accordingly, these results indicate that methods of treatment including both anti-B7-1 and anti-B7-2 antibodies will be even more effective than either of the antibodies alone, especially where both co-stimulatory molecules are functional. While the responder/stimulator pair, described herein, was not sensitive to inhibition by anti-B7-1 alone, some responder/stimulator pairs do exhibit moderate (0-50%) anti-B7-1 sensitivity.

To determine whether treatment with anti-B7 mAbs in the primary MLR had resulted in the development of T cell hyporesponsiveness or anergy, the responder T cells from the primary MLRs were tested in secondary MLRs where the stimulators were either from the same donor as the primary MLR or from a third party. Figure 14 shows that the responder T cells obtained from the primary MLR that was treated with anti-B7-1 alone show full proliferative responses to both the original sensitizing cells and to third party cells when tested in a secondary MLR with no immunosuppression indicating that blocking the B7-1 receptor alone by treatment with the anti-B7-1 mAb had no tolerizing effect on these responding T cells. This is in contrast to the lack of response to the primary stimulators seen in the secondary MLR when the primary MLR was treated with anti-B7-2 alone. The results in Figure 15 show that the responder T cells from the primary MLR treated with anti-B7-2 alone failed to respond to the same stimulators as used in the primary MLR but retained normal proliferative response to third party, unrelated stimulators indicating that these responder T cells were rendered tolerant to the original stimulator PBLs by treatment with anti-B7-2 and that the tolerization was specific for the stimulator antigens present in the primary MLR. With this responder/stimulator pair, treatment with anti-B7-2 alone resulted in tolerance to the stimulator cells; however, with other responder/ stimulator pairs, the induction of tolerance may not be complete.

Figure 16 shows that the responder T cells from the primary MLR treated with anti-B7-1 and anti-B7-2 failed to respond to the same stimulators as used in the

primary MLR, but retained normal proliferative response to third party, unrelated stimulators. This indicates that these responder T cells were rendered tolerant to the original stimulator PBLs by treatment with the combined anti-B7-1 and anti-B7-2. The results obtained with this responder/stimulator pair are typical for other responder/stimulator pairs in that tolerance induction is the rule.

Example 17: Inhibition of Primary and Secondary MLRs by treatment with Humanized Anti-B7-1 and Anti-B7-2

Abstract:

The ability of the anti-B7 mAbs to inhibit primary MLRs and to induce specific durable hyper-responsiveness or "tolerance" and secondary MLR were investigated. Primary MLRs were treated with the individual or combined anti-B7 mAbs or with CTLA4Ig and proliferation measured on days 3, 4, and 5. The combined anti-B7-1+anti-B7-2 mAbs and CTLA4Ig inhibited proliferation whereas the individual anti-B7 mAbs were less effective. Cells from the primary MLRs (after 48 hours of treatment) were then put into secondary MLRs with no inhibitors. Cells from primary MLRs treated with either the combined anti-B7 mAbs or CTLA4Ig showed minimal response to the original stimulators in the secondary MLR whereas medium or individual anti-B7 mAbs treated cells responded well. Cells form all conditions gave good responses against third party stimulators thereby demonstrating that the hyporesponsiveness seen in primary MLRs for cells treated with the combined anti-B7 mAbs or CTLA4Ig was both specific and durable.

Materials and Methods:

Experimental Design:

Cells:

Responders "A" Cells were PBLs prepared from freshly drawn blood. PBLs were purified by ficoll gradient centrifugation, the cells washed twice with culture

medium and suspended at $1x10^6$/mL in culture medium. Original Stimulator "B" Cells were PBLs prepared from leukophoresis. Cells purified by ficoll separation and washed twice with culture medium. Cells suspended at $2x10^6$/mL in culture medium for primary MLR and at $1x10^6$/mL for secondary MLR. Third Party Stimulator "C" Cells were PBLs prepared from a leukophoresis. Cells purified by ficoll separation and washed twice with culture medium. Cells suspended at $1x10^6$/mL in culture medium. "B" and "C" stimulators are PBLs obtained from two genetically different human donors.

Culture Medium:

RPMI 1640 containing 2mM glutamine, 10 mM HEPES, 10 % heat inactivated human AB serum, 100U/mL penicillin, 100 ug/mL strepamycin sulfate, and 5 ug/mL gentamicin sulfate.

Test Articles:

Control Ig is a chimeric mAb with the variable domains derived form a murine anti-HIV envelope protein mAb and the constant domains derived from human IgG1. All antibody, CTLA4Ig, and control Ig solutions were prepared in culture medium at the following concentration: anti-B7-1 (40 ug/mL), anti-B7-2 (40 ug/mL), anti-B7-1 + anti-B7-2 (40 ug/mL each), CTLA4Ig (40 ug/mL), CTLA4Ig (80 ug/mL), and control Ig (40 ug/mL).

Methods for primary MLR:

The following was performed:

- Irradiate "B" cells (stimulators) at 3.5 Gy
- In a 96 well "U" bottom microtiter plate, mix 50 uL of irradiated "B" cells + 50 uL of antibody, CTLA4Ig, Control Ig, or medium. Total volume = 100 uL containing $1x10^5$ B cells (stimulators) and 2X final concentration of inhibitory reagents. Incubate for 30 minutes on ice.
- Add 100 uL of "A" cells (responders)

- Incubate at 37C, 5%CO_2
- On day 2, 3, and 4, add 1 uCi [3H]-thymidine and continue incubation overnight
- Harvest cells and determine incorporated radioactivity by scintillation counting. Report results as day 3, 4, and 5.

Methods for Coupled Primary/Secondary MLR:

The following was performed:

- Irradiate B cells (stimulators) at 3.5 Gy
- Bulk primary MLR. In a T-25 flask, mix 2.5 mL of irradiated "B" cells ($2x10^6$/mL) + 2.5 mL of antibody, CTLA4Ig, Control Ig, or medium. Total volume = 5 mL containing $1x10^6$/mL "B" cells (stimulators) and 2X final concentration of inhibitory reagents. Incubate for 30 minutes on ice.
- Add 5 mL of "A" cells (responders). Total volume in T-25 = 10 mL
- Incubate at 37C, 5%CO_2 for ~ 48 hours
- Collect cells by ficoll gradient centrifugation, wash twice with ice cold medium and suspend at $1x10^6$/mL in culture medium. Hold for 8 hours on ice. Wash cells twice with ice cold medium and suspend at $1x10^6$/mL in culture medium.
- Irradiate "C" cells (third party stimulators) at 3.5 Gy. Irradiate "B" cells (original stimulators) at 3.5 Gy. Both "B" cells and "C" cells at $1x10^6$/mL.
- In a 96 well "U" bottom microtiter plate, mix 100 uL of irradiated "B" cells or irradiated "C" cells and 100 uL cells from bulk MLR.
- Incubate at 37C, 5%CO_2
- On day 2, 3, 4, and 5, add 1 uCi [3H]-thymidine and continue incubation overnight.
- Harvest cells and determine incorporated radioactivity by scintillation counting. Report results as day 3, 4, 5, and 6

Results:

Primary MLRs performed using one responder and two different stimulators were treated with the individual anti-B7 mAbs, the combined anti-B7 mAbs, CTLA4Ig, or control Ig and the culture proliferation measured on days 3, 4, and 5. Anti-B7-1 alone had minimal inhibitory effect on proliferation (1-35%). Anti-B7-2 alone had moderate inhibitory effect on proliferation (30-50%). The combined anti-B7 mAbs or CTLA4Ig had maximal inhibition of proliferation (86-92%, anti-B7s; 82-91%, CTLA4Ig; Figures 17 and 18).

After 48 hours of incubation in primary MLRs using one of the stimulators and containing inhibitors, the cells were washed, rested, and placed in secondary MLR using the original and third party stimulators in cultures lacking inhibitors. Culture proliferation was measured on days 3, 4, 5, & 6 (Figure 19 and 20). A compilation of the data from the secondary MLR experiments is presented in Figure 21.

Using data obtained form the peak proliferative response, regardless of the day of peak proliferative response, the response against the original and third party stimulators for cells from primary MLR treated with anti-B7-1 alone were as follows:

Table 7

Treatment in Primary MLR	Response Against Original Stimulators (%)	Response Against Third Party Stimulators (%)
Medium	100	100
anti-B7-1	70	89
anti-B7-2	47	89
anti-B7-1 + anti-B7-2	15	89
CTLA4Ig 10	19	90
CTLA4Ig 20	17	95
Control Ig	99	105

Primary MLRs treated with either the combined anti-B7 mAbs or CTLA4Ig resulted in cells that were refractory to proliferation against the same stimulators in a secondary MLR. Treatment with either of the anti-B7 mAbs alone was less effective. Regardless of the treatment in the primary MLR, cells form all treatment conditions responded normally to third party cells demonstrating that treatment with the combined anti-B7 mAbs or CTLA4Ig resulted in specific, durable hyporesponsiveness or anergy.

Conclusion:

Humanized anti-B7 mAbs h1F1 and 3D1 used in combination inhibit primary MLRs. This inhibition is specific and durable as responders from a primary MLR treated with the combined anti-B7 mAbs respond poorly to the same stimulators in a secondary MLR performed without inhibitors. Responders from a primary MLR treated with the combined anti-B7 mAbs respond normally to third party stimulators in the secondary MLR. The combined anti-B7 mAbs are as effective as CTLA4Ig in inhibiting a primary and secondary responses. The individual anti-B7 mAbs are less effective in inhibiting a primary MLR and

treatment with the individual anti-B7 mAbs did not lead to the development of anergy in the secondary MLR.

Example 18: Inhibition of Immune Responses in Non-Human Primates by Anti-B7 mAbs; Inhibition of Anti-Tetanus Responses

Abstract:

The ability of the anti-B7 mAbs to inhibit primary and secondary (recall) antibody responses was determined in a non-human primate tetanus immunization model. Four cohorts of cynomolgus monkeys (n=3) were immunized with tetanus toxoid on Day 0 and six weeks later on Day 42. Anti-tetanus titers were evaluated weekly. In groups administered a single 10 mg/kg dose of a combination of both human anti-B7-1 antibody (h1F1) and human anti-B7-2 antibody (h3D1) on Day 0, anti-tetanus antibody response was dramatically suppressed, with 0 of 6 treated animals developing a significant titer within six weeks of immunization. In addition, the animals that had been treated with the combination of antibodies on Day 0 did not respond to challenge with tetanus antigen on Day 42 regardless of whether they received saline or another dose of h1F1 and h3D1 on Day 42. In the cohort of animals that received saline on Day 0 and h1F1 and h3D1 on Day 42 only, the mean titer of the secondary antibody response to tetanus was lower than the mean titer observed in the saline control cohort. All cohorts of animals treated with h1F1 and h3D1 were re-immunized with a third dose of tetanus antigen 112 days after the last dose, when serum levels of h1F1 and h3D1 were below detectable limits. At this timepoint, the animals responded by making anti-tetanus antibodies with kinetics that appeared similar to a primary or secondary antibody response. These data show that inhibition of costimulation by blocking B7-1 and B7-2 with anti-B7-1 and anti-B7-2 antibodies is capable of dramatically suppressing the primary antibody response to tetanus as well as reducing the secondary antibody response. In addition, these results show recovery from immunosuppression, indicating that long-term tolerance against tetanus was not achieved in this study.

Materials and Methods:

Test and Control Articles:

Anti-B7-1 (h1F1):

Anti-B7-1 antibody was administered to non-human primates by slow IV infusion. Each animal in the treated groups received 10 mg/kg on Day 0 and/or Day 42 of the study.

Anti-B7 (h3D1):

Anti-B7-2 antibody was administered to non-human primates by slow IV infusion. Each animal in the treated groups received 10 mg/kg on Day 0 and/or Day 42 of the study.

Saline Control:

Saline (9% Sodium Chloride for injection, was administered to non-human primates by slow IV infusion. Each animal in the Saline control group and groups B and C received 15 ml of saline on Day 0 and/or Day 42 of the study.

Purified Tetanus Toxoid Antigen:

Tetanus toxoid antigen (University of Massachusetts Medical Center, Biologic Laboratories) was administered on Day 0. All animals were immunized with 10 limit of flocculation units (LfU) by intramuscular (IM) injection and 1 Lf unit by intradermal (ID) injection ninety minutes after saline or antibody administration. On Day 42, all animals were immunized with 10 Lf units by IM injection, 90 minutes after saline or antibody administration. On Day 84, all animals were injected with 1 Lf unit by ID injection to test for tetanus specific delayed type hypersensitivity (DTH). Animals in groups B, C, and D were immunized a third time with 10 Lf units by IM injection 112 days after the last dose (second immunization).

Experimental Design:

Twelve tetanus naïve, 4-6 kg male Cynomolgus macaques *(Macaca fasicularis)* were divided into four experimental groups of three animals per group:

Group A; received 2 immunizations with 10 Lf Units (Flocculation Units) i.m. tetanus toxoid on day 0 and 42 (controls).

Group B; received 10 mg/kg of each humanized anti-B7-1 (1F1) and anti-B7-2 (3D1) i.v., at least 90 minutes before 10 Lf units i.m. tetanus toxoid on day 0; tetanus toxoid immunization only (without mAb pretreatment) on day 42 and Day 112 (Costimulation blockade with primary immunization).

Group C; received tetanus toxoid immunization only (without mAb pretreatment) on day 0; 10 mg/kg of each humanized anti-B7-1 and anti-B7-2 i.v., at least 90 minutes before 10 Lf units i.m. tetanus toxoid on day 42 and day 154 (Costimulation blockade with secondary immunization).

Group D; received 10 mg/kg of each humanized anti-B7-1 (1F1) and anti-B7-2 (3D1) i.v., at least 90 minutes before 10 Lf units i.m. tetanus toxoid on day 0; received 10 mg/kg of each humanized anti-B7-1 and anti-B7-2 i.v., at least 90 minutes before 10 Lf units i.m. tetanus toxoid on day 42 and a tetunus toxoid immunization only (withoug mAb pretreatment) on Day 154 (Costimulation blockade with primary and secondary immunization).

All groups received tetanus immunization on Days 0 and 42. Groups B, C, D received a third tetanus immunization 112 days post anti-B7 dosing (Table 8).

Table 8 Treatment Groups

Group N=3/group	1° Tetanus Immunization Day 0	2° Tetanus Immunization Day 42	3° Tetanus Immunization
A Saline Control	Saline	Saline	None
B anti-B7 after 1° immunization	Anti B7-1/B7-2 10 mg/kg IV bolus	Saline	Day 112
C anti-B7 after 2° immunization	Saline	Anti B7-1/B7-2 10 mg/kg IV bolus	Day 154
D anti-B7 after 1° and 2° immunization	Anti B7-1/B7-2 10 mg/kg IV bolus	Anti B7-1/B7-2 10 mg/kg IV bolus	Day 154

All groups received tetanus immunization on Days 0 and 42. Groups B, C, and D received a third tetanus immunization 112 days post anti-B7 dosing.

Anti-tetanus antibody ELISA:

96-well ELISA plates were coated with tetanus toxoid at 4 μg/mL. A four-log titration of serum samples was performed starting at 1:100. Ab binding to tetanus was detected with a combination of monoclonal anti-human IgG and polyclonal goat anti-rhesus IgM HRP-conjugated antibodies, and developed with TNB substrate.

Results and Discussion:

Figure 22 shows the anti-tetanus IgM + IgG responses in monkeys immunized with tetanus toxoid and treated with the combined anti-B7-1 and anti-B7-2 mAbs.

Cynomolgus monkeys were administered either saline or a combination of 10 mg/kg anti-B7.1 antibody (h1F1) and 10 mg/kg anti-B7.2 antibody (h3D1) by IV infusion. Ninety minutes post antibody or saline infusion, each animal was

immunized with 10 Lf units of purified tetanus toxoid by IM injection and 1 Lf unit by ID injection. Anti-tetanus antibody titers were measured weekly. In the saline control group, mean log titers of anti-tetanus antibody were increased over baseline by Day 14, peaked at Day 49 and remained elevated over baseline throughout the study (Figure 22). In the groups receiving the combination of h1F1 and h3D1 on Day 0 (Groups B and D), 0 of 6 treated animals had a significant anti-tetanus antibody titer by day 42. Upon rechallange with tetanus toxoid on Day 42, animals in Group B, despite receiving no additional anti-B7 antibody did not mount a significant antibody response to tetanus (Figure 22). Analysis of serum showed significant levels of h1F1 and h3D1 remained in the serum (mean serum concentration >10 mg/mL) on Day 42. Animals in Group D, received another infusion of both h1F1 and h3D1 on Day 42 prior to re-immunization with tetanus toxoid. All 3 animals in this group had anti-tetanus titers that remained below detectable limits throughout the study. In Group C, animals that received saline on Day 0 and h1F1 and h3D1 on Day 42 only, had a primary anti-tetanus antibody responses similar to the Saline control group (Group A). The mean antibody titer observed in the secondary response of Group C, however, was lower than the mean antibody titer observed in the secondary response of Group A, (Figure 22).

Serum concentrations of h1F1 and h3D1 were monitored throughout the study and when they were below detectable limits, Groups B, C, and D were immunized a third time with tetanus toxoid. Each Group was re-immunized 112 days after their last anti-B7 antibody infusion and received no additional anti-B7 antibody treatment. At this time point, the animals in all groups responded by generating anti-tetanus antibodies with kinetics that appeared similar to a primary antibody response (Figure 22).

Conclusion:

These data show that inhibition of costimulation by blocking B7-1 and B7-2 with anti-B7-1 and anti-B7-2 antibodies is capable of dramatically suppressing the primary antibody response to tetanus as well as reducing the secondary antibody

response. In addition, these results show recovery from immunosuppression, indicating that long-term tolerance against tetanus was not achieved in this study.

Therefore, the administration of anti-B7 antibodies concurrent with exposure to a new antigen (tetanus immunization) can prevent the development of a new antibody response and can lessen the strength of a secondary response to the same antigen. Since many disease states are exacerbated by the development of such antibodies, treatment with the combined anti-B7 mAbs will prevent the development of such antibodies. One such disease state is the development of inhibitor antibodies to administered Factor VIII or Factor IX in hemophiliacs, thereby reducing the effectiveness of these life saving compounds. Treatment of hemophiliacs with anti-B7 mAbs prevents or reduces the formation of the inhibitor antibodies.

Example 19: Pharmacodynamics of Anti-B7-1 (h1F1) and Anti-B7-2 (h3D1) in Combination or Individually at Doses of 0.01, 0.1, 1, or 10 mg/kg in a Tetanus Toxoid Challenge Cynomolgus Monkey Model

Abstract:

The ability of varying doses of individual anti-B7-1 or anti-B7-2 antibodies, or the combined anti-B7-1 and anti-B7-2 antibodies to inhibit a primary and secondary (recall) response to tetanus was determined in a Cynomolgus monkey immunization model. Cynomolgus monkeys (total n=33) were administered a single intravenous dose of 10, 1, 0.1, or 0.01 mg/kg of h1F1 and h3D1 in combination, 10, 1, or 0.1 mg/kg of h1F1 or h3D1 alone, or vehicle control. All animals were immunized with purified tetanus toxoid one hour after administration of h1F1 and h3D1. Animals were immunized with tetanus toxoid again at 14 weeks to determine if normal immune function returned when h1F1 and h3D1 levels fell below detectable levels.

A complete suppression of a primary anti-tetanus antibody response was observed following a single IV dose of 10 or 1 mg/kg of h1F1 and h3D1 in combination, while a dose of 0.1 mg/kg resulted in only partial suppression. Treatment with h1F1 or h3D1 alone was not as effective as equal doses of h1F1 and

h3D1 in combination in suppressing the anti-tetanus antibody response. All animals in all groups produced a high anti-tetanus antibody titer following a second immunization with tetanus toxoid, after h1F1 and h3D1 concentrations fell below detection (<50 ng/mL). This indicated that normal immune function returned following cessation of treatment with h1F1 and h3D1, and that long-term tolerance against tetanus was not achieved in this study.

Materials and Methods:

Animals were administered a single IV dose of test or control (vehicle) article on Day 0 according to Table 9. The test or control article was infused via a peripheral vein using a syringe pump set at 0.1 mL/min/kg (maximum dose rate of 1 mg/min/kg). At t = 1 hour on Day 0 all animals received purified tetanus toxoid (Massachusetts Biologic Laboratories, UMass, Jamaica Plain, MA) at a dose of 10 Lf units (limit of flocculation) by IM injection and 10 Lf units by ID injection. At t = 14 weeks all animals received a second administration of purified tetanus toxoid at a dose of 10 Lf units by IM injection and 10 Lf units by ID injection. Blood samples were collected via venipuncture at specified timepoints up to 3024 hours (126 days) after dosing. Serum levels of anti-tetanus antibody (IgM and IgG) were determined by ELISA.

Table 9 Group Designations and Dose Levels

Group	Number of Animals	Dose Level of Antibody (mg/kg)	Test Agent(s) Administered
1	3	0	Vehicle
2	3	10	h1F1
3	3	1	h1F1
4	3	0.1	h1F1
5	3	10	h3D1
6	3	1	h3D1
7	3	0.1	h3D1
8	3	10[a]	h1F1 and h3D1
9	3	1[a]	h1F1 and h3D1
10	3	0.1[a]	h1F1 and h3D1
11	3	0.01[a]	h1F1 and h3D1

Anti-Tetanus Antibody Formation:

All animals treated with vehicle produced a detectable anti-tetanus antibody titer beginning 14 days after immunization (Table 10, Figures 23, 24, and 25). Treatment with the two highest doses (10 or 1 mg/kg) of h1F1 and h3D1 in combination completely suppressed the formation of a detectable antibody titer within the 14-week observation period in all animals. A partial suppression of the antibody response was observed in animals treated with 0.1 mg/kg, one out of three animals produced a detectable antibody titer. All animals treated with 0.01 mg/kg of h1F1 and h3D1 in combination produced a detectable antibody titer; however, this titer was lower in magnitude when compared to the titers observed in the vehicle control group, indicating that some suppression of the antibody response was achieved.

Table 10 Number of Cynomolgus Monkeys that Produced a Detectable Anti-Tetanus Antibody Titer Following a Primary Challenge with Tetanus Toxoid During Treatment with h1F1 and h3D1

Group	Treatment	Number of Animals that Produced a Detectable
1	Vehicle Control	3
2	10 mg/kg h1F1	1
3	1 mg/kg h1F1	3
4	0.1 mg/kg h1F1	2
5	10 mg/kg h3D1	1
6	1 mg/kg h3D1	1
7	0.1 mg/kg h3D1	3
8	10 mg/kg h1F1 and h3D1	0
9	1 mg/kg h1F1 and h3D1	0
10	0.1 mg/kg h1F1 and h3D1	1
11	0.01 mg/kg h1F1 and h3D1	3

When the 0.1, 1, and 10 mg/kg dose groups are taken together, treatment with either h1F1 (Figure 24) or h3D1 (Figure 25) alone is not as effective as treatment with h1F1 and h3D1 in combination (Figure 23). Treatment with h1F1 alone (Groups 2, 3, and 4) resulted in 6/9 (66%) animals with a detectable anti-tetanus antibody response. Treatment with h3D1 alone (Groups 5, 6, and 7) resulted in 5/9 (55%) animals with detectable antibodies, whereas only 1/9 (11%) animals had detectable antibodies following treatment with the combination of h1F1 and h3D1 (Groups 8, 9, and 10). Group 11 was not included in these comparisons since h1F1 and h3D1 were not administered individually at 0.01 mg/kg.

At 1 and 10 mg/kg, there was no detectable anti-tetanus antibody response in any of the animals treated with h1F1 and h3D1 in combination; however, at both of these doses, there were detectable antibodies in animals treated with h1F1 or h3D1 alone. When animals were treated with 0.1 mg/kg, there were detectable anti-tetanus antibodies in animals from all three treatment groups; however, there was a lower incidence (1/3) in the combination treatment group than in the h1F1 (2/3) or h3D1 (3/3) groups.

Figure 26 shows the area under the anti-tetanus antibody titer curve (AUC) for each treatment group. These AUC values were calculated using the antibody titer curves shown in Figures 23, 24, and 25. The AUC values were calculated from Weeks 0 to 14. To account for the number of responding animals in each group, the AUC values were weighted by the fraction of the number of animals in each group that produced detectable antibody titers. This plot gives an indication of the cumulative magnitude of the antibody titer or strength of the antibody response throughout the 14 week observation period. Animals treated with either 10 or 1 mg/kg of h1F1 and h3D1 in combination (Groups 8 or 9) showed 100% suppression (AUC = 0) of the antibody response relative to the control group. Treatment with 0.1 or 0.01 mg/kg of h1F1 and h3D1 in combination (Groups 10 or 11) suppressed the antibody response by 78% or 86% (AUC = 402 or 253 Log titer•hr), respectively, relative to the control group. The antibody response was suppressed between 8.6% and 99% (AUC = 1640 to 5.04 Log titer•hr) relative to the control

group in the animals treated with h1F1 or h3D1 alone (Groups 2-7). These data indicate that treatment with h1F1 or h3D1 alone was not as effective as equal doses of h1F1 and h3D1 in combination in suppressing the anti-tetanus antibody response.

Following the re-immunization with tetanus toxoid 14 weeks after administration of h1F1 and h3D1, all animals in all groups produced a high anti-tetanus antibody titer (>3 log titer), indicating that normal immune function returned following cessation of treatment with h1F1 and h3D1, and that long-term tolerance against tetanus was not achieved in this study. Animals treated with 10 mg/kg of h1F1 and h3D1 in combination showed a delayed antibody response with detectable antibody titers observed 14 days after immunization similar to a primary antibody response, indicating that a response to the first immunization was completely blocked. All other animals showed increased antibody titers 7 days after immunization, which resembles a memory response rather than a primary response.

Conclusions:

A complete suppression of a primary anti-tetanus antibody response was observed following a single IV dose of 10 or 1 mg/kg of h1F1 and h3D1 in combination, while a dose of 0.1 mg/kg resulted in only partial suppression. Treatment with h1F1 or h3D1 alone was not as effective as equal doses of h1F1 and h3D1 in combination in suppressing the anti-tetanus antibody response. All animals in all groups produced a high anti-tetanus antibody titer following a second immunization with tetanus toxoid after h1F1 and h3D1 concentrations fell below detection. This indicates that normal immune function returns following cessation of treatment with h1F1 and h3D1.

Example 20: Serum half-life of anti-B7 antibodies in non-human primates.

The murine-anti-hB7-1 and murine-anti-hB7-2 mAbs were tested in non-human primates for serum half-life and target cell saturation. Three Cynomolgus monkeys were dosed with one dose each of a combination of the anti-hB7-1 and anti-hB7-2 mAbs at 2, 8, or 20 mg each mAb/kg body weight. The monkeys were

analyzed for mAb binding to PBMC (Proliferative Blood Mononuclear Cells), serum mAb concentration, and primate anti-mouse antibody (PAMA) response (Table 11). PBMC saturation was determined by flow cytometry (FACS) where PBMCs isolated from the blood of mAb dosed primates were stained with goat-anti-murine Ig-PE (% in vivo) or the PBMC were first reacted with the anti-hB7-1 and anti-hB7-2 mAbs followed by detection with the goat-anti-murine Ig-PE (% ex vivo). The level of PBMC saturation at the various time points was calculated by (% in vivo/% ex vivo) x 100. This study shows that PBMC saturation for the anti-hB7-1 and anti-B7-2 mAbs falls below 80% between days 4 to 6 (mAbs @ 2mg/ks), days 6 to 8 (mAbs @ 8mg/kg), and days 13 to 20 (mAbs @ 20mg/kg) depending upon mAb dose. Although not measured directly, there was no apparent dramatic decrease in the numbers of circulating $B7^+$ cells.

Serum half-lives of the anti-hB7-1 and anti-hB7-2 mAbs were measured with a specific ELISA for each mAb using hB7-1Ig or hB7-2Ig as target and goat-anti-murine Ig HRP/ABTS for detection. These assays were sensitive to 400 ng/mL and 200 ng/mL for anti-hB7-2 and anti-hB7-1, respectively. PAMA responses were measured using a commercially available kit. The serum concentrations of the two anti-hB7 mAbs and the PAMA responses are shown at the individual dosage levels for each mAb. Both mAbs exhibit similar serum half lines of ~48 hours as determined at all three dosage levels. Increasing mAb dosage increased serum mAb concentrations by a comparable factor at all dosages and times tested. When dosed at 20 mg/kg, circulating mAb levels of >30 μg/mL were found for each mAb at 6 days post dosing.

PAMA responses to the anti-hB7-1 and anti-hB7-2 mAbs were low and were first measurable beginning 10 days after serum mAb levels had fallen below 10 μg/mL.

The serum half-life of humanized anti-human B7-2 and B7-1 antibodies were also determined in Cynomolgus monkeys (n=6) dosed once with 10 mg/kg of humanized anti-B7-2 antibody. Serum concentration was monitored by specific ELISA assay for each antibody using HRP-anti human IgG2 and ABTS.

Figure 27 shows the serum concentration of the humanized B7-2 and humanized B7-1 mAbs in Cynomolgus monkeys through 42 days after dosing.

The humanized anti-human B7-2 and anti-human B7-1 mAbs exhibited an extended serum half-life in Cynomolgus monkeys, as compared to a value of approximately 2 days for the murine anti-human B7-2 and anti-human B7-1 mAbs when dosed at the same level, demonstrating that the humanized anti-human B7 mAbs were retained in circulation much longer than the murine anti-B7 mAbs.

Table 11. Results from the Preclinical primate studies

Time	Dose @ 2 mg each mAb/kg			Dose @ 8 mg each mAb/kg			Dose @ 20 mg each mAb/kg		
Hours	Anti-hB7-2	PAMA	PBL Saturation	Anti-hB7-2	PAMA	PBL Saturation	Anti-hB7-2	PAMA	PBL Saturation
(Days)	µg/mL	ng/mL	%	µg/mL	ng/mL	%	µg/mL	ng/mL	%
0	BQL	Neg.	0	BQL	Neg.	0	BQL	Neg.	0
.167	61	NT		206	NT		580	NT	
.5	59	NT	100	229	NT	25	570	NT	65
1	52	NT		227	NT		527	NT	
3	52	NT	100	230	NT	100	548	NT	100
5	50	NT		139	NT		464	NT	
8	44	NT		169	NT		412	NT	
24 (1D)	26	NT	70	103	NT	100	286	NT	80
48 (2D)	15	NT	100	59	NT	100	196	NT	100
96 (4D)	2.4	NT	75	18	NT	100	83	NT	100
144 (6D)	BQL	NT	95	3.9	NT	100	32	NT	100
192 (8D)	BQL	NT	65	BQL	NT	100	13	NT	100
240 (10D)	BQL	NT		BQL	NT		3.9	NT	
312 (13D)	BQL	Neg.	5	BQL	Neg.	55	BQL	Neg.	80
480 (20D)		2908	10		4080	10		517	20
684 (27D)		1260			1460			1094	
816 (34D)									

BQL=Below Quantifiable Limit; NT=Not Tested

Example 21 Inhibition of Specific T-Cell Responses to Superantigens (Toxic shock syndrome toxin-1; TSST-1)

NODscid mice were populated with human lymphocytes by the administration of 10^8 human PBLs. After 28 days, the mice were treated with TSST-1 (10mg, I.P.) with or without the treatment with the combined antibodies to human B7-1 and B7-2 (500 mg, I.V.). After 14 additional days, the presence of human lymphocytes, T-cells, and TSST-1 specific T-cells (Vβ2-TCR-cells) in the peritoneal cavity was measured by FACS using antibodies specific for human CD45, CD4, and human Vβ2-TCR.

Table 12

Addition		Human T-cells (%)	
TSST-1	Anti-B7-1 + Anti-B7-2	Total	$V\beta2^+$
-	-	10.2	3.9
+	-	27.4	12.0
+	+	23.4	3.8

Results:

Table 12 shows the proportion of total human T cells and $V_\beta2^+$-TCR human T cells (TSST-1 specific) found in the peritoneal cavity of hu-NODscid mice. Treatment with TSST-1 greatly increased the percentage of human T cells and of TSST-1 specific human T cells ($V_\beta2^+$) in the huNOD-scid mice. Treatment with the anti-human B7-1 and B7-2 mAbs moderately diminished the total human T cell response and completely inhibited the expansion of the TSST-1 specific human T cells indicating that the anti-B-7 mAbs could effectively inhibit human T cell superantigen mediated responses.

Example 22: Evaluation of Anti-B7 Antibodies h1F1 (Anti-B7-1) and h3D1 (Anti-B7-2) in a Life-Supporting Renal Transplant Model in Cynomolgus Monkeys

Abstract:

This study was performed to evaluate the efficacy and compatibility of the monoclonal antibodies h1F1 (anti-B7-1) and h3D1 (anti-B7-2) in a life-supporting renal transplant model in Cynomolgus monkeys. The efficacy and compatibility of these antibodies were evaluated when they were given as a monotherapy as well as in combination with conventional immunosuppressive agents such as cyclosporin A (CsA), rapamycin, and steroids.

Twenty four male cynomolgus monkeys received blood group compatible, mixed lymphocyte reaction (MLR) mismatched, renal allografts. The recipients were studied consecutively in 6 treatment groups, which included the following combinations of immunosuppressive agents for the first 56 postoperative days (poDay) and then followed for an additional 65-66 days (total maximum follow-up period 119-120 days) without additional treatment:

· Group 1 received combined anti-B7 antibodies according to Schedule A: h1F1 20 mg/kg + h3D1 20 mg/kg preoperatively, then h1F1 5 mg/kg and h3D1 5 mg/kg postoperatively and h1F1 5 mg/kg and h3D1 5 mg/kg every 7 days repeated until poDay 56; 9 dosing time points.

· Group 2 received combined anti-B7 antibodies according to Schedule B: h1F1 5 mg/kg + h3D1 5 mg/kg preoperatively, then h1F1 10 mg/kg and h3D1 10 mg/kg immediately postoperatively, and again on poDay 3, then 5 mg/kg of each antibody for 8 consecutive doses given in weekly intervals starting poDay 7 and ending poDay 56.

· Group 3 received the combined anti-B7 antibodies according to Schedule A plus microemulsion CsA at a dose designed to reach 24-hour trough level concentrations of 200 to 300 ng/mL from poDay 0 to poDay 14, then at a

reduced dose to reach 24-hour CsA trough levels of 150 to 250 ng/mL from poDay 15 to the last dose on poDay 56.

· Group 4 received the combined anti-B7 antibodies (h1F1 and h3D1) according to Schedule A in combination with a tapering dose of steroids: methylprednisolone 2 mg/kg IV postoperatively and then daily on poDay 2, then prednisone 0.5 mg/kg reduced by 0.05 mg/kg every three days until 0.2 mg/kg was reached then 0.2 mg/kg was continued until poDay 56

· Group 5 was given combined anti-B7 antibodies (h1F1 and h3D1) according to Schedule A in combination with rapamycin: rapamycin 1 mg/kg postoperatively and then daily through poDay 13, then 0.5 mg/kg daily was continued until poDay 56

· Group 6 was given microemulsion CsA at a dose designed to reach 24-hour trough level concentrations of 200 to 300 ng/mL from poDay 0 to poDay 14, then at a reduced dose to reach 24-hour CsA trough levels of 150 to 250 ng/mL from poDay 15 to the last dose on poDay 56.

· Group 7 was given rapamycin at 1 mg/kg from poDay 0 to poDay 13, and then at 0.5 mg/kg from poDay 14 through poDay 56.

Animals were euthanized before poDay 120 if the creatinine levels rose above 8.0 mg/dL, which was interpreted as evidence of terminal renal rejection.

The survival of transplanted, treated cynomolgus monkeys is depicted in Table 13.

Table 13. Survival and Diagnosis of Transplanted, Treated Cynomolgus Monkeys

Treatment Group	Treatment Schedule anti-B7-1 + anti-B7-2/Other Therapies	Survival (poDay)*
historical controls	none	10
1	schedule A / none	9, 48, 119, 119
2	schedule B / none	12, 14, 18, 120
3	schedule A / CsA	96, 119, 119, 119
4	schedule A / steroids	6, 77, 111, 119
5	schedule A / rapamycin	69, 73, 81, 114
6	no mAbs / CsA	22, 25, 38, 71
7	no mAbs/rapamycin	11, 18, 27, 35

* kidney recipients surviving until day 119/120 were sacrificed as per study protocol

The results of this study show that treatment of non-human primate, renal allograft recipients with the combination of the B7-1 and B7-2 monoclonal antibodies can lead to long-term graft survival. Graft survival was observed for up to 66 days post termination of treatment in 50% of the Group 1 animals. In contrast, antibodies dosed according to Schedule B, used in Group 2 were not sufficient to overcome the early acute rejection episode in 3 of the 4 animals treated, and only one animal of this group survived 120 days. Combining Schedule A with microemulsion CsA resulted in long-term survival in all animals with no evidence of an antagonistic immunosuppressive effect of CsA on the previously demonstrated efficacy of the antibodies. In Group 4, coadministration of high dose steroids with the antibodies did not antagonize the immunosuppressive effect of the antibodies in this non-human primate model. In Group 5, coadministration of rapamycin with the antibodies did not antagonize the immunosuppressive effect of the antibodies in this non-human primate model. In Group 6, treatment with CsA alone resulted in early

rejection in most kidney recipients. The results with this treatment group were inferior to those obtained with the combined treatment with the anti-B7 antibodies alone (Group 1) or with the combined antibodies + CsA (Group 4). In group 7, treatment with rapamycin alone resulted in early rejection in all kidney recipients. The result in this treatment group was inferior to that obtained with the combined treatment with the anti-B7 antibodies alone (Group 1) or with the combined antibodies and rapamycin (Group 5).

Thus, the monoclonal antibodies h1F1 and h3D1 are efficacious in avoiding early terminal rejection in a life-supporting renal transplant model in non-human primates. The antibodies are compatible with other immunosuppressants and their efficacy appears to be increased by combining them with microemulsion CsA rapamycin, or steroids.

The following study was divided into two phases. The purpose of the first phase was to define the efficacy of the novel immunosuppressive antibodies h1F1 and h3D1 when given as a monotherapy to cynomolgus monkeys that had received a renal transplant in a life supporting model. Animals transplanted without concurrent immunosuppressant treatment reject their grafts within 10 days of transplant. The study was designed to test two different schedules of antibody therapy. The antibody treatment was discontinued after poDay 56 in both schedules and animals were then followed until graft rejection or until Day 119-120.

The second phase of this study was designed to test if: a) h1F1 and h3D1, when given in combination with either CsA, rapamycin, or steroids, would antagonize the immunosuppressive effect of CsA, rapamycin, or steroids, thus resulting in shortened graft survival, or b) CsA, rapamycin, or steroids would antagonize the immunosuppressive efficacy of h1F1 and h3D1 either during the time when treatment was given simultaneously (poDay 0 to poDay 56) or following termination of all immunosuppressive therapy (> poDay 56).

Materials and Methods:

Animals:

Male cynomolgus monkeys, Macaca fascicularis, with a weight between 5 and 8 kg were used in this study. The animals underwent blood group typing. Donor and recipient monkeys were paired based on an ABO blood group match, a negative cross-match, and a stimulation index of at least 2.5 in a two-way MLR.

Test and Control Articles:

h1F1 and h3D1:

The anti-B7 antibodies were administered in a syringe connected to a syringe pump. h1F1 and h3D1 were always given in combination and administered at a maximum infusion rate of 1 mg/kg/min through a peripheral venous catheter.

The antibodies were administered according to different protocols as defined for each group.

Microemulsion CsA (Neoral):

The microemulsion CsA (Neoral; 100 mg/mL (Novartis)) was kept at room temperature and protected from exposure to light. The calculated volume of Neoral based on the required dose (in mg/kg) was drawn directly into the smallest appropriate syringe and administered without any dilution through a nasogastric tube directly into the stomach.

Methylprednisolone and Prednisone:

Methylprednisolone (Solu-Medrol, Pharmacia & Upjohn Co., Kalamazoo, MI) was supplied in vials with self-contained sterile water for reconstitution and was reconstituted following manufacturer's instructions. The drug was kept refrigerated after reconstitution and discarded after 48 hours.

Prednisone (Mutual Pharmaceutical Co., Inc., Philadelphia, PA) was supplied in tablets (5 mg). Tablets were dissolved in sterile water, one tablet per 5 mL water, with a resulting solution concentration of 1 mg/mL. The remaining drug was discarded at the end of dosing each day.

Experimental Design:

A total of 24 monkeys received unilateral renal transplants in a life-supporting model.

Group 1: h1F1 and h3D1 Monotherapy (Schedule A):

Group 1 received combination anti-B7-1 + anti-B7-2 antibody monotherapy according to Schedule A: h1F1 20 mg/kg + h3D1 20 mg/kg preoperatively, then h1F1 5 mg/kg and h3D1 5 mg/kg postoperatively and h1F1 5 mg/kg and h3D1 5 mg/kg every 7 days repeated until poDay 56.

Group 2: h1F1 and h3D1 Monotherapy (Schedule B):

The main differences between Schedules A and B were that in Schedule B, the first preoperative dose was reduced from 20 mg/kg to 5 mg/kg, the immediate postoperative dose was increased from 5 mg/kg to 10 mg/kg, and an additional dose of 10 mg/kg was given on poDay 3. The remainder of the postoperative dosing schedule was the same as Schedule A. Therefore, the total amount of antibody given over the postoperative course for each animal was the same in Schedules A and B.

Group 3: h1F1 and h3D1 (Schedule A) plus CsA:

In Group 3, antibody treatment (Schedule A) was combined with daily oral administration of microemulsion CsA. Group 3 received the antibodies according to Schedule A plus microemulsion CsA at a dose designed to reach 24-hour trough level concentrations of 200 to 300 ng/mL from poDay 0 to poDay 14, then at a reduced dose to reach 24-hour CsA trough levels of 150 to 250 ng/mL from poDay 15 to the last dose on poDay 56.

Microemulsion CsA was administered daily by gavage following short Ketamine sedation by gavage. Twenty-four hour trough levels of CsA were measured three times per week and the daily dose was adjusted to meet the target CsA trough levels. Additional modifications of the CsA dose were performed to prevent excessive weight loss or when renal function impairment was thought to be related to CsA trough levels.

Group 4: h1F1 and h3D1 (Schedule A) plus Steroids:

In Group 4, h1F1 and h3D1 were administered according to Schedule A in combination with a tapering dose of steroids: methylprednisolone 2 mg/kg IV postoperatively and then daily on poDay 2, then prednisone 0.5 mg/kg reduced by 0.05 mg/kg every three days until 0.2 mg/kg was reached then 0.2 mg/kg was continued until poDay 56

For the first 3 postoperative days (poDay 0 through 2), the animals received methylprednisolone at a dose of 2 mg/kg given intravenously as a bolus. From poDay 3 to poDay 56, prednisone was given to the ketamine sedated animals by gavage in a tapering schedule (see Figure 4) starting at 0.5 mg/kg and ending at a dose of 0.2 mg/kg.

Group 5: h1F1 and h3D1 (Schedule A) plus Rapamycin:

In Group 5, h1F1 and h3D1 were administered according to Schedule A in combination with rapamycin: rapamycin 1 mg/kg postoperatively and then daily through poDay 13, then 0.5 mg/kg daily was continued until poDay 56

Group 6: CsA alone:

In Group 6, microemulsion CsA was administered at a dose designed to reach 24-hour trough level concentrations of 200 to 300 ng/mL from poDay 0 to poDay 14, then at a reduced dose to reach 24-hour CsA trough levels of 150 to 250 ng/mL from poDay 15 to the last dose on poDay 56.

Microemulsion CsA was administered daily by gavage following short Ketamine sedation by gavage. Twenty-four hour trough levels of CsA were

measured three times per week and the daily dose was adjusted to meet the target CsA trough levels. Additional modifications of the CsA dose were performed to prevent excessive weight loss or when renal function impairment was thought to be related to CsA trough levels.

Group 7: Rapamycin

Rapamycin was administered daily by gavage following short Ketamine sedation by gavage. Group 7 was given rapamycin at 1 mg/kg from poDay 0 to poDay 13, and then at 0.5 mg/kg from poDay 14 through poDay 56.

Results and Discussion:

Historical control group: Renal transplantation with no treatment:

Historical data shows that cynomolgus monkeys receiving renal transplants and no treatment uniformly reject the transplanted kidney by day 10 post transplant.

Group 1: Monotherapy with h1F1 and h3D1 according to Schedule A:

Two of four animals treated with the combined anti-B7-1 + anti-B7-2 mAbs according to schedule A retained a functional allograft until the end of the study period (120 days) whereas the other two rejected the transplanted kidney during the treatment period (day 9 and day 48). Therefore, three out of four kidney recipients treated according to schedule A retained the transplanted kidney for much longer than historical controls demonstrating the efficacy of anti-B7 therapy in preventing the rejection of transplanted kidneys.

Group 2: Monotherapy with h1F1 and h3D1 according to Schedule B:

In contrast to Group 1, only 1 of the 4 animals treated with the anti-B7 antibodies according to schedule B retained a functional renal allograft beyond poDay 18. However, this animal retained the transplanted kidney until the end of the study period (poDay 120). This data suggests that treatment with anti-B7 mAbs according to schedule A results in better immunosuppression of kidney rejection than animals treated according to schedule B. This result may be due to the higher

concentrations of anti-B7 mAbs present early after transplantation in animals treated according to schedule A.

Group 3: Therapy with h1F1 and h3D1 according to Schedule A plus microemulsion CsA:

All animals treated with the anti-B7 mAbs according to schedule A in combination with CsA exhibited greatly delayed rejection of the transplanted kidneys with three out of four animals retaining the functional allograft until the study end (poDay 119). These results are superior to those obtained after treatment with the anti-B7 mAbs alone (Group 1) or with CsA alone (Group 6) demonstrating that there is no antagonism between treatment with the anti-B7 mAbs + CsA and that cotreatment with these agents provides and additional benefit of delayed organ rejection.

Group 4: Therapy with h1F1 and h3D1 according to Schedule A plus methylprednisolone/prednisone:

One of four animals treated with the combined anti-B7 mAbs + steroids retained the transplanted kidnet until the end of the study period (poDay 119). Of the remaining three kidney recipients, two exhibited delayed rejection of the transplanted kidneys. The third monkey rejected the transplanted kidney early (poDay 6) due to ureter necrosis believed to be associated with the administration of the high dose steroids. Taken together, these data suggest that the combination of the anti-B7 mAbs + steroiods are effective in preventing the rejection of the transplanted kidney and the use of these agents in combination is not contraindicated.

Group 5: Therapy with h1F1 and h3D1 according to Schedule A plus rapamycin:

All four animals treated with the combined anti-B7 mAbs according to schedule A + rapamycin retained functional renal allografts beyond the treatment period for a duration ranging from 69 to 114 poDay. This demonstrated that the treatment with the combined anti-B7 mAbs + rapamycin is beneficial in delaying organ rejection and does not antagonize the treatment with the anti-B7 mabs alone.

Group 6: Therapy with CsA alone:

All four animals treated with CsA alone rejected their transplanted kidneys with three out of four animals rejecting during the CsA treatment period. These results are inferior to those obtained with in animals treated with the combined anti-B7 mAbs + CsA.

Group 7: Therapy with Rapamycin alone:

All four animals treated with rapamycin alone rejected their transplanted kidneys during the treatment period.

Conclusion:

The purpose of the study was to evaluate the efficacy and compatibility of the novel monoclonal antibodies, h1F1 and h3D1, in a life-supporting non-human primate kidney transplant model. Efficacy was assessed by evaluating the incidence of terminal acute rejection and ultimately allograft (animal) survival. Terminal rejection in untreated animals would be expected to occur within 10 days post-transplantation in the model used. Compatibility and efficacy were tested in a total of 7 different treatment groups. The treatment regimens used in later groups evolved as the study progressed and were based on the results of previous groups.

Evaluation of Efficacy:

Group 1: h1F1 and h3D1 Monotherapy (Schedule A):

Animals in the first therapeutic group were treated with only h1F1 and h3D1, given weekly for 56 days without any additional immunosuppressive drug therapy. The results showed that monotherapy with h1F1 and h3D1 was able to prevent terminal acute rejection in 2 of the 4 animals of this group and delay the occurrence of terminal rejection in an additional monkey until poDay 48. The administration of the antibodies did not prolong graft survival in one monkey beyond that seen in the historical control group.

Group 2: h1F1 and h3D1 Monotherapy (Schedule B):

The regimen and schedule of antibody administration was changed in treatment Group 2 in attempt to prevent the early acute rejection episodes seen in Group 1 between poDay 5 and 7. The preoperative dose in this group was reduced from 20 mg/kg of each mAb to 5 mg/kg of each mAb and the first postoperative dose was increased from 5 to 10 mg/kg of each mAb. The total amount of antibody administered perioperatively was therefore reduced from 25 mg/kg to 15 mg/kg. An additional dose of 10 mg/kg was given on poDay 3. Thereafter, the administration schedule in Group 1 and 2 were identical.

Three of the 4 animals did not recover from this first rejection episode. Only one animal survived for the entire follow-up period. This demonstrates the critical aspect of timing and dosing of the antibodies during the early peri- and postoperative periods. The reduction in the perioperative dose most likely resulted in a more vigorous rejection response, and the administration of the additional dose of antibodies on poDay 3 did not affect this outcome.

In both Groups 1 and 2, there were animals with long-term survival without any additional immunosuppressive therapy.

A direct comparison of graft outcome following Schedule A (Group 1) and Schedule B (Group 2) favored Schedule A. The combination of antibodies were administered according to Schedule A for the next two groups.

Group 3: h1F1 and h3D1 (Schedule A) plus CsA:

A dose of microemulsion CsA, that aimed at delivering 24-hour trough levels in the 200 to 300 ng/mL range was administered in Group 3. All animals in this group survived long-term (> 56 days) and 3 of the 4 animals showing no evidence of terminal acute rejection until the end of the follow-up period. Therefore, neither the anti-B7 antibodies nor the CsA negatively affected the efficacy of the other. The median survival was 119 days, and the mean survival was 113 days in this group, which was at least as good as, if not better than, the length of survival in Group 1 (median: 84 days; mean: 74 days). An additional treatment group in which the

recipients are treated with only microemulsion CsA (Group 6) dosed to achieve similar trough levels as in Group 3 (for 56 days) showed that all four transplant recipients rejected their transplanted kidneys and that three out of four rejected during the treatment period.

The combination of the monoclonal antibodies with microemulsion CsA was sufficient to avoid an early acute rejection episode.

Group 4: h1F1 and h3D1 (Schedule A) plus Steroids:

The combination of h1F1 and h3D1 was administered with a tapered dose of steroids in Group 4. Monotherapy with steroids alone is generally not sufficient to prevent terminal allograft rejection in this model. A high dose of steroids was chosen for this group to determine if the steroids and antibodies affected the efficacy of each other. One of four animals treated with the combined anti-B7 mAbs + steroids retained the transplanted kidney until the end of the study period (poDay 119). Of the remaining three kidney recipients, two exhibited delayed rejection of the transplanted kidneys. The third monkey rejected the transplanted kidney early (poDay 6) due to ureter necrosis believed to be associated with the administration of the high dose steroids.

There did not appear to be any negative effect of co-administration of high dose steroids on the efficacy of the antibodies in this group. It is unclear if the antibody administration had an effect on the immunosuppressive efficacy of steroids as there are no data available on the efficacy of a similar steroid regimen used as monotherapy in a non-human primate renal transplant model.

Group 5: Therapy with h1F1 and h3D1 according to Schedule A plus rapamycin:

All four animals treated with the combined anti-B7 mAbs according to schedule A + rapamycin retained functional renal allografts beyond the treatment period for a duration ranging from 69 to 114 poDay. This demonstrated that the treatment with the combined anti-B7 mAbs + rapamycin is beneficial in delaying organ rejection and does not antagonize the treatment with the anti-B7 mabs alone. An additional treatment group in which the recipients were treated only with

rapamycin (Group 7) dosed to achieve similar levels as in Group 5 showed that all four transplant recipients rejected their transplanted kidneys during the treatment period.

Group 6: Therapy with CsA alone:

All four animals treated with CsA alone rejected their transplanted kidneys with three out of four animals rejecting during the CsA treatment period. These results are inferior to those obtained in animals treated with the combined anti-B7 mAbs + CsA.

Group 7: Rapamycin alone:

All four animals treated with rapamycin alone rejected their transplanted kidneys during the treatment period. These results are inferior to those obtained in animals treated with the combined anti-B7 mAbs and rapamycin (Group 5).

Example 23: Induction Therapy with Monoclonal Antibodies Against B7-1 and B7-2 Delays the Onset of Renal Allograft Rejection in Non-human Primates

Abstract:

In this study, the administration of monoclonal antibodies anti-B7-1 (h1F1) and anti-B7-2 (h3D1) alone and in combination were tested for their ability to delay the onset of acute renal allograft rejection in rhesus monkeys. The most durable effect resulted from simultaneous blockade of both B7 ligands. The mechanism of action did not involve global depletion of T or B cells.

Materials and Methods:

Experimental Design:

MHC Typing and Donor-Recipient Selection:

Donor-recipient combinations were selected based on genetic non-identity at major histocompatibility complex (MHC) class II. This was established by

denaturing gradient gel electrophoresis and direct sequencing of the second exon of the major histocompatibility antigen, HLA-DRB. T cell responsiveness of the recipient towards the donor was confirmed *in vitro* for all donor-recipient pairs using the mixed lymphocyte reaction (MLR) assay. Each animal was tested against all potential donors to establish the highest responder pairs for transplantation.

Renal Allografts:

Renal allotransplantation was performed as previously described. Knechtle SJ, *et al., Transplantation,* 63:1-6 (1997); Kirk AD, *et al., Proc Natl Acad Sci USA.* 94:8789-8794 (1997); Kirk AD, *et al., Nature Medicine,* 5:686-693 (1999). Briefly, outbred juvenile rhesus monkeys (age 18 to 36 months, male), seronegative for simian immunodeficiency virus, and herpes B virus, were obtained from LABS of Virginia, Inc. (Yemassee, SC).

All procedures were performed under general anesthesia. Renal transplantation was performed between genetically distinct donor-recipient pairs as determined by MHC analysis. The animals were heparinized (100 units/kg) during organ harvest and implantation. The allograft was implanted using standard microvascular techniques to create an end to side anastomosis between the donor renal artery and recipient distal aorta as well as the donor renal vein and recipient vena cava. A primary ureteroneocystostomy was then created. Bilateral native nephrectomy was completed prior to closure. Skin sutures were removed after 7 to 10 days.

Anti-B7-1 and/or anti-B7-2 antibodies were administered intravenously at doses detailed below. Animals were euthanized at the time of renal failure as determined by a rising serum creatinine level or if a weight loss of 15% of pre-transplant body weight occurred in accordance with AAALAC standards. Complete gross and histopathological analysis was performed at necropsy on all sacrificed animals.

Results and Discussion:

Group I: Control Animals:

Five animals received renal allografts without any treatment to prevent rejection. All five lost their grafts to acute rejection within 8 days (Table 14, Figure 28).

Group II: Monotherapy with h1F1 Alone:

Two animals were treated solely with h1F1 (Table 14, Figure 28). The antibody was given at a dose of 20 mg/kg beginning prior to graft reperfusion. Subsequent doses of 5 mg/kg were given every seven days until rejection. These two animals had trivial prolongation of their graft survival with rejection occurring in 8 and 9 days.

Group III: Monotherapy with h3D1 Alone:

Two animals were treated solely with h3D1 (Table 14, Figure 28). The antibody was given at a dose of 20 mg/kg beginning prior to graft reperfusion. Subsequent doses of 5 mg/kg were given every seven days until rejection. These two animals also had minimal prolongation of their graft survival with rejection occurring in 8 and 28 days.

Group IV: Combination Therapy with h1F1 Together with h3D1:

Four animals were treated with both h1F1 and h3D1 (Table 14, Figure 28). The antibodies were given at a dose of 20 mg/kg beginning prior to graft reperfusion. In one animal (AT48) a dose of 5 mg/kg was given immediately post transplant. In all animals doses of 5 mg/kg were given every seven days for a fixed period of time or until rejection. Antibody administration was scheduled to be halted after 60 days in three of the four animals in Group IV. One animal was dosed out to 80 days (AC2B). All animals had prolonged graft survivals of 47, 67, 227, and >365 days. One animal remained alive and well without rejection at a time point of one year post-transplant. This animal was not sacrificed; however, for the purposes of

this study, monitoring was discontinued. Creatinine began to rise from baseline approximately one week prior to sacrifice in those animals who rejected.

The animals receiving combination therapy with both h1F1 and h3D1 had significantly prolonged allograft function (p=0.016) compared to Group I. The prolongation of survival was also markedly increased in the combination group when compared to both of the monotherapy groups.

Table 14 Survival and Diagnosis of Rhesus Monkeys

Date of Transplant	Group	Treatment	Recipient	Survival (poDay)
12/1/96	I	none	X9X	5
11/30/96	I	none	1FE	7
11/15/96	I	none	T4T	7
4/2/97	I	none	95052	8
5/3/99	I	none	AT5H	8
11/2/98	II	h1F1	AC74	9
2/10/99	II	h1F1	2WN	8
2/3/99	III	h3D1	AT5J	8
2/16/99	III	h3D1	2WF	28
10/26/98	IV	h1F1 + h3D1	AC2B	>365[a]
10/28/98	IV	h1F1 + h3D1	AC8V	47
3/1/99	IV	h1F1 + h3D1	AT5P	67[b]
3/2/99	IV	h1F1 + h3D1	AT48	227[b]

[a] 80 days of post-operative treatment

[b] 60 days of post-operative treatment

The treatment regimen and outcomes of all animals transplanted are shown.

Humanized antibodies were given at an initial dose of 20 mg/kg followed by 5 mg/kg and then weekly doses of 5 mg/kg for between 60-80 days. Animal AT48 also received a 5 mg/kg dose immediately post transplant.

The teachings of all the references, patents and/or patent applications cited herein are incorporated herein by reference in their entirety.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

Applicant's or agent's file reference 1290.1019003	International application No.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on pages 2;22;112;117 , line s 22,30; 3,10; 20; 8

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

10801 University Boulevard
Manassas, Virginia 20110-2209

Date of deposit	Accession Number
05 May 1998 22 June 1999	CRL-12524** PTA-263**

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet xx

In respect of those designations for which a European patent is sought, the Applicant(s) hereby informs the International Bureau that the Applicant wishes that, until the publication of the mention of the grant of a European patent or for 20 years from the date of filing if the application is refused or withdrawn or deemed to be withdrawn, the biological material deposited with the American Type Culture Collection under Accession No. **see above

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM (Additional Sheet)

C. ADDITIONAL INDICATIONS (Continued)

shall be made available as provided in Rule 28(3) EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

In respect of the designation of Australia in the subject PCT application, and in accordance with Regulation 3.25(3) of the Australian Patents Regulations, the Applicant hereby gives notice that the furnishing of a sample of the biological material deposited with the American Type Culture Collection under Accession No. **see page 1 shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention and who is nominated in a request for the furnishing of a sample.

In respect of the designation of Canada in the subject PCT application, the Applicant hereby informs the International Bureau that the Applicant wishes that, until either a Canadian patent has been issued on the basis of an application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the Commissioner of Patents only authorizes the furnishing of a sample of the biological material deposited with the American Type Culture Collection under Accession No. ** see page 1 and referred to in the application to an independent expert nominated by the Commissioner.

* C L M S *

CLAIMS

What is claimed is:

1. A humanized immunoglobulin having binding specificity to B7 molecules, said immunoglobulin comprising an antigen binding region of non-human origin and at least a portion of an immunoglobulin of human origin.

2. The humanized immunoglobulin of Claim 1, wherein the B7 molecules are B7-1 and/or B7-2.

3. A pharmaceutical composition comprising a humanized B7-1 antibody and a humanized B7-2 antibody.

4. A host cell comprising nucleic acid that encodes a humanized B7-1 antibody and/or a humanized B7-2 antibody.

5. A humanized immunoglobulin having binding specificity for B7-1, said immunoglobulin comprising an antigen binding region of non-human origin and at least a portion of an immunoglobulin of human origin.

6. The humanized immunoglobulin of Claim 5, wherein the portion of an immunoglobulin of human origin is derived from a human constant region.

7. The humanized immunoglobulin of Claim 6, wherein the human constant region comprises an IgG constant region.



8. The humanized immunoglobulin of Claim 7, wherein the human constant region contains a mutation capable of reducing the effector function of the immunoglobulin.

9. The humanized immunoglobulin of Claim 8, wherein the human constant region comprises an IgG2 constant region and a Valine amino acid at position 234 is substituted with Alanine and/or a Glycine amino acid at position 237 is substituted with Alanine.

10. The humanized immunoglobulin of Claim 7, wherein the IgG constant region is selected from the group consisting of: an IgG4 constant region and an IgG2 constant region.

11. The humanized immunoglobulin of Claim 5, wherein the antigen binding region is of rodent origin.

12. The humanized immunoglobulin of Claim 5, wherein the antigen binding region comprises a complementarity determining region of rodent origin, and the portion of an immunoglobulin of human origin is derived from a human framework region.

13. The humanized immunoglobulin of Claim 12, wherein the complementarity determining region is derived from 1F1 monoclonal antibody.

14. A humanized immunoglobulin having binding specificity for B7-1 derived from the cell line deposited with the ATCC, Accession No. PTA-263.

15. A humanized immunoglobulin light chain having binding specificity for B7-1 comprising CDR1, CDR2 and CDR3 of the light chain of murine 1F1 antibody, and a human light chain framework region.

16. The humanized immunoglobulin light chain of Claim 15, wherein the light chain comprises a variable region of SEQ ID NO: 28.

17. An isolated nucleic acid comprising a nucleic acid selected from the group consisting of:

a) SEQ ID NO:27,

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:28,

c) a nucleic acid which hybridizes to the nucleic acid according to a) or b) under stringent hybridization conditions, and

d) a nucleic acid which is the complement of the nucleic acid according to a) or b).

18. A humanized immunoglobulin heavy chain specific for B7-1 comprising CDR1, CDR2 and CDR3 of the heavy chain of the 1F1 antibody, and a human heavy chain framework region.

19. The humanized immunoglobulin heavy chain of Claim 18, wherein the heavy chain comprises a variable region of SEQ ID NO:26.

20. An isolated nucleic acid comprising a nucleic acid selected from the group consisting of:

a) SEQ ID NO: 25,

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:26,

c) a nucleic acid which hybridizes to the nucleic acid according to a) or b) under stringent hybridization conditions, and

d) a nucleic acid which is the complement of the nucleic acid according to a) or b).

21. A host cell comprising nucleic acid encoding the humanized immunoglobulin of Claim 5.

22. A fused gene encoding a humanized immunoglobulin light or heavy chain comprising:

a) a first nucleic acid sequence encoding an antigen binding region derived from murine 1F1 monoclonal antibody; and

b) a second nucleic acid sequence encoding at least a portion of a constant region of an immunoglobulin of human origin.

23. A method of inhibiting the interaction of a first cell bearing a B7-1 receptor with a second cell bearing B7-1, comprising contacting said first cell with an effective amount of a humanized immunoglobulin of Claim 5.

24. A method for treating an individual having a transplanted organ, tissue or cell, comprising administering a therapeutically effective amount of the humanized antibody of Claim 5.

25. A method of treating a disease that is modulated by B7-1 comprising administering a therapeutically effective amount of the humanized antibody of Claim 5.

26. A method of making a humanized immunoglobulin having binding specificity for B7-1, said immunoglobulin comprising an antigen binding region of non-human origin and at least a portion of an immunoglobulin of human origin, comprising the steps of:

(a) determining the complementarity determining regions of an antibody of non-human origin which has binding specificity for B7-1;

(b) obtaining a human antibody having a framework region amino acid sequence suitable for grafting of the complementarity determining regions determined in (a), and

(c) grafting the complementarity determining regions of (a) into the framework region of the human antibody of (b),

wherein a humanized immunoglobulin having binding specificity for B7-1 is made.

27. A method for determining the presence or absence of B7-1 in a sample comprising the steps of:

a) contacting said sample with a humanized antibody specific to B7-1 sufficiently to allow formation of a complex between B7-1 and the anti-B7-1 antibody, and

b) detecting the presence or absence of said complex formation.

28. A humanized immunoglobulin having binding specificity for B7-2, said immunoglobulin comprising an antigen binding region of non-human origin and at least a portion of an immunoglobulin of human origin.

29. The humanized immunoglobulin of Claim 28, wherein the portion of an immunoglobulin of human origin is derived from a human constant region.

30. The humanized immunoglobulin of Claim 29, wherein the human constant region comprises an IgG constant region.

31. The humanized immunoglobulin of Claim 30, wherein the human constant region contains a mutation capable of reducing the effector function of the immunoglobulin.

32. The humanized immunoglobulin of Claim 31, wherein the human constant region comprises an IgG2 constant region and a Valine amino acid at position 234 is substituted with Alanine and/or a Glycine amino acid at position 237 is substituted with Alanine.

33. The humanized immunoglobulin of Claim 30, wherein the IgG constant region is selected from the group consisting of an IgG4 constant region and an IgG2 constant region.

34. The humanized immunoglobulin of Claim 28, wherein the antigen binding region is of rodent origin.

35. The humanized immunoglobulin of Claim 28, wherein the antigen binding region comprises a complementarity determining region of rodent origin, and the portion of an immunoglobulin of human origin is derived from a human framework region.

36. The humanized immunoglobulin of Claim 35, wherein the complementarity determining region is derived from 3D1 monoclonal antibody.

37. A humanized immunoglobulin having binding specificity for B7-2 derived from the cell line deposited with the ATCC, Accession No. CRL-12524.

38. A humanized immunoglobulin light chain having binding specificity for B7-2 comprising CDR1, CDR2 and CDR3 of the light chain of murine 3D1 antibody, and a human light chain framework region.

39. The humanized immunoglobulin light chain of Claim 38, wherein the light chain comprises a variable region of SEQ ID NO: 8.

40. An isolated nucleic acid comprising a nucleic acid selected from the group consisting of:

a) SEQ ID NO:7,

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:8,

c) a nucleic acid which hybridizes to the nucleic acid according to a) or b) under stringent hybridization conditions, and

d) a nucleic acid which is the complement of the nucleic acid according to a) or b).

41. A humanized immunoglobulin heavy chain specific for B7-2 comprising CDR1, CDR2 and CDR3 of the heavy chain of the 3D1 antibody, and a human heavy chain framework region.

42. The humanized immunoglobulin heavy chain of Claim 41, wherein the heavy chain comprises a variable region of SEQ ID NO:6.

43. An isolated nucleic acid comprising a nucleic acid selected from the group consisting of:

b) SEQ ID NO: 5,

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:6,

c) a nucleic acid which hybridizes to the nucleic acid according to a) or b) under stringent hybridization conditions, and

d) a nucleic acid which is the complement of the nucleic acid according to a) or b).

44. A host cell comprising nucleic acid encoding the humanized immunoglobulin of Claim 28.

45. A fused gene encoding a humanized immunoglobulin light or heavy chain comprising:

a) a first nucleic acid sequence encoding an antigen binding region derived from murine 3D1 monoclonal antibody; and

b) a second nucleic acid sequence encoding at least a portion of a constant region of an immunoglobulin of human origin.



46. A method of inhibiting the interaction of a first cell bearing a B7-2 receptor with a second cell bearing B7-2, comprising contacting said first cell with an effective amount of a humanized immunoglobulin of Claim 28.

47. A method for treating an individual having a transplanted organ, tissue or cell, comprising administering a therapeutically effective amount of the humanized antibody of Claim 28.

48. A method of treating a disease that is modulated by B7-2 comprising administering a therapeutically effective amount of the humanized antibody of Claim 28.

49. A method of making a humanized immunoglobulin having binding specificity for B7-2, said immunoglobulin comprising an antigen binding region of non-human origin and at least a portion of an immunoglobulin of human origin, comprising the steps of:

(a) determining the complementarity determining regions of an antibody of non-human origin which has binding specificity for B7-2;

(b) obtaining a human antibody having a framework region amino acid sequence suitable for grafting of the complementarity determining regions determined in (a), and

(c) grafting the complementarity determining regions of (a) into the framework region of the human antibody of (b),

wherein a humanized immunoglobulin having binding specificity for B7-2 is made.



50. A method for determining the presence or absence of B7-2 in a sample comprising the steps of:

a) contacting said sample with a humanized antibody specific to B7-2 sufficiently to allow formation of a complex between B7-2 and the anti-B7-2 antibody, and

b) detecting the presence or absence of said complex formation.

51. A method for transplanting cells to an individual in need thereof, comprising:

a) obtaining cells from a donor,

b) contacting the cells with an immunoglobulin specific to B7-1, an immunoglobulin specific to B7-2 and recipient cells from the individual for a period of time sufficient for tolerance induction, thereby obtaining a mixture, and

c) introducing the mixture to the individual.

52. The method of Claim 51, wherein the cells from the donor are derived from bone marrow or blood.

53. The method of Claim 51, wherein the recipient cell is a lymphocyte.

54. The method of Claim 51, wherein the period of time is between about 12 hours and about 96 hours.

55. The method of Claim 54, wherein the period of time is between about 36 and 48 hours.



56. The method of Claim 55, wherein the individual has a disease that is selected from the group consisting of: a proliferative disease, anemia, inborn errors of metabolism, congenital immunodeficiency diseases, and myeloid dysplasia syndrome.

57. The method of Claim 56, wherein the proliferative disease is selected from the group consisting of: leukemia, lymphoma and cancer.

58. The method of Claim 57, wherein the anemia is selected from the group consisting of: sickle-cell anemia, thalassemia and aplastic anemia.

59. The method of Claim 51, comprising administering to the individual a drug that is used to modulate the immune response.

60. The method of Claim 59, wherein the drug is selected from the group consisting of: methotrexate, rapamycin, cyclosporin, steroids, CD40 pathway inhibitors, transplant salvage pathway inhibitors, IL-2 receptor antagonists and analogs thereof.

61. A method of treating a transplant recipient or preventing transplant rejection in a transplant recipient, comprising administering to the recipient an effective amount of an immunoglobulin specific to B7-1 and an effective amount of an immunoglobulin specific to B7-2.



62. The method of Claim 61, further including administering a composition selected from the group consisting of: calcineurin inhibitors, steroids, and immunosuppressive agents that arrest the growth of immune cells, methotrexate, CD40 pathway inhibitors, transplant salvage pathway inhibitors, IL-2 receptor antagonists and analogs thereof.

63. The method of Claim 62, wherein the calcineurin inhibitor is cyclosporin A or FK506.

64. The method of Claim 62, wherein the steroid is methyl prednisone or prednisone.

65. The method of Claim 62, wherein the immunosuppressive agent that arrests the growth of immune cells is rapamycin.

66. The method of Claim 61, wherein the immunoglobulin specific to B7-1 is administered in an amount between about 1 mg/kg and 25 mg/kg, and the immunoglobulin specific to B7-2 is administered in an amount between about 1 mg/kg and 25 mg/kg.

67. The method of Claim 66, wherein the immunoglobulin specific to B7-1 and the immunoglobulin specific to B7-2 are administered on the day the recipient receives the transplantation.



68. The method of Claim 67, wherein the humanized immunoglobulin specific to B7-1 is administered between about 1 mg/kg and 25 mg/kg, and the humanized immunoglobulin specific to B7-2 is administered between about 1 mg/kg and 25 mg/kg on the day the recipient receives the transplantation.

69. The method of Claim 68, wherein the humanized immunoglobulin specific to B7-1 and the humanized immunoglobulin specific to B7-2 are further administered periodically after the recipient receives the transplantation.

70. The method of Claim 69, wherein the humanized immunoglobulin specific to B7-1 and the humanized immunoglobulin specific to B7-2 are further administered at least weekly after the recipient receives the transplantation.

71. The method of Claim 70, wherein the humanized immunoglobulin specific to B7-1 is administered between about 1 mg/kg and 5 mg/kg, and the humanized immunoglobulin specific to B7-2 is administered between about 1 mg/kg and 5 mg/kg at least weekly after the recipient receives the transplantation.

72. A method for treating an individual with a disease selected from the group consisting of: autoimmune diseases, infectious diseases, inflammatory disorders, systemic lupus erythematosus, diabetes mellitus, insulitis, asthma, arthritis, inflammatory bowel disease, inflammatory dermatitis, and multiple sclerosis, comprising administering a therapeutically effective amounts of a humanized immunoglobulin specific to B7-1 and/or a therapeutically effective amount of a humanized immunoglobulin specific to B7-2.



73. A method of modulating an immune response of an individual having a transplanted organ, tissue, cell or the like comprising administering an effective amount of the humanized immunoglobulin specific to B7-1 and/or an effective amount of a humanized immunoglobulin specific to B7-2 in a carrier.

74. The method of Claim 73, comprising administering a drug that is used to modulate the immune response of an individual having a transplanted organ, tissue, cell or the like, wherein the drug is selected from the group consisting of: methotrexate, rapamycin, cyclosporin, steroids, CD40 pathway inhibitors, transplant salvage pathway inhibitors, IL-2 receptor antagonists and analogs thereof.

75. A method of decreasing an antibody response to an antigen in a mammal, comprising administering to the individual an effective amount of a humanized immunoglobulin specific to B7-1 and/or a humanized immunoglobulin specific to B7-2, in the presence of the antigen.

76. The method of Claim 75, further including administering the antigen to the individual.

77. The method of Claim 75, wherein the antigen is selected from the group consisting of: tetanus toxoid, Factor VIII, Factor IX, insulin, growth hormone, and a gene delivery vector.

78. Use of a humanized immunoglobulin having binding specificity to B7-1 and/or B7-2 for use in therapy, e.g., autoimmune diseases, infectious diseases, inflammatory disorders, systemic lupus erythematosus, diabetes mellitus, insulitis, asthma, arthritis, inflammatory bowel disease, inflammatory dermatitis, and multiple sclerosis, comprising the composition of Claim 1.

79. Use of a humanized immunoglobulin having binding specificity to B7-1 and/or B7-2, for the manufacture of a medicament, for transplanting cells, tissue or organs an individual.

80. Use of a humanized immunoglobulin having binding specificity to B7-1 and/or B7-2 in the presence of an antigen, for the manufacture of a medicament, for decreasing an antibody response to an antigen in a mammal.