HUMAN CTLA-4 ANTIBODIES AND THEIR USES

REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional patent application Serial No. 60/150,452, the disclosure of which is incorporated herein in its entirety.

FIELD OF THE INVENTION

The present invention relates generally to molecular immunology and the treatment of human diseases. In particular, it relates to novel human sequence antibodies against human CTLA-4 and methods of treating human diseases and infections using these antibodies.

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BACKGROUND OF THE INVENTION

The vertebrate immune system requires multiple signals to achieve optimal immune activation; *see, e.g.*, Janeway, Cold Spring Harbor Symp. *Quant. Biol.* 54:1-14 (1989); Paul William E., ed. Raven Press, N.Y., *Fundamental Immunology*, 4th edition (1998), particularly chapters 12 and 13, pages 411 to 478. Interactions between T lymphocytes (T cells) and antigen presenting cells (APC) are essential to the immune response. Levels of many cohesive molecules found on T cells and APC's increase during an immune response (Springer *et al..*, *A. Rev. Immunol.* 5:223-252 (1987); Shaw and Shimuzu, *Current Opinion in Immunology*, Eds. Kindt and Long, 1:92-97 (1988)); and Hemler, *Immunology Today* 9:109-113 (1988)). Increased levels of these molecules may help explain why activated APC's are more effective at stimulating antigen-specific T cell proliferation than are resting APC's (Kaiuchi *et al..*, *J. Immunol.* 131:109-114 (1983); Kreiger *et al..*, *J. Immunol.* 135:2937-2945 (1985); McKenzie, *J. Immunol.* 141:2907-2911 (1988); and Hawrylowicz and Unanue, *J. Immunol.* 141:4083-4088 (1988)).

T cell immune response is a complex process that involves cell-cell interactions (Springer *et al..*, *A. Rev. Immunol.* 5:223-252 (1987)), particularly between T and accessory cells such as APC's, and production of soluble immune mediators (cytokines or lymphokines) (Dinarello (1987) *New Engl. Jour. Med* 317:940-945; Sallusto (1997) *J. Exp. Med.* 179:1109-1118). This response is regulated by several T-cell surface receptors, including the T-cell receptor complex (Weiss (1986) *Ann. Rev. Immunol.* 4:593-619) and other "accessory" surface molecules (Allison (1994) *Curr.*

Opin. Immunol. 6:414-419; Springer (1987) *supra*). Many of these accessory molecules are naturally occurring cell surface differentiation (CD) antigens defined by the reactivity of monoclonal antibodies on the surface of cells (McMichael, Ed., *Leukocyte Typing III*, Oxford Univ. Press, Oxford, N.Y. (1987)).

Early studies suggested that B lymphocyte activation requires two signals (Bretscher (1970) *Science* 169:1042-1049) and now it is believed that all lymphocytes require two signals for their optimal activation, an antigen specific or clonal signal, as well as a second, antigen non-specific signal. (Janeway, *supra*). Freeman (1989) *J. Immunol.* 143:2714-2722) isolated and sequenced a cDNA clone encoding a B cell activation antigen recognized by MAb B7 (Freeman (1987) *J. Immunol.* 138:3260). COS cells transfected with this cDNA have been shown to stain by both labeled MAb B7 and MAb BB-1 (Clark (1986) Human Immunol. 16:100-113; Yokochi (1981) *J. Immunol.* 128:823; Freeman *et al..*, (1989) *supra*; Freeman *et al..* (1987), *supra*). In addition, expression of this antigen has been detected on cells of other lineages, such as monocytes (Freeman *et al..*, *supra*).

T helper cell (Th) antigenic response requires signals provided by APC's. The first signal is initiated by interaction of the T cell receptor complex (Weiss, *J. Clin. Invest.* 86:1015 (1990)) with antigen presented in the context of class II major histocompatibility complex (MHC) molecules on the APC (Allen, *Immunol. Today* 8:270 (1987)). This antigen-specific signal is not sufficient to generate a full response, and in the absence of a second signal may actually lead to clonal inactivation or anergy (Schwartz, *Science* 248:1349 (1990)). The requirement for a second "costimulatory" signal provided by the MHC has been demonstrated in a number of experimental systems (Schwartz, *supra*; Weaver and Unanue, *Immunol. Today* 11:49 (1990)). The molecular nature of this second signal is not completely understood, although it is clear in some cases that both soluble molecules such as interleukin (IL)-1 (Weaver and Unanue, *supra*) and membrane receptors involved in intercellular adhesion (Springer, *Nature* 346:425 (1990)) can provide costimulatory signals.

CD28 antigen, a homodimeric glycoprotein of the immunoglobulin superfamily (Aruffo and Seed, *Proc. Natl. Acad. Sci.* 84:8573-8577 (1987)), is an accessory molecule found on most mature human T cells (Damle *et al..*, *J. Immunol.* 131:2296-2300 (1983)). Current evidence suggests that this molecule functions in an alternative T cell activation pathway distinct from that initiated by the T-cell receptor complex (June *et al..*, *Mol. Cell. Biol.* 7:4472-4481 (1987)). Monoclonal antibodies

(MAbs) reactive with CD28 antigen can augment T cell responses initiated by various polyclonal stimuli (reviewed by June *et al.*, *supra*). These stimulatory effects may result from MAb-induced cytokine production (Thompson *et al.*, *Proc. Natl. Acad. Sci* 86:1333-1337 (1989); and Lindsten *et al.*, *Science* 244:339-343 (1989)) as a consequence of increased mRNA stabilization (Lindsten *et al.*. (1989), *supra*). Anti-CD28 mAbs can also have inhibitory effects, *i.e.*, they can block autologous mixed lymphocyte reactions (Damle *et al.*, *Proc. Natl. Acad. Sci.* 78:5096-6001 (1981)) and activation of antigen-specific T cell clones (Lesslauer *et al.*, Eur. *J. Immunol.* 16:1289-1296 (1986)).

Some studies have indicated that CD28 is a counter-receptor for the B cell activation antigen, B7/BB-1 (Linsley *et al.*, *Proc. Natl. Acad. Sci.* USA 87:5031-5035 (1990)). The B7/BB-1 antigen is hereafter referred to as the "B7 antigen". The B7 ligands are also members of the immunoglobulin superfamily but have, in contrast to CD28, two Ig domains in their extracellular region, an N-terminal variable (V)-like domain followed by a constant (C)-like domain.

Delivery of a non-specific costimulatory signal to the T cell requires at least two homologous B7 family members found on APC's, B7-1 (also called B7, B7.1, or CD80) and B7-2 (also called B7.2 or CD86), both of which can deliver costimulatory signals to T cells via CD28. Costimulation through CD28 promotes T cell activation.

Using genetic fusions of the extracellular portions of B7 antigen and CD28 receptor, and Immunoglobulin (Ig) C.gamma.1 (constant region heavy chains), interactions between CD28 and B7 antigen have been characterized (Linsley *et al.*, *J. Exp. Med.* 173:721-730 (1991)). Immobilized B7Ig fusion protein, as well as B7 positive CHO cells, have been shown to costimulate T cell proliferation.

T cell stimulation with B7 positive CHO cells also specifically stimulates increased levels of transcripts for IL-2. Additional studies have shown that anti-CD28 MAb inhibited IL-2 production induced in certain T cell leukemia cell lines by cellular interactions with a B cell leukemia line (Kohno *et al.*, Cell. Immunol. 131-1-10 (1990)).

CD28 has a single extracellular variable region (V)-like domain (Aruffo and Seed, *supra*). A homologous molecule, CTLA-4 has been identified by differential screening of a murine cytolytic-T cell cDNA library (Brunet (1987) *Nature* 328:267-270).

CTLA-4 is a T cell surface molecule that was originally identified by differential screening of a murine cytolytic T cell cDNA library (Brunet *et al.*, *Nature* 328:267-270(1987)). CTLA-4 is also a member of the immunoglobulin (Ig) superfamily; CTLA-4 comprises a single extracellular Ig domain. CTLA-4 transcripts have been

found in T cell populations having cytotoxic activity, suggesting that CTLA-4 might function in the cytolytic response (Brunet *et al..*, *supra* ; Brunet *et al..*, Immunol. Rev. 103-21-36 (1988)). Researchers have reported the cloning and mapping of a gene for the human counterpart of CTLA-4 (Dariavach *et al..*, *Eur. J. Immunol.* 18:1901-1905 (1988)) to the same chromosomal region (2q33-34) as CD28 (Lafage-Pochitaloff *et al..*, *Immunogenetics* 31:198-201 (1990)). Sequence comparison between this human CTLA-4 DNA and that encoding CD28 proteins reveals significant homology of sequence, with the greatest degree of homology in the juxtamembrane and cytoplasmic regions (Brunet *et al..*, 1988, *supra*; Dariavach *et al..*, 1988, *supra*).

Some studies have suggested that CTLA-4 has an analogous function as a secondary costimulator (Linsley *et al.*, *J Exp. Med.* 176:1595-1604 (1992); Wu *et al..*, *J Exp. Med.* 185:1327-1335 (1997) Lindsley, P. *et al..* U.S. Patent Nos. 5,977,318; 5,968,510; 5,885,796; and 5,885,579). However, others have reported that CTLA-4 has an opposing role as a dampener of T cell activation (Krummel (1995) *J. Exp. Med.* 182:459-465); Krummel *et al..*, *Int'l Immunol.* 8:519-523(1996); Chambers *et al..*, *Immunity.* 7:885-895(1997)). It has been reported that CTLA-4 deficient mice suffer from massive lymphoproliferation (Chambers *et al..*, *supra*). It has been reported that CTLA-4 blockade augments T cell responses *in vitro* (Walunas *et al..*, *Immunity.* 1:405-413 (1994)) and *in vivo* (Kearney (1995) *J. Immunol.* 155:1032-1036), exacerbates antitumor immunity (Leach (1996) *Science.* 271:1734-1736), and enhances an induced autoimmune disease (Luhder (1998) *J Exp. Med.* 187:427-432). It has also been reported that CTLA-4 has an alternative or additional impact on the initial character of the T cell immune response (Chambers (1997) *Curr. Opin. Immunol.* 9:396-404; Bluestone (1997) *J. Immunol.* 158:1989-1993; Thompson (1997) *Immunity 7:445-450*). This is consistent with the observation that some autoimmune patients have autoantibodies to CTLA-4. It is possible that CTLA-4 blocking antibodies have a pathogenic role in these patients (Matsui (1999) J. Immunol. 162:4328-4335).

Non-human CTLA-4 antibodies have be used in the various studies discussed above. However, one of the major impediments facing the development of *in vivo* therapeutic and diagnostic applications for antibodies in humans is the intrinsic immunogenicity of non-human immunoglobulins. For example, when immunocompetent human patients are administered therapeutic doses of rodent monoclonal antibodies, the patients produce antibodies against the rodent immunoglobulin sequences; these human anti-mouse antibodies (HAMA) neutralize the therapeutic antibodies and can cause acute

toxicity. These and other deficiencies in the previous antibodies are overcome by the provision of human antibodies to CTLA-4 by the present invention.

SUMMARY OF THE INVENTION

The present invention provides a human sequence antibody that specifically binds to human CTLA-4 and a human sequence antibody that specifically binds to human CTLA-4 which is substantially free of non-immunoglobulin associated human proteins.

In a related aspect, the invention also provides a therapeutically-effective human sequence antibody that specifically binds to human CTLA-4. In some embodiments, the therapeutically-effective human sequence antibody binds to CTLA-4 on the cell surface of normal human T cells. In other embodiments, the T cell subpopulations marked by CD antigens CD4, CD8, CD25, and CD69 remain stable during and subsequent to the administration of the therapeutically-effective human sequence antibody. In other embodiments, the therapeutically-effective human sequence antibody binds CTLA-4 on the cell surface of normal human T cells. In other embodiments, the human sequence antibody well-tolerated in a patient. In a related embodiment,

Also provided is a composition of polyclonal antibodies comprising a plurality of human sequence antibodies that specifically bind to human CTLA-4. The composition of polyclonal antibodies can comprise at least about 2, 5, 10, 50, 100, 500 or 1000 different human sequence antibodies that specifically bind to human CTLA-4.

The invention also provides human sequence antibodies that specifically bind to human CTLA-4 and which block binding of human CTLA-4 to human B7 or do not block binding of human CTLA-4 to human B7.

The invention also provides human sequence antibodies that bind to human CTLA-4 with an equilibrium association constant (Ka) of at least 10^8 M^{-1}. Also provided are human sequence antibodies that bind to human CTLA-4 with an equilibrium association constant (Ka) of at least 10^9 M^{-1}.

The invention also provides human sequence antibodies that specifically bind to human CTLA-4 that block binding of human CTLA-4 to human B7 by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 99%, or 100%.

The invention also provides human sequence antibodies that specifically bind to human CTLA-4 having an antibody heavy chain of either IgG or IgM. The IgG

antibody heavy chain can be IgG1, IgG2, IgG3 or IgG4. The invention also provides human sequence antibodies wherein the antibody light chain is a kappa light chain. The human sequence antibody can be encoded by human IgG heavy chain and human kappa light chain nucleic acids that comprise nucleotide sequences in their variable regions as set forth in SEQ ID NO:2 through SEQ ID NO:23, respectively.

The invention also provides a human sequence antibody wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids that comprise nucleotide sequences in their variable regions as set forth in SEQ ID NO:16 and SEQ ID NO:6, respectively.

The invention also provides a human sequence antibody wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids that comprise nucleotide sequences in their variable regions as set forth in SEQ ID NO:18 and SEQ ID NO:8, respectively.

The invention also provides a human sequence antibody wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids that comprise nucleotide sequences in their variable regions as set forth in SEQ ID NO:22 and SEQ ID NO:12, respectively.

The invention also provides a human sequence antibody wherein the human sequence antibody is encoded by heavy chain and light chain variable region amino acid sequences as set for the in SEQ ID NO:17 and SEQ ID NO:7, respectively.

The invention provides a human sequence antibody wherein the human sequence antibody is encoded by heavy chain and light chain variable region amino acid sequences as set for the in SEQ ID NO:19 and SEQ ID NO:9, respectively.

The invention also provides a human sequence antibody wherein the human sequence antibody is encoded by heavy chain and light chain variable region amino acid sequences as set for the in SEQ ID NO:23 and SEQ ID NO:13, respectively.

The invention provides a human sequence antibody wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids comprising variable heavy and light chain sequences from V gene segments VH 3-30.3 and VK A-27, respectively.

The invention also provides a human sequence antibody wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids comprising variable heavy and light chain sequences from V gene segments VH 3-33 and VK L-15, respectively.

Some human sequence antibodies of the invention comprise heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGNNKYYADSVKG (SEQ ID NO:32) and TGWLGPFDY (SEQ ID NO:37), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVGSSYLA (SEQ ID NO:24), GAFSRAT (SEQ ID NO:29), and QQYGSSPWT (SEQ ID NO:35), respectively.

Some human sequence antibodies of the invention comprise heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGSNKHYADSVKG (SEQ ID NO:33) and TGWLGPFDY (SEQ ID NO:38), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVSSSFLA (SEQ ID NO:25), GASSRAT (SEQ ID NO:30), and QQYGSSPWT (SEQ ID NO:35), respectively.

Other human sequence antibodies of the invention comprise heavy chain CDR1, CDR2, and CDR3 sequences, SYGMH (SEQ ID NO:28), VIWYDGSNKYYADSVKG (SEQ ID NO:34) and APNYIGAFDV (SEQ ID NO:39), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQGISSWLA (SEQ ID NO:26), AASSLQS (SEQ ID NO:31), and QQYNSYPPT (SEQ ID NO:36), respectively.

The invention also provides human sequence antibodies that specifically bind to human CTLA-4, wherein said human sequence antibody is produced by a transgenic non-human animal. The transgenic non-human animal can be a mouse.

The invention also provides a human sequence antibody that specifically bind to human CTLA-4 that is a Fab fragment.

The invention provides a polyvalent complex comprising at least two human sequence antibodies each of which specifically binds to human CTLA-4. The two different antibodies can be linked to each other covalently or non-covalently.

The invention provides a nucleic acid encoding a heavy chain of a human sequence antibody. The nucleic acid can comprise a nucleotide sequence as set forth in SEQ ID NO:1.

The invention provides a transgenic non-human animal having a genome comprising a human sequence heavy chain transgene and a human sequence light chain transgene, which animal has been immunized with a human CTLA-4, or a fragment or an analog thereof, whereby the animal expresses human sequence antibodies to the human

CTLA-4. The transgenic non-human animal can be a transgenic mouse. The transgenic mouse can comprise HCo7 or HCo12.

The invention provides a hybridoma cell line comprising a B cell obtained from a transgenic non-human animal having a genome comprising a human sequence heavy chain transgene and a human sequence light chain transgene, wherein the hybridoma produces a human sequence antibody that specifically binds to human CTLA-4. In a related embodiment,the hybridoma secretes a human sequence antibody that specifically binds human CTLA-4 or binding fragment thereof, wherein the antibody is selected from the group consisting of: a human sequence antibody comprising heavy chain heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGNNKYYADSVKG (SEQ ID NO:32) and TGWLGPFDY (SEQ ID NO:37), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVGSSYLA (SEQ ID NO:24), GAFSRAT (SEQ ID NO:29), and QQYGSSPWT (SEQ ID NO:35), respectively, and heavy chain and light chain variable region amino acid sequences as set forth in SEQ ID NO:17 and SEQ ID NO:7, respectively; a human sequence antibody comprising heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGSNKHYADSVKG (SEQ ID NO:33) and TGWLGPFDY (SEQ ID NO:38), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVSSSFLA (SEQ ID NO:25), GASSRAT (SEQ ID NO:30), and QQYGSSPWT (SEQ ID NO:35), respectively, and heavy chain and light chain variable region amino acid sequences as set forth in SEQ ID NO:19 and SEQ ID NO:9, respectively; or a human sequence antibody of claim 1, comprising heavy chain CDR1, CDR2, and CDR3 sequences, SYGMH (SEQ ID NO:28), VIWYDGSNKYYADSVKG (SEQ ID NO:34) and APNYIGAFDV (SEQ ID NO:39), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQGISSWLA (SEQ ID NO:26), AASSLQS (SEQ ID NO:31), and QQYNSYPPT (SEQ ID NO:36), respectively, and heavy chain and light chain variable region amino acid sequences as set forth in SEQ ID NO:23 and SEQ ID NO:13, respectively.

The invention provides a pharmaceutical composition comprising a human sequence antibody that specifically binds to human CTLA-4 and a pharmaceutically acceptable carrier. The pharmaceutical composition can further comprise an agent effective to induce an immune response against a target antigen. Also provided are chemotherapeutic agents. In addition, antibodies to immunosuppressive molecules are also provided.

The invention provides a method for inducing, augmenting or prolonging an immune response to an antigen in a patient, comprising administering to the patient an effective dosage of a human sequence antibody that specifically binds to human CTLA-4, wherein the antibody blocks binding of human CTLA-4 to human B7. The antigen can be a tumor antigen, or the antigen can be from a pathogen. The tumor antigen can also be telomerase. The pathogen can be a virus, a bacterium, a fungus or a parasite. The pathogen can also be an HIV. This method can further comprise administering the antigen, or a fragment or an analog thereof, to the patient, whereby the antigen in combination with the human sequence antibody induces, augments or prolongs the immune response. The antigen can be a tumor antigen or a component of an amyloid formation in the patient, such as a patient suffering from Alzheimer's disease and the antigen is AB peptide. This method can further comprise administering a cytokine to the patient.

The invention provides a method of suppressing an immune response in a patient, comprising administering to the patient an effective dosage of a polyvalent preparation comprising at least two human sequence antibodies to human CTLA-4 linked to each other. The invention also provides a method of suppressing an immune response in a patient, comprising administering to the patient an effective dosage of a polyclonal preparation comprising at least two human sequence antibodies to human CTLA-4.

The present invention further provides isolated or recombinant human sequence antibodies and human monoclonal antibodies which specifically bind to human CTLA-4, as well as compositions containing one or a combination of such antibodies. Some of the human sequence antibodies of the invention are characterized by binding to human CTLA-4 with high affinity, and/or by blocking the interaction of human CTLA-4 with its ligand, the human B7-1 and B7-2 molecules. Accordingly, the human sequence antibodies and the human monoclonal antibodies of the invention can be used as diagnostic or therapeutic agents *in vivo* and *in vitro*.

The human sequence antibodies of the invention can encompass various antibody isotypes, or mixtures thereof, such as IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD, and IgE. Typically, they include IgG1 (*e.g.*, IgG1k) and IgM isotypes. The human sequence antibodies can be full-length (*e.g.*, an IgG1 or IgG4 antibody) or can include only an antigen-binding portion (*e.g.*, a Fab, F(ab')2, Fv or a single chain Fv fragment). Some human sequence antibodies are recombinant human

sequence antibodies. Some human sequence antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a human light chain transgene. The hybridoma can be made by, *e.g.*, fusing the B cell to an immortalized cell. Some human sequence antibodies of the invention are produced by hybridomas referred to as 4C8, 4E10, 4E10.5, 5A8, 5C4, 5C4.1.3, 5D7, 5D7.1, 5E10, 5E10.12, 5G1, 5G1.4, 6A10, 6C9, 6C9.6, 6D9, 6D9.7, 6G4, 7E4, 7E4.4, 7E6, 7H8, 8E8, 8E8.4, 8F8, 8F8.19, 8H1, 9810, 9A10.1, 9B9, 9C1, 9G5, 105B, 10B5.8, 10B9, 10B9.2, 10D1, 10D1.3, 10E11, 10E4, 10E4.5, 11B4, 11D10, 11E4, 11E4.1, 11E8, 11F10, 11F11, 11F9, 11G1, 11G1.5, 1C7, 1H8.8, 2A7, 2A7.6, 2E2, 2E2.7, 2E7, 2E7.2, 2G1, 2G1.2, 3C12, 3E10, 3E10.5, 3E6, 3E6.0, 3F10, 4A1, 4B6 and 4B6.12. Suffixes after the decimal point indicate different clonal isolates of the same hybridoma cell lines.

Some human sequence anti-CTLA-4 antibodies of the present invention can be characterized by one or more of the following properties: a) specificity for human CTLA-4 (specifically binding to human CTLA-4); b) a binding affinity to human CTLA-4 with an equilibrium association constant (K_a) of at least about $10^7\ M^{-1}$, or about $10^9\ M^{-1}$, or about $10^{10}\ M^{-1}$ to $10^{11}\ M^{-1}$ or higher; c) a kinetic association constant (k_a) of at least about 10^3, about 10^4, or about $10^5\ m^{-1}s^{-1}$; and/or, d) a kinetic disassociation constant (k_d) of at least about 10^3, about 10^4, or about $10^5\ m^{-1}s^{-1}$.

In another aspect, the invention provides nucleic acid molecules encoding the human sequence antibodies, or antigen-binding portions, of the invention. Accordingly, recombinant expression vectors that include the antibody-encoding nucleic acids of the invention, and host cells transfected with such vectors, are also encompassed by the invention, as are methods of making the antibodies of the invention by culturing these host cells.

In yet another aspect, the invention provides isolated B-cells from a transgenic non-human animal, *e.g.*, a transgenic mouse, which are capable of expressing various isotypes (*e.g.*, IgG, IgA and/or IgM) of human monoclonal antibodies that specifically bind to human CTLA-4. The isolated B cells can be obtained from a transgenic non-human animal, *e.g.*, a transgenic mouse, which has been immunized with a purified or enriched preparation of human CTLA-4 antigen (or antigenic fragment thereof) and/or cells expressing human CTLA-4. The transgenic non-human animal, *e.g.*, a transgenic mouse, can have a genome comprising a human heavy chain transgene and a

human light chain transgene. The isolated B-cells can be immortalized to provide a source (*e.g.*, a hybridoma) of human monoclonal antibodies to human CTLA-4.

Accordingly, the present invention also provides a hybridoma capable of producing human monoclonal antibodies that specifically bind to human CTLA-4. The hybridoma can include a B cell obtained from a transgenic non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a human light chain transgene fused to an immortalized cell. The transgenic non-human animal can be immunized with a purified or enriched preparation of human CTLA-4 antigen and/or cells expressing human CTLA-4 to generate antibody-producing hybridomas.

In yet another aspect, the invention provides a transgenic non-human animal, such as a transgenic mouse, which express human monoclonal antibodies (also referred to herein as a "HuMAb-Mouse™") that specifically bind to human CTLA-4. The transgenic non-human animal can be a transgenic mouse having a genome comprising a human heavy chain transgene and a human light chain transgene. The transgenic non-human animal can be immunized with a purified or enriched preparation of CTLA-4 antigen (or antigenic fragment thereof) and/or cells expressing the human CTLA-4. The transgenic non-human animal, *e.g.*, the transgenic mouse, can be capable of producing multiple isotypes of human monoclonal antibodies to human CTLA-4 (*e.g.*, IgG, IgA and/or IgM) by undergoing V-D-J recombination and isotype switching. Isotype switching may occur by, *e.g.*, classical or non-classical isotype switching.

In another aspect, the present invention provides methods for producing human sequence antibodies and human sequence monoclonal antibodies that specifically react with human CTLA-4. Some methods of the invention include immunizing a transgenic non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a human light chain transgene, with a purified or enriched preparation of human CTLA-4 antigen and/or cells expressing human CTLA-4. B cells (*e.g.*, splenic B cells) of the animal can then be obtained and fused with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies against human CTLA-4.

Anti- human CTLA-4 human monoclonal antibodies of the invention, or antigen binding portions thereof (*e.g.*, Fab), can be derivatized or linked to another functional molecule, *e.g.*, another peptide or protein (*e.g.*, an Fab' fragment). For

example, an antibody or antigen-binding portion of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities. For example, the human sequence anti-CTLA-4 antibody, or antigen binding fragment thereof, can be conjugated to a therapeutic moiety, *e.g.*, a cytotoxic drug, an enzymatically active toxin, or a fragment thereof, a radioisotope, or a small molecule anti-cancer drug. The antibodies of the invention can also be conjugated to cytotoxic pharmaceuticals, *e.g.*, radiolabeled with a cytotoxic agents, such as, *e.g.*, ^{131}I (*e.g.*, Shen (1997) Cancer 80(12 Suppl):2553-2557), copper-67 (*e.g.*, Deshpande (1988) J. Nucl. Med. 29:217-225) or, *e.g.*, conjugation to the ribosome inactivating protein gelonin (*e.g.*, Boyle (1996) J. of Immunol. 18:221-230).

In another aspect, the present invention provides compositions, *e.g.*, pharmaceutical and diagnostic compositions, comprising a pharmaceutically acceptable carrier and at least one human monoclonal antibody of the invention, or an antigen-binding portion thereof, which specifically binds to human CTLA-4. Some compositions comprise a combination of the human sequence antibodies or antigen-binding portions thereof, preferably each of which binds to a distinct epitope. Compositions, *e.g.*, pharmaceutical compositions, comprising a combination of at least one human sequence antibodies or at least one human monoclonal antibody of the invention, or antigen-binding portions thereof, and at least one bispecific or multispecific molecule of the invention, are also within the scope of the invention.

For *in vivo* methods, the antibody, or antigen-binding portion thereof (or a bispecific or multispecific molecule of the invention), can be administered to a human subject suffering from a T-cell-related disease, or a disease that can be ameliorated or prevented by augmenting or suppressing or prolonging an immune response.

Human sequence monoclonal antibody and human sequence antibody compositions of the invention also can be administered in combination with other known therapies, *e.g.*, an anti-cancer therapy. Accordingly, the invention provides a method for treating cancer in a subject comprising administering a therapeutically effective amount of a pharmaceutical composition of a human sequence antibody together with a pharmaceutical carrier to the subject. Some such methods include a vaccine. Some such vaccines include a tumor cell vaccine, a GM-CSF-modified tumor cell vaccine, or an antigen-loaded dendritic cell vaccine. In some such methods, the cancer is prostate cancer, melanoma, or epithelial cancer.

Human sequence antibodies to human CTLA-4 can be used in methods of treatment requiring either stimulation of immune responses or suppression. The former indication is treated using antibodies that block binding of human CTLA-4 to human B7. Diseases amenable to treatment by stimulation, augmentation of prolonging of immune responses including cancer, including cancers of the prostate, kidney or colon, pathogenic infections, diseases associated with auto-antigens, *e.g.*, amyloidogenic diseases, including Alzheimer's disease, and diseases with inflammatory or allergic components. Immunosuppression is achieved using a polyvalent preparation comprising at least two different antibodies to human CTLA-4 that are linked to each other. Diseases amenable to treatment include graft versus host disease, host versus graft disease, autoimmune diseases and inflammation.

In yet another aspect, the present invention provides a method for detecting *in vitro* or *in vivo* the presence of human CTLA-4 antigen in a sample, *e.g.*, for diagnosing a human CTLA-4-related disease. In some methods, this is achieved by contacting a sample to be tested, along with a control sample, with a human sequence antibody or a human monoclonal antibody of the invention, or an antigen-binding portion thereof (or a bispecific or multispecific molecule), under conditions that allow for formation of a complex between the antibody and human CTLA-4. Complex formation is then detected (*e.g.*; using an ELISA) in both samples, and any statistically significant difference in the formation of complexes between the samples is indicative the presence of human CTLA-4 antigen in the test sample.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims.

All publications, figures, GenBank Accession references (sequences), ATCC Deposits, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes to the same extent as if each was so individually denoted.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematics illustrating the targeted insertion of a neo cassette into the Sma I site of the μ1 exon. Fig. 1A) Schematic diagram of the genomic structure of the μ locus. The filled boxes represent the μ exons; Fig. 1B) Schematic

diagram of the CmD targeting vector. The dotted lines denotes those genomic μ sequences included in the construct. Plasmid sequences are not shown; Fig. 1C) Schematic diagram of the targeted μ locus in which the neo cassette has been inserted into μ1. The box at the lower right shows those RFLP's diagnostic of homologous recombination between the targeting construct and the μ locus. The RFLP's were detected by Southern blot hybridization using probe A, the 915 bp Sac I fragment is shown in Fig. 1C.

Figure 2 shows the results of experiments demonstrating that soluble human sequence antibodies against human CTLA-4 inhibit the binding of recombinant soluble human CTLA-4 to cells expressing mouse B7.1, as described in detail, below.

Figure 3 shows the results of a competitive binding assay to identify human sequence antibodies of the invention that recognize non-overlapping epitopes on human CTLA-4, as described in detail, below.

Figure 4 shows preliminary nucleotide sequence data for the heavy and light chain fragment of the anti-CTLA-4 antibody 10D1.3.

Figure 5 shows the nucleotide sequences of the light chain variable Regions (V_K) of Anti-Human CTLA-4 Antibodies. The anti-CTLA-4 antibodies 10D1 (SEQ ID NO:6) and 4B6 (SEQ ID NO:8) derived from the V_K A-27 germline sequence (SEQ ID NO:4) are depicted at the top of the Figure. The anti-CTLA-4 antibody 1E2 (SEQ ID NO:12) derived from the V_K L-15 germline sequence (SEQ ID NO:10) is shown at the bottom of the Figure. The V_K sequences of three anti-CTLA-4 antibodies are aligned with their germline encoded V_K gene sequences. The complementary determining residues (CDR) are labeled. Dashes denote sequence identity.

Figure 6 shows the nucleotide sequences of the heavy chain variable Regions (V_H) of Anti-Human CTLA-4 Antibodies. The anti-CTLA-4 antibodies 10D1 (SEQ ID NO:16) and 4B6 (SEQ ID NO:18) derived from the V_H 3-30.3 germline sequence (SEQ ID NO:14) are depicted at the top of the Figure. The anti-CTLA-4 antibody 1E2 (SEQ ID NO:22) derived from the V_H 3-33 germline sequence (SEQ ID NO:20) is shown at the bottom of the Figure. The V_H sequences of three anti-CTLA-4 antibodies are aligned with their germline encoded sequences. The complementary determining residues (CDR) are labeled. Dashes denote sequence identity.

Figure 7 shows the predicted amino acid sequences of the light chain Variable Regions of Anti-Human CTLA-4 Antibodies. The predicted amino acid V_K

sequences of the anti-CTLA-4 antibodies described in **Figure 5** are shown. The anti-CTLA-4 antibodies 10D1 (SEQ ID NO:7) and 4B6 (SEQ ID NO:9) derived from the V_K A-27 germline sequence (SEQ ID NO:5) are depicted at the top of the Figure. The anti-CTLA-4 antibody 1E2 (SEQ ID NO:13) derived from the V_K L-15 germline sequence (SEQ ID NO:11) is shown at the bottom of the Figure.

Figure 8 shows the predicted amino acid sequences of the heavy chain Variable Regions of Anti-Human CTLA-4 Antibodies. The predicted amino acid V_H sequences of the anti-CTLA-4 antibodies described in **Figure 6** are shown. The anti-CTLA-4 antibodies 10D1 (SEQ ID NO:17) and 4B6 (SEQ ID NO:19) derived from the V_H 3-30.3 germline sequence (SEQ ID NO:15) are depicted at the top of the Figure. The anti-CTLA-4 antibody 1E2 (SEQ ID NO:23) derived from the V_H 3-33 germline sequence (SEQ ID NO:21) is shown at the bottom of the Figure.

Figure 9 shows the results of binding experiments of MAb 10D1 to recombinant human CTLA-4 by ELISA. MAb 10D1 binds with dose-dependent and saturating kinetics to purified recombinant CTLA-4.

Figure 10 shows the binding of 10D1 to a CTLA4-expressing T-cell line. These data show that MAb 10D1 binds with dose-dependent and saturating kinetics to cells expressing CTLA-4.

Figure 11 shows inhibition of binding of human B7.2 Ig to CTLA4-expressing T-cells. These data show that MAb 10D1 can efficiently block B7.2 binding to CTLA-4 as compared to a control human MAb.

Figure 12 shows the results for blocking CTLA4-FITC binding to murine B7.1-expressing cells. These data show that MAb 10D1 can efficiently block CTLA-4 binding to B7.1 as compared to a control human MAb.

Figure 13 shows competitive ELISAs of anti-CTLA-4 human MAbs demonstrating epitope group classifications.

Figure 14 shows CTLA-4 expression on PHA-stimulated T-cells. Activated, but not resting T cells, express low but detectable levels of CTLA-4 at the cell surface.

Figure 15 shows the results of MAb 10D1 in Complement Dependent Lysis of Activated T Cells. No lysis of PHA-activated T cells is observed.

Figure 16 shows the results of MAb 10D1 in Antibody-Dependent Lysis of Activated T Cells. No lysis of PHA-activated T cells is observed with 10D1 and mononuclear cells.

Figure 17 shows anti-10D1 IgM and IgG responses in cynomolgus monkeys injected with 10D1 antibody. No significant antibody response to 10D1 is observed.

DETAILED DESCRIPTION

The present invention provides novel antibody-based therapies for treating and diagnosing diseases characterized by expression, particularly over-expression, or activation of, particularly overactivation, of human CTLA-4 and/or related molecules. Therapies of the invention employ human sequence antibodies, human sequence monoclonal antibodies, or antigen-binding portions thereof, which bind to an epitope present on human CTLA-4. These human sequence anti-CTLA-4 antibodies can act as functional antagonists (*e.g.*, inhibiting the ability of CTLA-4 to bind ligand or to activate the cell, *e.g.*, by inhibiting its ability to transmit a signal to the cell) or agonists (*e.g.*, to simulate the effect of ligand).

The human sequence antibodies of the invention can be produced in a non-human transgenic animal, *e.g.*, a transgenic mouse, capable of producing multiple isotypes of human (*e.g.*, monoclonal or polyclonal) antibodies to human CTLA-4 (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J recombination and isotype switching. Accordingly, various aspects of the invention include antibodies and antibody fragments, and pharmaceutical compositions thereof, as well as non-human transgenic animals, and B-cells and hybridomas for making such monoclonal antibodies. Methods of using the antibodies of the invention to detect a cell expressing human CTLA-4 or a related, cross-reactive growth factor receptor, or to inhibit growth, differentiation and/or motility of a cell expressing human CTLA-4, either *in vitro* or *in vivo*, are also encompassed by the invention.

Except when noted, the terms "patient" or "subject" are used interchangeably and refer to mammals such as human patients and non-human primates, as well as experimental animals such as rabbits, rats, and mice, and other animals.

The term "treating" includes the administration of the compounds or agents of the present invention to prevent or delay the onset of the symptoms, complications, or biochemical indicia of a disease, alleviating the symptoms or arresting or inhibiting further development of the disease, condition, or disorder (*e.g.*, autoimmune disease). Treatment may be prophylactic (to prevent or delay the onset of the disease, or

to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease.

In general, the phrase "well tolerated" refers to the absence of adverse changes in health status that occur as a result of the treatment and would affect treatment decisions.

The term "lymphocyte" as used herein has the normal meaning in the art, and refers to any of the mononuclear, nonphagocytic leukocytes, found in the blood, lymph, and lymphoid tissues, *i.e.*, B and T lymphocytes.

The phrase "subpopulations of T lymphocytes" or "T cell subset(s)" refers to T lymphocytes or T cells characterized by the expression of particular cell surface markers (*see* Barclay, A. N. *et al.*. (eds.), 1997, The Leukocyte Antigen Facts Book, 2nd. edition, Academic Press, London, United Kingdom). The term "stable" in reference to T cells refers to the fact that the frequency or percentage of a T cell subset does not change over the course or duration of the administration of an agent.

The terms "cytotoxic T lymphocyte-associated antigen-4," "CTLA-4," "CTLA4," "CTLA-4 antigen" and "CD152" (*see, e.g.*, Murata (1999) Am. J. Pathol. 155:453-460) are used interchangeably, and include variants, isoforms, species homologs of human CTLA-4, and analogs having at least one common epitope with CTLA-4 (*see, e.g.*, Balzano (1992) Int. J. Cancer Suppl. 7:28-32).

The complete cDNA sequence of human CTLA-4 has the Genbank accession number L15006. The region of amino acids 1-37 is the leader peptide; 38-161 is the extracellular V-like domain; 162-187 is the transmembrane domain; and 188-223 is the cytoplasmic domain. Variants of the nucleotide sequence have been reported, including a G to A transition at position 49, a C to T transition at position 272, and an A to G transition at position 439. The complete DNA sequence of mouse CTLA-4 has the EMBL accession number X05719 (Brunet *et al.* (1987) Nature 328:267-270). The region of amino acids 1-35 is the leader peptide.

The complete DNA sequence of human B7-1 (CD80) has the Genbank accession number X60958; the accession number for the mouse sequence is X60958; the accession number for the rat sequence is U05593. The complete cDNA sequence of human B7-2 (CD86) has the Genbank accession number L25259; the accession number for the mouse sequence is L25606.

The genes encoding CD28 have been extensively characterized. The chicken mRNA sequence has the Genbank accession number X67915. The rat mRNA

sequence has the Genbank accession number X55288. The human mRNA sequence has the Genbank accession number J02988. The mouse mRNA sequence has the Genbank accession number M34536.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

An intact "antibody" comprises at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxyl-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. The term antibody includes antigen-binding portions of an intact antibody that retain capacity to bind CTLA-4. Examples of binding include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*., (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as

a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); *See, e.g.*, Bird *et al.*. (1988) *Science* 242:423-426; and Huston *et al.*. (1988) *Proc. Natl. Acad. Sci.* USA 85:5879-5883). Such single chain antibodies are included by reference to the term "antibody" Fragments can be prepared by recombinant techniques or enzymatic or chemical cleavage of intact antibodies.

A bispecific antibody has two different binding specificities, see. *e.g.*, U.S. Patents. 5,922,845 and 5,837,243; Zeilder (1999) J. Immunol. 163:1246-1252; Somasundaram (1999) Hum. Antibodies 9:47-54; Keler (1997) Cancer Res. 57:4008-4014. For example, the invention provides bispecific antibodies having one binding site for a cell surface antigen, such as human CTLA-4, and a second binding site for an Fc receptor on the surface of an effector cell. The invention also provides multispecific antibodies, which have at least three binding sites. The term "bispecific antibodies" further includes diabodies. Diabodies are bivalent, bispecific antibodies in which the VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (*See, e.g.*, Holliger, P., *et al.*. (1993) *Proc. Natl. Acad. Sci.* USA 90:6444-6448; Poljak, R.J., *et al.*. (1994) Structure 2:1121-1123).

The term "human sequence antibody" includes antibodies having variable and constant regions (if present) derived from human germline immunoglobulin sequences. The human sequence antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human sequence antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences (*i.e.*, humanized antibodies).

The terms "monoclonal antibody" or "monoclonal antibody composition" refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions (if present) derived from human germline immunoglobulin sequences. In one

embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The term "diclonal antibody" refers to a preparation of at least two antibodies to human CTLA-4. Typically, the different antibodies bind different epitopes.

The term "oligoclonal antibody" refers to a preparation of 3 to 100 different antibodies to human CTLA-4. Typically, the antibodies in such a preparation bind to a range of different epitopes.

The term "polyclonal antibody" refers to a preparation of more than 1 (two or more) different antibodies to human CTLA-4. Such a preparation includes antibodies binding to a range of different epitopes.

The invention provides human sequence antibodies to human CTLA-4 which block or antagonize signals transduced by the human CTLA-4 receptor. Some of these antibodies can bind to an epitope on human CTLA-4 so as to inhibit CTLA-4 from interacting with a human B7 counterreceptor. Because interaction of human CTLA-4 with human B7 transduces a signal leading to inactivation of T-cells bearing the human CTLA-4 receptor, antagonism of the interaction effectively induces, augments or prolongs the activation of T cells bearing the human CTLA-4 receptor, thereby prolonging or augmenting an immune response. A "blocking antibody" refers to an antibody that reduces the binding of soluble human CTLA-4 to cell-expressed human B7 ligand by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 99% or 99.9% under conditions in which the ratio of antibody combining site to human CTLA-4 ligand binding site is greater than 1:1 and the concentration of antibody is greater than 10^{-8} M.

Other antibody preparations, sometimes referred to as multivalent preparations, bind to human CTLA-4 in such a manner as to crosslink multiple human CTLA-4 receptors on the same cell. Cross-linking of receptor has the same or similar effect to binding of human CTLA-4 to human B7. Thus, cross-linking of receptors effectively agonizes the human CTLA-4 response resulting in immunosuppression.

Cross-linking can also be accomplished by combining soluble divalent antibodies having different epitope specificities. These polyclonal antibody preparations comprise at least two pairs of heavy and light chains binding to different epitopes on human CTLA-4 such that an immunosuppressing signal can be transduced as a result of human CTLA-4 crosslinking.

The term "recombinant human antibody" includes all human sequence antibodies of the invention that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (described further in Section I, below); antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial human antibody library, or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions (if present) derived from human germline immunoglobulin sequences. Such antibodies can, however, be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

A "heterologous antibody" is defined in relation to the transgenic non-human organism producing such an antibody. This term refers to an antibody having an amino acid sequence or an encoding nucleic acid sequence corresponding to that found in an organism not consisting of the transgenic non-human animal, and generally from a species other than that of the transgenic non-human animal.

A "heterohybrid antibody" refers to an antibody having a light and heavy chains of different organismal origins. For example, an antibody having a human heavy chain associated with a murine light chain is a heterohybrid antibody. Examples of heterohybrid antibodies include chimeric and humanized antibodies, discussed *supra*.

The term "substantially pure" or "isolated" means an object species (*e.g.*, an antibody of the invention) has been identified and separated and/or recovered from a component of its natural environment such that the object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition); a "substantially pure" or "isolated" composition also means where the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. A substantially pure or isolated composition can also comprise more than about 80 to 90 percent by weight of all macromolecular species present in the composition. An isolated object species (*e.g.*, antibodies of the invention) can also be purified to essential homogeneity (contaminant species cannot be detected in

the composition by conventional detection methods) wherein the composition consists essentially of derivatives of a single macromolecular species. An isolated antibody to human CTLA-4 can be substantially free of other antibodies that lack binding to human CTLA-4 and bind to a different antigen. An isolated antibody that specifically binds to an epitope, isoform or variant of human CTLA-4 may, however, have cross-reactivity to other related antigens, *e.g.*, from other species (*e.g.*, CTLA-4 species homologs). Moreover, an isolated antibody of the invention be substantially free of other cellular material (*e.g.*, non-immunoglobulin associated proteins) and/or chemicals.

"Specific binding" refers to antibody binding to a predetermined antigen. The phrase "specifically (or selectively) binds" to an antibody refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics.Typically, the antibody binds with an association constant (K_a) of at least about 1 x 10^6 M^{-1} or 10^7 M^{-1}, or about 10^8 M^{-1} to 10^9 M^{-1}, or about 10^{10} M^{-1} to 10^{11} M^{-1} or higher, and binds to the predetermined antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen. The phrases "an antibody recognizing an antigen" and " an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen".

The phrase "specifically bind(s)" or "bind(s) specifically" when referring to a peptide refers to a peptide molecule which has intermediate or high binding affinity, exclusively or predominately, to a target molecule. The phrases "specifically binds to" refers to a binding reaction which is determinative of the presence of a target protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated assay conditions, the specified binding moieties bind preferentially to a particular target protein and do not bind in a significant amount to other components present in a test sample. Specific binding to a target protein under such conditions may require a binding moiety that is selected for its specificity for a particular target antigen. A variety of assay formats may be used to select ligands that are specifically reactive with a particular protein. For example, solid-phase ELISA immunoassays, immunoprecipitation, Biacore and Western blot are used to identify peptides that specifically react with CTLA-4. Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 times background.

The term "high affinity" for an IgG antibody refers to an equilibrium association constant (K_a) of at least about 10^7M^{-1}, at least about 10^8M^{-1}, at least about 10^9M^{-1}, at least about $10^{10}M^{-1}$, at least about $10^{11}M^{-1}$, or at least about $10^{12}M^{-1}$ or greater, *e.g.*, up to $10^{13}M^{-1}$ or $10^{14}M^{-1}$ or greater. However, "high affinity" binding can vary for other antibody isotypes.

The term "K_a", as used herein, is intended to refer to the equilibrium association constant of a particular antibody-antigen interaction. This constant has units of 1/M.

The term "K_d", as used herein, is intended to refer to the equilibrium dissociation constant of a particular antibody-antigen interaction. This constant has units of M.

The term "k_a", as used herein, is intended to refer to the kinetic association constant of a particular antibody-antigen interaction. This constant has units of 1/Ms

The term "k_d", as used herein, is intended to refer to the kinetic dissociation constant of a particular antibody-antigen interaction. This constant has units of 1/s.

"Particular antibody-antigen interactions" refers to the experimental conditions under which the equilibrium and kinetic constants are measured.

"Isotype" refers to the antibody class (*e.g.*, IgM or IgGl) that is encoded by heavy chain constant region genes.

"Isotype switching" refers to the phenomenon by which the class, or isotype, of an antibody changes from one Ig class to one of the other Ig classes.

"Nonswitched isotype" refers to the isotypic class of heavy chain that is produced when no isotype switching has taken place; the CH gene encoding the nonswitched isotype is typically the first CH gene immediately downstream from the functionally rearranged VDJ gene. Isotype switching has been classified as classical or non-classical isotype switching. Classical isotype switching occurs by recombination events which involve at least one switch sequence region in the transgene. Non-classical isotype switching may occur by, for example, homologous recombination between human σ_μ and human Σ_μ (δ-associated deletion). Alternative non-classical switching mechanisms, such as intertransgene and/or interchromosomal recombination, among others, may occur and effectuate isotype switching.

The term "switch sequence" refers to those DNA sequences responsible for switch recombination. A "switch donor" sequence, typically a μ switch region, are 5' (*i.e.*, upstream) of the construct region to be deleted during the switch recombination. The "switch acceptor" region are between the construct region to be deleted and the replacement constant region (*e.g.*, γ, ε, etc.). As there is no specific site where recombination always occurs, the final gene sequence is not typically predictable from the construct.

"Glycosylation pattern" is defined as the pattern of carbohydrate units that are covalently attached to a protein, more specifically to an immunoglobulin protein. A glycosylation pattern of a heterologous antibody can be characterized as being substantially similar to glycosylation patterns which occur naturally on antibodies produced by the species of the non-human transgenic animal, when one of ordinary skill in the art would recognize the glycosylation pattern of the heterologous antibody as being more similar to said pattern of glycosylation in the species of the non-human transgenic animal than to the species from which the CH genes of the transgene were derived.

The term "naturally-occurring" as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "rearranged" refers to a configuration of a heavy chain or light chain immunoglobulin locus wherein a V segment is positioned immediately adjacent to a D-J or J segment in a conformation encoding essentially a complete VH or VL domain, respectively. A rearranged immunoglobulin gene locus can be identified by comparison to germline DNA; a rearranged locus has at least one recombined heptamer/nonamer homology element.

The term "unrearranged" or "germline configuration" in reference to a V segment refers to the configuration wherein the V segment is not recombined so as to be immediately adjacent to a D or J segment.

The term "nucleic acid" is intended to include DNA molecules and RNA molecules. A nucleic acid can be single-stranded or double-stranded.

The term "isolated nucleic acid" in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3) that bind to CTLA-4, is intended to

refer to a nucleic acid in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than CTLA-4, which other sequences may naturally flank the nucleic acid in human genomic DNA. SEQ ID NOs: 4-23 comprise the nucleotide and amino acid sequences comprising the heavy chain (VH) and light chain (VL) variable regions of the 10D1, 4B6 and 1E2 human anti-CTLA-4 monoclonal antibodies of the invention.

The term "substantially identical," in the context of two nucleic acids or polypeptides refers to two or more sequences or subsequences that have at least about 80%, about 90, about 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. For example, the invention provides nucleic acids having sequences that are substantially identical to SEQ ID NO:1, SEQ ID NO:2. Such "substantially identical" sequences are typically considered to be homologous. The "substantial identity" can exist over a region of sequence that is at least about 50 residues in length, over a region of at least about 100 residues, or over a region 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 to generate a consensus sequence. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. Kabat's scheme is extendible to other antibodies not included in his compendium by aligning the antibody in question with one of the consensus sequences in Kabat by reference to conserved amino acids. 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. Likewise,

nucleic acids encoding antibody chains are aligned when the amino acid sequences encoded by the respective nucleic acids are aligned according to the Kabat numbering convention.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (*e.g.*, total cellular or library DNA or RNA), wherein the particular nucleotide sequence is detected at least at about 10 times background. In one embodiment, a nucleic acid can be determined to be within the scope of the invention (*e.g.*, is substantially identical to SEQ ID NO:1 or SEQ ID NO:2) by its ability to hybridize under stringent conditions to a nucleic acid otherwise determined to be within the scope of the invention (such as the exemplary sequences described herein).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but not to other sequences in significant amounts (a positive signal (*e.g.*, identification of a nucleic acid of the invention) is about 10 times background hybridization). Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found An extensive guide to the hybridization of nucleic acids is found in *e.g.*, Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes

(*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide as described in Sambrook (cited below). For high stringency hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary high stringency or stringent hybridization conditions include: 50% formamide, 5x SSC and 1% SDS incubated at 42° C or 5x SSC and 1% SDS incubated at 65° C, with a wash in 0.2x SSC and 0.1% SDS at 65° C. . For selective or specific hybridization, a positive signal (*e.g.*, identification of a nucleic acid of the invention) is about 10 times background hybridization. Stringent hybridization conditions that are used to identify nucleic acids within the scope of the invention include, *e.g.*, hybridization in a buffer comprising 50% formamide, 5x SSC, and 1% SDS at 42°C, or hybridization in a buffer comprising 5x SSC and 1% SDS at 65°C, both with a wash of 0.2x SSC and 0.1% SDS at 65°C. In the present invention, genomic DNA or cDNA comprising nucleic acids of the invention can be identified in standard Southern blots under stringent conditions using the nucleic acid sequences disclosed here. Additional stringent conditions for such hybridizations (to identify nucleic acids within the scope of the invention) are those which include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C.

However, the selection of a hybridization format is not critical - it is the stringency of the wash conditions that set forth the conditions which determine whether a nucleic acid is within the scope of the invention. Wash conditions used to identify nucleic acids within the scope of the invention include, *e.g.*: a salt concentration of about 0.02 molar at pH 7 and a temperature of at least about 50°C or about 55°C to about 60°C; or, a salt concentration of about 0.15 M NaCl at 72°C for about 15 minutes; or, a salt concentration of about 0.2X SSC at a temperature of at least about 50°C or about 55°C to about 60°C for about 15 to about 20 minutes; or, the hybridization complex is washed twice with a solution with a salt concentration of about 2X SSC containing 0.1% SDS at room temperature for 15 minutes and then washed twice by 0.1X SSC containing 0.1% SDS at 68°C for 15 minutes; or, equivalent conditions. See Sambrook, Tijssen and Ausubel for a description of SSC buffer and equivalent conditions.

The nucleic acids of the invention be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, *e.g.*, other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel

electrophoresis and others well known in the art. *see, e.g.*, Sambrook, Tijssen and Ausubel. The nucleic acid sequences of the invention and other nucleic acids used to practice this invention, whether RNA, cDNA, genomic DNA, or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed recombinantly. Any recombinant expression system can be used, including, in addition to bacterial, *e.g.*, yeast, insect or mammalian systems. Alternatively, these nucleic acids can be chemically synthesized *in vitro*. Techniques for the manipulation of nucleic acids, such as, *e.g.*, subcloning into expression vectors, labeling probes, sequencing, and hybridization are well described in the scientific and patent literature, *see, e.g.*, Sambrook, Tijssen and Ausubel. Nucleic acids can be analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, *e.g.*, analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological metho such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, dot-blot analysis, gel electrophoresis (*e.g.*, SDS-PAGE), RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

The nucleic acid compositions of the present invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures may be mutated, thereof in accordance with standard techniques to provide gene sequences. For coding sequences, these mutations, may affect amino acid sequence as desired. In particular, DNA sequences substantially homologous to or derived from native V, D, J, constant, switches and other such sequences described herein are contemplated (where "derived" indicates that a sequence is identical or modified from another sequence).

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding

regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

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

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

The term "minilocus transgene" refers to a transgene that comprises a portion of the genomic immunoglobulin locus having at least one internal (*i.e.*, not at a terminus of the portion) deletion of a non-essential DNA portion (*e.g.*, intervening sequence; intron or portion thereof) as compared to the naturally-occurring germline Ig locus.

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include

^{32}P, fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (*e.g.*, the polypeptides of the invention can be made detectable, *e.g.*, by incorporating a radiolabel into the peptide, and used to detect antibodies specifically reactive with the peptide).

The term "sorting" in the context of cells as used herein to refers to both physical sorting of the cells, as can be accomplished using, *e.g.*, a fluorescence activated cell sorter, as well as to analysis of cells based on expression of cell surface markers, *e.g.*, FACS analysis in the absence of sorting.

The phrase "immune cell response" refers to the response of immune system cells to external or internal stimuli (*e.g.*, antigen, cytokines, chemokines, and other cells) producing biochemical changes in the immune cells that result in immune cell migration, killing of target cells, phagocytosis, production of antibodies, other soluble effectors of the immune response, and the like.

The terms "T lymphocyte response" and "T lymphocyte activity" are used here interchangeably to refer to the component of immune response dependent on T lymphocytes (*i.e.*, the proliferation and/or differentiation of T lymphocytes into helper, cytotoxic killer, or suppressor T lymphocytes, the provision of signals by helper T lymphocytes to B lymphocytes that cause or prevent antibody production, the killing of specific target cells by cytotoxic T lymphocytes, and the release of soluble factors such as cytokines that modulate the function of other immune cells).

The term "immune response" refers to the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the human body of invading pathogens, cells or tissues infected with pathogens, cancerous cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

Components of an immune response may be detected in vitro by various methods that are well known to those of ordinary skill in the art. For example, (1) cytotoxic T lymphocytes can be incubated with radioactively labeled target cells and the lysis of these target cells detected by the release of radioactivity, (2) helper T lymphocytes can be incubated with antigens and antigen presenting cells and the synthesis and secretion of cytokines measured by standard methods (Windhagen A; *et*

al.., 1995, Immunity 2(4): 373-80), (3) antigen presenting cells can be incubated with whole protein antigen and the presentation of that antigen on MHC detected by either T lymphocyte activation assays or biophysical methods (Harding *et al.*., 1989, Proc. Natl. Acad. Sci., 86: 4230-4), (4) mast cells can be incubated with reagents that cross-link their Fc-epsilon receptors and histamine release measured by enzyme immunoassay (Siraganian, *et al.*., 1983, TIPS 4: 432-437).

Similarly, products of an immune response in either a model organism (*e.g.*, mouse) or a human patient can also be detected by various methods that are well known to those of ordinary skill in the art. For example, (1) the production of antibodies in response to vaccination can be readily detected by standard methods currently used in clinical laboratories, *e.g.*, an ELISA; (2) the migration of immune cells to sites of inflammation can be detected by scratching the surface of skin and placing a sterile container to capture the migrating cells over scratch site (Peters *et al.*., 1988, Blood 72: 1310-5); (3) the proliferation of peripheral blood mononuclear cells in response to mitogens or mixed lymphocyte reaction can be measured using ^{3}H-thymidine; (4) the phagocitic capacity of granulocytes, macrophages, and other phagocytes in PBMCs can be measured by placing PMBCs in wells together with labeled particles (Peters *et al.*., 1988); and (5) the differentation of immune system cells can be measured by labeling PBMCs with antibodies to CD molecules such as CD4 and CD8 and measuring the fraction of the PBMCs expressing these markers.

As used herein, the phrase "signal transduction pathway" or "signal transduction event" refers to at least one biochemical reaction, but more commonly a series of biochemical reactions, which result from interaction of a cell with a stimulatory compound or agent. Thus, the interaction of a stimulatory compound with a cell generates a "signal" that is transmitted through the signal transduction pathway, ultimately resulting in a cellular response, *e.g.*, an immune response described above.

A signal transduction pathway refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. Signal transduction molecules of the present invention include, for example, MAb 147.1 of the invention. As used herein, the phrase "cell surface receptor" includes molecules and complexes of molecules capable of receiving a signal and the transmission of such a signal across the plasma membrane of a cell. An example of a "cell surface receptor" of the present invention is the T cell receptor (TCR) or the B7 ligands of CTLA-4.

A signal transduction pathway in a cell can be initiated by interaction of a cell with a stimulator that is inside or outside of the cell. If an exterior (*i.e.*, outside of the cell) stimulator (*e.g.*, an MHC-antigen complex on an antigen presenting cell) interacts with a cell surface receptor (*e.g.*, a T cell receptor), a signal transduction pathway can transmit a signal across the cell's membrane, through the cytoplasm of the cell, and in some instances into the nucleus. If an interior (*e.g.*, inside the cell) stimulator interacts with an intracellular signal transduction molecule, a signal transduction pathway can result in transmission of a signal through the cell's cytoplasm, and in some instances into the cell's nucleus.

Signal transduction can occur through, *e.g.*, the phosphorylation of a molecule; non-covalent allosteric interactions; complexing of molecules; the conformational change of a molecule; calcium release; inositol phosphate production; proteolytic cleavage; cyclic nucleotide production and diacylglyceride production. Typically, signal transduction occurs through phosphorylating a signal transduction molecule.

The term "nonspecific T cell activation" refers to the stimulation of T cells independent of their antigenic specificity.

PRODUCTION OF HUMAN ANTIBODIES TO CTLA-4

The monoclonal antibodies (mAbs) and the human sequence antibodies of the invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* 256: 495 (1975). Any technique for producing monoclonal antibody can be employed *e.g.*, viral or oncogenic transformation of B lymphocytes. One animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known (*see, e.g.*, Harlow and Lane (1988), *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York).

Human monoclonal antibodies and human sequence antibodies directed against human CTLA-4 can be generated using transgenic mice carrying a human immune system rather than the mouse system. These transgenic mice, also referred to herein as "HuMAb-Mouse™", contain a human immunoglobulin gene miniloci that

encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N. *et al.*. (1994) *Nature* 368(6474): 856-859 and US patent 5,770,429). Accordingly, the mice exhibit reduced expression of mouse IgM or κ, and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgGκ monoclonal (Lonberg, N. *et al.*. (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764:536-546). The preparation of transgenic mice is described in detail Section II below and in Taylor, L. *et al.*. (1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. *et al.*. (1993) *International Immunology* 5: 647-656; Tuaillon *et al.*. (1993) *Proc. Natl. Acad. Sci USA* 90:3720-3724; Choi *et al.*. (1993) *Nature Genetics* 4:117-123; Chen, J. *et al.*. (1993) EMBO J. 12: 821-830; Tuaillon *et al.*. (1994) *J. Immunol.* 152:2912-2920; Lonberg *et al.*., (1994) *Nature* 368(6474): 856-859; Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Taylor, L. *et al.*. (1994) *International Immunology* 6: 579-591; Lonberg, N. and Huszar, D. (1995) Intern. Rev. Immunol. Vol. 13: 65-93; Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764:536-546; Fishwild, D. *et al.*. (1996) *Nature Biotechnology* 14: 845-851. *See further*, U.S. Patent Nos. 5,625,126 and 5,770,429, both to Lonberg and Kay, and GenPharm International; U.S. Patent No. 5,545,807 to Surani *et al.*.; International Publication Nos. WO 98/24884, published on June 11, 1998; WO 94/25585, published November 10, 1994; WO 93/1227, published June 24, 1993; WO 92/22645, published December 23, 1992; WO 92/03918, published March 19, 1992. Alternatively, the CMD and HCo12 transgenes, described in Examples 1 and 2, below, can be used to generate human anti-CTLA-4 antibodies.

Detailed procedures to generate fully human monoclonal antibodies to CTLA-4 are described in the Examples below. Cumulative experience with various antigens has shown that the transgenic mice respond when initially immunized intraperitoneally (IP) with antigen in complete Freund's adjuvant, followed by every other week IP immunizations (up to a total of 6) with antigen in incomplete Freund's adjuvant. However, adjuvants other than Freund's are also found to be effective. In addition, whole cells in the absence of adjuvant are found to be highly immunogenic. The immune response can be monitored over the course of the immunization protocol

with plasma samples being obtained by retroorbital bleeds. The plasma can be screened by ELISA (as described below), and mice with sufficient titers of anti-CTLA-4 human immunoglobulin can be used for fusions. Mice can be boosted intravenously with antigen 3 days before sacrifice and removal of the spleen. It is expected that 2-3 fusions for each immunization may need to be performed. Between 6 and 24 mice are typically immunized for each antigen. Usually both HCo7 and HCo12 strains are used. In addition, both HCo7 and HCo12 transgene can be bred together into a single mouse having two different human heavy chain transgenes.

To purify human anti-CTLA-4 antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (Pharmacia, Piscataway, NJ). Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by OD280 using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80 °C.

To determine if the selected human anti-CTLA-4 monoclonal antibodies bind to unique epitopes, each antibody can be biotinylated using commercially available reagents (Pierce, Rockford, IL). Competition studies using unlabeled monoclonal antibodies and biotinylated monoclonal antibodies can be performed using CTLA-4 coated-ELISA plates as described above. Biotinylated MAb binding can be detected with a strep-avidin-alkaline phosphatase probe.

To determine the isotype of purified antibodies, isotype ELISAs can be performed. Wells of microtiter plates can be coated with 1 μg/ml of anti-human IgG overnight at 4°C. After blocking with 1% BSA, the plates are reacted with 1 μg/ml or less of monoclonal antibodies or purified isotype controls, at ambient temperature for one to two hours. The wells can then be reacted with either human IgGl or human IgM-specific alkaline phosphatase-conjugated probes. Plates are developed and analyzed as described above.

To demonstrate binding of monoclonal antibodies to live cells expressing the CTLA-4, flow cytometry can be used. Briefly, cell lines expressing CTLA-4 (grown under standard growth conditions) are mixed with various concentrations of monoclonal antibodies in PBS containing 0.1% BSA and 10% fetal calf serum, and incubated at 37°C for 1 hour. After washing, the cells are reacted with Fluorescein-labeled anti-human IgG

antibody under the same conditions as the primary antibody staining. The samples can be analyzed by FACScan instrument using light and side scatter properties to gate on single cells. An alternative assay using fluorescence microscopy may be used (in addition to or instead of) the flow cytometry assay. Cells can be stained exactly as described above and examined by fluorescence microscopy. This method allows visualization of individual cells, but may have diminished sensitivity depending on the density of the antigen.

Anti-CTLA-4 human IgGs can be further tested for reactivity with CTLA-4 antigen by Western blotting. Briefly, cell extracts from cells expressing CTLA-4 can be prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the separated antigens are transferred to nitrocellulose membranes, blocked with 10% fetal calf serum, and probed with the monoclonal antibodies to be tested. Human IgG binding can be detected using anti-human IgG alkaline phosphatase and developed with BCIP/NBT substrate tablets (Sigma Chem. Co., St. Louis, MO).

PRODUCTION OF TRANSGENIC NON-HUMAN ANIMALS THAT GENERATE HUMAN MONOCLONAL ANTI-CTLA-4 ANTIBODIES

The present invention also provides transgenic non-human animals, *e.g.*, a transgenic mice, which are capable of expressing human monoclonal antibodies that specifically bind to CTLA-4. High affinity human sequence antibodies are also provided. Some transgenic non-human animals, *e.g.*, the transgenic mice, have a genome comprising a human heavy chain transgene and a light chain transgene. Some transgenic non-human animals are immunized with a purified or enriched preparation of CTLA-4 antigen and/or cells expressing CTLA-4. Some transgenic non-human animals are capable of producing multiple isotypes of human monoclonal antibodies to CTLA-4 (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J recombination and isotype switching. Isotype switching may occur by, *e.g.*, classical or non-classical isotype switching.

The design of a transgenic non-human animal that responds to foreign antigen stimulation with a heterologous antibody repertoire, requires that the heterologous immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B-cell development. In some mice, correct function of a heterologous heavy chain transgene includes isotype switching. Accordingly, the transgenes of the invention are constructed so as to produce isotype switching and one or more of the following: (1) high level and cell-type specific expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a

sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

Not all of the foregoing criteria need be met. For example, in transgenic animal in which the endogenous immunoglobulin loci of the transgenic animals are functionally disrupted, the transgene need not activate allelic exclusion. Further, in transgenic animals in which the transgene comprises a functionally rearranged heavy and/or light chain immunoglobulin gene, the second criteria of functional gene rearrangement is unnecessary, at least for that transgene which is already rearranged. For background on molecular immunology, *See, e.g., Fundamental Immunology*, 4th edition (1998), Paul, William E., ed. Lippencott-Raven Press, N.Y.

Some transgenic non-human animals used to generate the human monoclonal antibodies of the invention contain rearranged, unrearranged or a combination of rearranged and unrearranged heterologous immunoglobulin heavy and light chain transgenes in the germline of the transgenic animal. Each of the heavy chain transgenes comprises at least one CH gene. In addition, the heavy chain transgene can contain functional isotype switch sequences, which are capable of supporting isotype switching of a heterologous transgene encoding multiple CH genes in the B-cells of the transgenic animal. Such switch sequences can be those which occur naturally in the germline immunoglobulin locus from the species that serves as the source of the transgene CH genes, or such switch sequences can be derived from those which occur in the species that is to receive the transgene construct (the transgenic animal). For example, a human transgene construct that is used to produce a transgenic mouse may produce a higher frequency of isotype switching events if it incorporates switch sequences similar to those that occur naturally in the mouse heavy chain locus, as presumably the mouse switch sequences are optimized to function with the mouse switch recombinase enzyme system, whereas the human switch sequences are not. Switch sequences can be isolated and cloned by conventional cloning methods, or can be synthesized de novo from overlapping synthetic oligonucleotides designed on the basis of published sequence information relating to immunoglobulin switch region sequences (Mills *et al..*, *Nucl. Acids Res.* 15:7305-7316 (1991); Sideras *et al..*, *Intl. Immunol.* 1:631-642 (1989).

For each of the foregoing transgenic animals, functionally rearranged heterologous heavy and light chain immunoglobulin transgenes are found in a significant fraction of the B-cells of the transgenic animal (at least 10 percent).

The transgenes used to generate the transgenic animals of the invention include a heavy chain transgene comprising DNA encoding at least one variable gene segment, one diversity gene segment, one joining gene segment and at least one constant region gene segment. The immunoglobulin light chain transgene comprises DNA encoding at least one variable gene segment, one joining gene segment and at least one constant region gene segment. The gene segments encoding the light and heavy chain gene segments are heterologous to the transgenic non-human animal in that they are derived from, or correspond to, DNA encoding immunoglobulin heavy and light chain gene segments from a species not consisting of the transgenic non-human animal. In one aspect of the invention, the transgene is constructed such that the individual gene segments are unrearranged, *i.e.*, not rearranged so as to encode a functional immunoglobulin light or heavy chain. Such unrearranged transgenes support recombination of the V, D, and J gene segments (functional rearrangement) and preferably support incorporation of all or a portion of a D region gene segment in the resultant rearranged immunoglobulin heavy chain within the transgenic non-human animal when exposed to CTLA-4 antigen.

Such transgenes typically comprise a substantial portion of the C, D, and J segments as well as a subset of the V gene segments. In such transgene constructs, the various regulatory sequences, *e.g.* promoters, enhancers, class switch regions, splice-donor and splice-acceptor sequences for RNA processing, recombination signals and the like, comprise corresponding sequences derived from the heterologous DNA. Such regulatory sequences may be incorporated into the transgene from the same or a related species of the non-human animal used in the invention. For example, human immunoglobulin gene segments may be combined in a transgene with a rodent immunoglobulin enhancer sequence for use in a transgenic mouse. Alternatively, synthetic regulatory sequences may be incorporated into the transgene, wherein such synthetic regulatory sequences are not homologous to a functional DNA sequence that is known to occur naturally in the genomes of mammals. Synthetic regulatory sequences are designed according to consensus rules, such as, for example, those specifying the permissible sequences of a splice-acceptor site or a promoter/enhancer motif. The transgene may comprise a minilocus.

Some transgenic animals used to generate human antibodies to CTLA-4 contain at least one, typically 2-10, and sometimes 25-50 or more copies of the transgene described in Example 37 of US patent 5,770,429, or the transgene described in Example 2

below (*e.g.*, HCo12), at least one copy of a light chain transgene described in Examples 38 of US patent 5,770,429, two copies of the Cmu deletion described in Example 1 below, and two copies of the Jkappa deletion described in Example 9 of US patent 5,770,429. The resultant animals are injected with antigens and used for production of human monoclonal antibodies against these antigens.

Some transgenic animals exhibit immunoglobulin production with a significant repertoire, ideally substantially similar to that of a native mouse. Thus, for example, animals in which the endogenous Ig genes have been inactivated, the total immunoglobulin levels range from about 0.1 to about 10 mg/ml of serum.

The immunoglobulins expressed by the transgenic mice typically recognize about one-half or more of highly antigenic proteins, *e.g.*, staphylococcus protein A. Typically, the immunoglobulins exhibit an association constant for preselected antigens of at least about 10^7M^{-1}, 10^8M^{-1}, 10^9M^{-1}, $10^{10}M^{-1}$, $10^{11}M^{-1}$, $10^{12}M^{-1}$, $10^{13}M^{-1}$, or greater.

The transgenic mice of the present invention can be immunized with a purified or enriched preparation of human CTLA-4 antigen (or antigenic fragment thereof) and/or cells expressing human CTLA-4 as described previously. The mice produce B cells that undergo class-switching via intratransgene switch recombination (*cis*-switching) and express immunoglobulins reactive with CTLA-4. The immunoglobulins can be human sequence antibodies, wherein the heavy and light chain polypeptides are encoded by human transgene sequences, which may include sequences derived by somatic mutation and V region recombinatorial joints, as well as germline-encoded sequences; these human sequence immunoglobulins can be referred to as being substantially identical to a polypeptide sequence encoded by a human VL or VH gene segment and a human JL or JH segment, even though other non-germline sequences may be present as a result of somatic mutation and differential V-J and V-D-J recombination joints. With respect to such human sequence antibodies, the variable regions of each chain are typically at least 80 percent encoded by human germline V, J, and, in the case of heavy chains, D, gene segments; frequently at least 85 percent of the variable regions are encoded by human germline sequences present on the transgene; often 90 or 95 percent or more of the variable region sequences are encoded by human germline sequences present on the transgene. However, since non-germline sequences are introduced by somatic mutation and VJ and VDJ joining, the human sequence antibodies frequently have some variable region sequences (and less frequently constant region sequences) which are not

encoded by human V, D, or J gene segments as found in the human transgene(s) in the germline of the mice. Typically, such non-germline sequences (or individual nucleotide positions) cluster in or near CDRs, or in regions where somatic mutations are known to cluster.

The human sequence antibodies which bind to the predetermined antigen can result from isotype switching, such that human antibodies comprising a human sequence γ chain (such as γ1, γ2, γ3, or γ4) and a human sequence light chain (such as kappa or lambda) are produced. Such isotype-switched human sequence antibodies often contain one or more somatic mutation(s), typically in the variable region and often in or within about 10 residues of a CDR) as a result of affinity maturation and selection of B cells by antigen, particularly subsequent to secondary (or subsequent) antigen challenge. Some high affinity human sequence antibodies have equilibrium association constants of at least about $1 \times 10^7\ M^{-1}$, or at least about $1 \times 10^8\ M^{-1}$, or more than about $1 \times 10^9\ M^{-1}$, or $5 \times 10^9\ M^{-1}$ to $1 \times 10^{11}\ M^{-1}$ or greater.

Another aspect of the invention pertains to the B cells from such mice which can be used to generate hybridomas expressing human monoclonal antibodies which bind with high affinity (*e.g.*, having association constant of greater than $10^7 M^{-1}$) to CTLA-4. These hybridomas are used to generate a composition comprising an immunoglobulin having an association constant (Ka) of at least $10^7\ M^{-1}$ for binding CTLA-4. Such immunoglobulin contains a human sequence light chain composed of a light chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human Vk or Vλ gene segment and a human Jk or Jλ segment, and a light chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human Ck or Cλ gene segment. It also contains a human sequence heavy chain composed of a heavy chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human VH gene segment, optionally a D region, and a human JH segment, and a constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human CH gene segment.

The invention also provides human monoclonal antibodies and human sequence antibodies to human CTLA-4 derivatized or linked to another functional molecule, *e.g.*, another peptide or protein (*e.g.*, a cytokine, a cytotoxic agent, an immune stimulatory or inhibitory agent, a Fab' fragment, and the like, as discussed above) to

generate a bispecific or multispecific molecule which binds to multiple binding sites or target epitopes. For example, an antibody or antigen-binding portion of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, antibody fragment, peptide or binding mimetic.

Accordingly, the present invention includes bispecific and multispecific composition comprising at least one human sequence antibody or antigen binding fragment with a first binding specificity for human CTLA-4 and a second binding specificity for a second target epitope. The second target epitope can be an Fc receptor, *e.g.*, human FcγRI or a human Fcγ receptor. Therefore, the invention includes bispecific and multispecific molecules capable of binding both to FcγR1, FcγR or FcεR expressing effector cells (*e.g.*, monocytes, macrophages or polymorphonuclear cells (PMNs)), and to target cells expressing human CTLA-4. These multi-specific (*e.g.*, bispecific or multispecific) molecules target human CTLA-4 expressing cells to effector cells, and trigger Fc receptor-mediated effector cell activities, such as phagocytosis of a human CTLA-4-expressing cells, antibody dependent cell-mediated cytotoxicity (ADCC), cytokine release, or generation of superoxide anion.

The bispecific and multispecific molecules of the invention can comprise a binding specificity at least one antibody, or an antibody fragment thereof, including, *e.g.*, an Fab, Fab', $F(ab')_2$, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in, e.g, Ladner *et al.*. U.S. Patent No. 4,946,778. Bispecific and multispecific molecules of the invention can comprise a binding specificity for an FcγR or an FcγR present on the surface of an effector cell, and a second binding specificity for a target cell antigen, *e.g.*, human CTLA-4.

The binding specificity for an Fc receptor is provided by a monoclonal antibody, the binding of which is not blocked by human immunoglobulin G (IgG). As used herein, the term "IgG receptor" refers to any of the eight γ-chain genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fcγ receptor classes: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). For example, the Fcγ receptor can be a human high affinity FcγRI. The human FcγRI is a 72 kDa molecule, which shows high affinity for monomeric IgG (10^8 to $10^9 M^{-1}$).

The production and characterization of these preferred monoclonal antibodies are described by Fanger *et al.*. in PCT application WO 88/00052 and in U.S. Patent No. 4,954,617. These antibodies bind to an epitope of FcγRI, FcγRII or FcγRIII at a site which is distinct from the Fcγ binding site of the receptor and, thus, their binding is not blocked substantially by physiological levels of IgG. Specific anti-FcγRI antibodies useful in this invention are MAb 22, MAb 32, MAb 44, MAb 62 and MAb 197. The hybridoma producing MAb 32 is available from the American Type Culture Collection, ATCC Accession No. HB9469. Anti-FcγRI MAb 22, $F(ab')_2$ fragments of MAb 22, and can be obtained from Medarex, Inc. (Annandale, N.J.). In other embodiments, the anti-Fc γ receptor antibody is a humanized form of monoclonal antibody 22 (H22). The production and characterization of the H22 antibody is described in Graziano (1995) J. Immunol 155:4996-5002 and PCT/US93/10384. The H22 antibody producing cell line was deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the accession no. CRL 11177.

The binding specificity for an Fc receptor can also be provided by an antibody that binds to a human IgA receptor, *e.g.*, an Fc-alpha receptor (FcαR (CD89)). Preferably, the antibody binds to a human IgA receptor at a site that is not blocked by endogenous IgA. The term "IgA receptor" is intended to include the gene product of one α-gene (FcαRI) located on chromosome 19. This gene is known to encode several alternatively spliced transmembrane isoforms of 55 to 110 kDa. FcαRI (CD89) is constitutively expressed on monocytes/ macrophages, eosinophilic and neutrophilic granulocytes, but not on non-effector cell populations. FcαRI has medium affinity ($\approx 5 \times 10^7\ M^{-1}$) for both IgA1 and IgA2, which is increased upon exposure to cytokines such as G-CSF or GM-CSF (Morton (1996) Critical Reviews in Immunology 16:423-440). Four FcαRI-specific monoclonal antibodies, identified as A3, A59, A62 and A77, which bind FcαRI outside the IgA ligand binding domain, have been described by, e.g, Monteiro (1992) *J. Immunol.* 148:1764.

Bispecific and multispecific molecules of the invention can further comprise a binding specificity which recognizes, *e.g.*, binds to, a target cell antigen, *e.g.* human CTLA-4. The binding specificity is provided by a human sequence antibody or a human monoclonal antibody of the present invention.

An "effector cell specific antibody" as used herein refers to an antibody or functional antibody fragment that binds the Fc receptor of effector cells. Preferred

antibodies for use in the subject invention bind the Fc receptor of effector cells at a site which is not bound by endogenous immunoglobulin.

As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, *e.g.*, lymphocytes (*e.g.*, B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, neutrophils, polymorphonuclear cells, granulocytes, mast cells, and basophils. Effector cells express specific Fc receptors and carry out specific immune functions. An effector cell can induce antibody-dependent cell-mediated cytotoxicity (ADCC), *e.g.*, a neutrophil capable of inducing ADCC. For example, monocytes, macrophages, neutrophils, eosinophils, and lymphocytes which express FcαR are involved in specific killing of target cells and presenting antigens to other components of the immune system, or binding to cells that present antigens. An effector cell can also phagocytose a target antigen, target cell, or microorganism.

The expression of a particular FcR on an effector cell can be regulated by humoral factors such as cytokines. For example, expression of FcγRI has been found to be up-regulated by interferon gamma (IFN-γ). This enhanced expression increases cytotoxic activity (including, *e.g.*, phagocytosis) by FcγRI-bearing cells against target cells.

"Target cell" shall mean any undesirable cell in a subject (*e.g.*, a human or animal) that can be targeted by a composition (*e.g.*, a human sequence antibody or a human monoclonal antibody of the invention, a bispecific or a multispecific molecule of the invention). The target cell can be a cell expressing or overexpressing human CTLA-4. Cells expressing human CTLA-4 can include tumor cells, *e.g.* lymphomas.

In addition to human sequence antibodies and human monoclonal antibodies of the invention, other antibodies can be also be employed in the bispecific or multispecific molecules of the invention, including, *e.g.*, murine, chimeric and humanized monoclonal antibodies.

Chimeric mouse-human monoclonal antibodies (*i.e.*, chimeric antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the Fc constant region of a murine (or other species) monoclonal antibody molecule is digested with restriction enzymes to remove the region encoding the murine

Fc, and the equivalent portion of a gene encoding a human Fc constant region is substituted. (*See, e.g.*, Robinson *et al.*., International Patent Publication PCT/US86/02269; Akira, *et al.*., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.*., European Patent Application 173,494; Neuberger *et al.*., International Application WO 86/01533; Cabilly *et al.*. U.S. Patent No. 4,816,567; Cabilly *et al.*., European Patent Application 125,023; Better (1988) Science 240:1041-1043; Liu (1987) PNAS 84:3439-3443; Liu (1987) J. Immunol. 139:3521-3526; Sun (1987) PNAS 84:214-218; Nishimura (1987) Canc. Res. 47:999-1005; Wood (1985) Nature 314:446-449; Shaw (1988) J. Natl. Cancer Inst. 80:1553-1559).

The chimeric antibody can be further humanized by replacing sequences of the Fv variable region which are not directly involved in antigen binding with equivalent sequences from human Fv variable regions. General reviews of humanized chimeric antibodies are provided by Morrison (1985) *Science* 229:1202-1207 and by Oi (1986) *BioTechniques* 4:214. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from 7E3, an anti-$GPIIb_bIII_a$ antibody producing hybridoma. The recombinant DNA encoding the chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable humanized antibodies can alternatively be produced by CDR substitution U.S. Patent 5,225,539; Jones (1986) Nature 321:552-525; Verhoeyan *et al.*. 1988 Science 239:1534; and Beidler (1988) J. Immunol. 141:4053-4060.

All of the CDRs of a particular human antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the humanized antibody to the Fc receptor. An antibody can be humanized by any method, which is capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention, see UK Patent Application GB 2188638A, filed on March 26, 1987. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed mutagenesis as

described in, *e.g.*, WO 94/10332 entitled, *Humanized Antibodies to Fc Receptors for Immunoglobulin G on Human Mononuclear Phagocytes.*

Chimeric and humanized antibodies in which specific amino acids have been substituted, deleted or added are also within the scope of the invention. For example, humanized antibodies can have amino acid substitutions in the framework region, such as to improve binding to the antigen. In a humanized antibody having mouse CDRs, amino acids located in the human framework region can be replaced with the amino acids located at the corresponding positions in the mouse antibody. Such substitutions are known to improve binding of humanized antibodies to the antigen in some instances. Antibodies in which amino acids have been added, deleted, or substituted are referred to herein as modified antibodies or altered antibodies.

Bispecific and multispecific molecules of the invention can further include a third binding specificity, in addition to an anti-Fc binding specificity and an anti-human CTLA-4 binding specificity. The third binding specificity can be an anti-enhancement factor (EF) portion, *e.g.*, a molecule which binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against the target cell. The "anti-enhancement factor portion" can be an antibody, functional antibody fragment or a ligand that binds to a given molecule, *e.g.*, an antigen or a receptor, and thereby results in an enhancement of the effect of the binding determinants for the Fc receptor or target cell antigen. The "anti-enhancement factor portion" can bind an Fc receptor or a target cell antigen. Alternatively, the anti-enhancement factor portion can bind to an entity that is different from the entity to which the first and second binding specificities bind. For example, the anti-enhancement factor portion can bind a cytotoxic T-cell via, *e.g.*, CD2, CD3, CD8, CD28, CD4, CD40, ICAM-1 or other immune cell molecules that are involved in an increased immune response against the target cell.

Bispecific and multispecific molecules of the present invention can be made using chemical techniques (*see, e.g.*, Kranz (1981) *Proc. Natl. Acad. Sci.* USA 78:5807), "polydoma" techniques (*see, e.g.*, U.S. Patent 4,474,893), or recombinant DNA techniques. Bispecific and multispecific molecules of the present invention can also be prepared by conjugating the constituent binding specificities, *e.g.*, the anti-FcR and anti-human CTLA-4 binding specificities, using methods known in the art and as described herein. For example, each binding specificity of the bispecific and multispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be

used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohaxane-1-carboxylate (sulfo-SMCC) (*see, e.g.*, Karpovsky (1984) J. Exp. Med. 160:1686; Liu (1985) Proc. Natl. Acad. Sci. USA 82:8648). Other methods include those described by Paulus (Behring Ins. Mitt. (1985) No. 78, 118-132; Brennan (1985) Science 229:81-83), Glennie (1987) J. Immunol. 139: 2367-2375). Other conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies (*e.g.*, two humanized antibodies), they can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. The hinge region can be modified to contain an odd number of sulfhydryl residues, *e.g.*, one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific and multispecific molecule is a MAb x MAb, MAb x Fab, Fab x $F(ab')_2$ or ligand x Fab fusion protein. A bispecific and multispecific molecule of the invention, *e.g.*, a bispecific molecule can be a single chain molecule, such as a single chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific and multispecific molecules can also be single chain molecules or may comprise at least two single chain molecules. Methods for preparing bi- and multispecific molecules are described for example in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

Binding of the bispecific and multispecific molecules to their specific targets can be confirmed by enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), or a Western Blot Assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using *e.g.*, an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR

complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

Also included in the invention are modified antibodies. The term "modified antibody" includes antibodies, such as monoclonal antibodies, chimeric antibodies, and humanized antibodies which have been modified by, *e.g.*, deleting, adding, or substituting portions of the antibody. For example, an antibody can be modified by deleting the constant region and replacing it with a constant region meant to increase half-life, *e.g.*, serum half-life, stability or affinity of the antibody.

The antibody conjugates of the invention can be used to modify a given biological response or create a biological response (*e.g.*, to recruit effector cells). The drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon-alpha; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, *see, e.g.*, Arnon *et al.*., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.*. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.*. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.*. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.*. (eds.), pp.

303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

PHARMACEUTICAL COMPOSITIONS

The invention provides pharmaceutical compositions comprising one or a combination of human monoclonal antibodies and/or human sequence antibodies (intact or binding fragments) formulated together with a pharmaceutically acceptable carrier. Some compositions include a combination of multiple (*e.g.*, two or more) isolated human antibodies and/or human sequence antibody or antigen-binding portions thereof of the invention. In some compositions, each of the antibodies or antigen-binding portions thereof of the composition is a monoclonal antibody or a human sequence antibody that binds to a distinct, pre-selected epitope of human CTLA-4.

A. Effective Dosages

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors.

A physician or veterinarian can start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a compositions of the invention is that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose generally depends upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, or administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic compositions can be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

Effective doses of the compositions of the present invention, for the treatment of immune-related conditions and diseases described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient; whether the patient is human or an animal, other medications

administered, and whether treatment is prophylactic or therapeutic. Treatment dosages need to be titrated to optimize safety and efficacy.

For administration with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per every two weeks or once a month or once every 3 to 6 months. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated. Antibody is usually administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of antibody to CTLA-4 in the patient. In some methods, dosage is adjusted to achieve a plasma antibody concentration of 1–1000 μg/ml and in some methods 25 – 300 μg/ml. Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the antibody in the patient. In general, human antibodies show the longest half life, followed by humanized antibodies, chimeric antibodies, and nonhuman antibodies. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patent can be administered a prophylactic regime.

Doses for nucleic acids encoding immunogens range from about 10 ng to 1 g, 100 ng to 100 mg, 1 μg to 10 mg, or 30-300 μg DNA per patient. Doses for infectious viral vectors vary from 10-100, or more, virions per dose.

Some human sequence antibodies and human monoclonal antibodies of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be

formulated, for example, in liposomes. For methods of manufacturing liposomes, *See, e.g.*, U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (*See, e.g.*, V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (*See, e.g.*, U.S. Patent 5,416,016 to Low *et al.*.); mannosides (Umezawa *et al.*., (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.*. (1995) *FEBS Lett.* 357:140; M. Owais *et al.*. (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.*. (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.*. (1994) *J. Biol. Chem.* 269:9090); *See also* K. Keinanen; M.L. Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273. In some methods, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In some methods, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the tumor or infection. The composition should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms such as bacteria and fungi.

For therapeutic applications, the pharmaceutical compositions are administered to a patient suffering from established disease in an amount sufficient to arrest or inhibit further development or reverse or eliminate, the disease, its symptoms or biochemical markers. For prophylactic applications, the pharmaceutical compositions are administered to a patient susceptible or at risk of a disease in an amount sufficient to delay, inhibit or prevent development of the disease, its symptoms and biochemical markers.. An amount adequate to accomplish this is defined as a "therapeutically-" or "prophylactically-effective dose." Dosage depends on the disease being treated, the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected. Specifically, in treatment of tumors, a "therapeutically effective dosage" can inhibit tumor growth by at least about 20%, or at least about 40%, or at least about 60%, or at least about 80% relative to untreated subjects. The ability of a compound to inhibit cancer can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit by conventional assays in vitro. A

therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject.

The composition should be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

B. Routes of Administration

Pharmaceutical compositions of the invention also can be administered in combination therapy, *i.e.*, combined with other agents. For example, in treatment of cancer, the combination therapy can include a composition of the present invention with at least one anti-tumor agent or other conventional therapy, such as radiation treatment.

Pharmaceutically acceptable carriers includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. The carrier can be suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (*See, e.g.*, Berge, S.M., *et al.*. (1977) J. Pharm. Sci. 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric,

phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A composition of the present invention can be administered by a variety of methods known in the art. The route and/or mode of administration vary depending upon the desired results. The active compounds can be prepared with carriers that protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are described by *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978. Pharmaceutical compositions are preferably manufactured under GMP conditions.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*. (1984) *J. Neuroimmunol.* 7:27).

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile, substantially isotonic, and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to

high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.Therapeutic compositions can also be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in, *e.g.*, U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4.,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known .

C. Formulation

For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations can conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount range from about 0.01 per cent to about ninety-nine percent of active ingredient, from about 0.1 per cent to about 70 per cent, or from about 1 per cent to about 30 per cent.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate. Dosage forms for the topical or transdermal administration of compositions of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The phrases "parenteral administration" and "administered parenterally" mean modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, *supra*, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.01 to 99.5% (or 0.1 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The pharmaceutical compositions are generally formulated as sterile, substantially isotonic and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

METHODS AND USES OF THE INVENTION

A. Methods

The compositions (*e.g.*, human sequence antibodies and human monoclonal antibodies to human CTLA-4 and derivatives/conjugates thereof) of the present invention have *in vitro* and *in vivo* diagnostic and therapeutic utilities. For example, these molecules can be administered to cells in culture, *e.g. in vitro* or ex vivo, or in a subject, *e.g.*, *in vivo*, to treat, prevent or diagnose a variety of disorders. The term "subject" includes human and non-human animals. Non-human animals includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, and reptiles. The methods are particularly suitable for treating human patients having a disorder that can be treated by augmenting or reducing the T-cell mediated immune response.

When antibodies to CTLA-4 are administered together with another agent, the two can be administered in either order or simultaneously. The methods can be used to treat any kind of cancer including melanoma, colon cancer, prostate cancer, and renal cancer.

For example, latex microspheres coated with anti-CTLA-4 (to increase the valency of the antibody) can inhibit T cell proliferation and activation. Agents having the same antibody combining site may act as a CTLA-4 antagonist when presented as an Fab or a soluble IgG, and a CTLA-4 agonist when highly cross-linked. Thus multivalent forms of anti-CTLA-4 antibodies are useful therapeutic agents for down-modulating immune responses.

In addition to linking to latex microspheres or other insoluble particles, the antibodies can be cross-linked to each other or genetically engineered to form multimers. Cross-linking can be by direct chemical linkage, or by indirect linkage such as an antibody-biotin-avidin complex. Cross-linking can be covalent, where chemical linking groups are employed, or non-covalent, where protein-protein or other protein-ligand interactions are employed. Genetic engineering approaches for linking include, *e.g.*, the re-expression of the variable regions of high-affinity IgG antibodies in IgM expression vectors or any protein moiety (*e.g.*, polylysine, and the like). Converting a high affinity IgG antibody to an IgM antibody can create a decavalent complex with very high avidity. IgA_2 expression vectors may also be used to produce multivalent antibody complexes. IgA_2 can form polymers together with J chain and secretory component. IgA_2 may have the added advantage that it can be additionally crosslinked by the IgA receptor CD89, which is expressed on neutrophils, macrophages, and monocytes.

Agonism can also be obtained using some preparations of polyclonal antibodies to CTLA-4 comprising antibodies to at least two non-overlapping epitopes on CTLA-4. One antibody in such a preparation containing two binding sites can bind to two molecules of CTLA-4 to form a small cluster. A second antibody possessing different binding sites can then link (aggregate) these small clusters to form large clusters, thereby forming a complex of CTLA-4 (on the cell surface) that can transduce a signal to the T cell to inhibit, reduce or prevent activation of the T-cell bearing (expressing) CTLA-4. Thus, some preparations of polyclonal antibodies show similar agonism to the polyvalent preparations described above.

Therefore, polyvalent or polyclonal preparations of anti CTLA-4 antibodies are useful for agonizing the CTLA-4 receptor, thereby suppressing immune responses otherwise mediated by T cells bearing the CTLA-4 receptor. Some examples of diseases that can be treated using such polyvalent or polyclonal preparations of antibodies induce autoimmune disease, transplant rejection, and inflammation.

B. Uses

1. Activating immune responses

a. Cancer

Some therapeutic methods treat patients with cancer. Blockade of CTLA-4 by antibodies can enhance the immune response to cancerous cells in the patient. Optionally, antibodies to CTLA-4 can be combined with an immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells, and cells transfected with genes encoding immune stimulating cytokines and cell surface antigens such as B7 (*see, e.g.*, Hurwitz, A. *et al.* (1998) *Proc. Natl. Acad. Sci U.S.A.*. 95, 10067-10071).

In murine experimental systems, implantation of some tumors followed by the administration of anti-CTLA-4 antibodies can result in the rejection of tumors. In some cases tumor rejection of established tumors occurs; in other cases the growth of the tumor is slowed by the use of anti-CTLA-4 antibodies. In general CTLA-4 blockade is effective against immunogenic tumors. Operationally this is defined as those tumors for which vaccination using the tumor itself can lead to immunity to tumor challenge. In humans, some tumors have been shown to be immunogenic such as melanomas. It is anticipated that by raising the threshold of T cell activation by CTLA-4 blockade, we may expect to activate tumor responses in the host.

CTLA-4 blockade is most effective when combined with a vaccination protocol. Many experimental strategies for vaccination against tumors have been devised (*see* Rosenberg, S., 2000, Development of Cancer Vaccines, ASCO *Educational Book* Spring: 60-62; Logothetis, C., 2000, ASCO *Educational Book* Spring: 300-302; Khayat, D. 2000, ASCO *Educational Book* Spring: 414-428; Foon, K. 2000, ASCO *Educational Book* Spring: 730-738; *see also* Restifo, N. and Sznol, M., Cancer Vaccines, Ch. 61, pp. 3023-3043 in DeVita, V. *et al.*. (eds.), 1997, Cancer: Principles and Practice of Oncology, Fifth Edition). In one of these strategies, a vaccine is prepared using autologous or allogeneic tumor cells. These cellular vaccines have been shown to be most effective when the tumor cells are transduced to express GM-CSF. GM-CSF has been shown to be a potent activator of antigen presentation for tumor vaccination (Dranoff *et al.* (1993) *Proc. Natl. Acad. Sci U.S.A.* 90 (80: 3539-43).

Anti-CTLA-4 blockade together with the use of GMCSF-modified tumor cell vaccines has been shown to be effective in a number of experimental tumor models such as mammary carcinoma (Hurwitz *et al.*. (1998) *supra*), primary prostate cancer

(Hurwitz A. *et al.*. (2000) *Cancer Research* 60 (9): 2444-8) and melanoma (van Elsas, A *et al.* (1999) *J. Exp. Med.* 190: 355-66). In these instances, non-immunogenic tumors, such as the B16 melanoma, have been rendered susceptible to destruction by the immune system. The tumor cell vaccine may also be modified to express other immune activators such as IL2, and costimulatory molecules, among others.

The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens (Rosenberg, SA (1999) *Immunity* 10: 281-7). In many cases, these tumor specific antigens are differentiation antigens expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp 100, MAGE antigens, Trp-2. More importantly, many of these antigens can be shown to be the targets of tumor specific T cells found in the host. CTLA-4 blockade may be used in conjunction with a collection of recombinant proteins and/or peptides expressed in a tumor in order to generate an immune response to these proteins. These proteins are normally viewed by the immune system as self antigens and are therefore tolerant to them. The tumor antigen may also include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim, N *et al.* (1994) *Science* 266, 2011-2013). (These somatic tissues may be protected from immune attack by various means). Tumor antigen may also be "neo-antigens" expressed in cancer cells because of somatic mutations that alter protein sequence or create fusion proteins between two unrelated sequences (ie. bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors. Other tumor vaccines may include the proteins from viruses implicated in human cancers such a Human Papilloma Viruses (HPV), Hepatitis Viruses (HBV and HCV) and Kaposi's Herpes Sarcoma Virus (KHSV). Another form of tumor specific antigen which may be used in conjunction with CTLA-4 blockade is purified heat shock proteins (HSP) isolated from the tumor tissue itself. These heat shock proteins contain fragments of proteins from the tumor cells and these HSPs are highly efficient at delivery to antigen presenting cells for eliciting tumor immunity (Suot, R & Srivastava, P (1995) *Science* 269: 1585-1588; Tamura, Y. *et al.*. (1997) *Science* 278: 117-120.

Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DC's can be produced ex vivo and loaded with various protein and peptide antigens as well as tumor cell extracts (Nestle, F. *et al.*. (1998) *Nature Medicine* 4: 328-332). DCs may also be transduced by genetic means to express these

tumor antigens as well. DCs have also been fused directly to tumor cells for the purposes of immunization (Kugler, A. *et al.* (2000) *Nature Medicine* 6:332-336). As a method of vaccination, DC immunization may be effectively combined with CTLA-4 blockade to activate more potent anti-tumor responses.

CTLA-4 blockade may also be combined with standard cancer treatments. CTLA-4 blockade may be effectively combined with chemotherapeutic regimes. In these instances, it may be possible to reduce the dose of chemotherapeutic reagent administered (Mokyr, M. *et al.* (1998) *Cancer Research* 58: 5301-5304). The scientific rationale behind the combined use of CTLA-4 blockade and chemotherapy is that cell death, that is a consequence of the cytotoxic action of most chemotherapeutic compounds, should result in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with CTLA-4 blockade through cell death are radiation, surgery, and hormone deprivation (Kwon, E. *et al.* (1999) *Proc. Natl. Acad. Sci U.S.A.* 96 (26): 15074-9. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors may also be combined with CTLA-4 blockade. Inhibition of angiogenesis leads to tumor cell death which may feed tumor antigen into host antigen presentation pathways.

CTLA-4 blocking antibodies can also be used in combination with bispecific antibodies that target Fc alpha or Fc gamma receptor-expressing effectors cells to tumor cells (*see, e.g.*, U.S. Patent Nos. 5,922,845 and 5,837,243). Bispecific antibodies can be used to target two separate antigens. For example anti-Fc receptor/anti tumor antigen (*i.e.*, Her-2/neu) bispecific antibodies have been used to target macrophages to sites of tumor. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would by augmented by the use of CTLA-4 blockade. Alternatively, antigen may be delivered directly to DCs by the use of bispecific antibodies which bind to tumor antigen and a dendritic cell specific cell surface marker.

Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of proteins which are expressed by the tumors and which are immunosuppressive. These include among others Tgfβ (Kehrl, J. *et al.*. (1986) *J. Exp. Med.* 163: 1037-1050), IL-10 (Howard, M. & O'Garra, A. (1992) *Immunology Today* 13: 198-200), and Fas ligand (Hahne, M. *et al.*. (1996) *Science* 274: 1363-1365). Antibodies to each of these entities may be used in

combination with anti-CTLA-4 to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

Other antibodies which may be used to activate host immune responsiveness can be used in combination with anti-CTLA-4. These include molecules on the surface of dendritic cells which activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity (Ridge, J. *et al.*. (1998) *Nature* 393: 474-478). and can be used in conjuction with CTLA-4 antibodies (Ito, N. *et al.* (2000) *Immunobiology* 201 (5) 527-40). Activating antibodies to T cell costimulatory molecules such as OX-40 (Weinberg, A. *et al.* (2000) *J Immunol* 164: 2160-2169), 4-1BB (Melero, I. *et al.* (1997) *Nature Medicine* 3: 682-685 (1997), and ICOS (Hutloff, A. *et al.*. (1999) *Nature* 397: 262-266) may also provide for increased levels of T cell activation.

Bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. While graft versus host disease is a consequence of this treatment, therapeutic benefit may be obtained from graft vs. tumor responses. CTLA-4 blockade can be used to increase the effectiveness of the donor engrafted tumor specific T cells (Blazar, B. *et al.*. (1999) *J Immunol* 162: 6368-6377).

There are also several experimental treatment protocols that involve ex vivo activation and expansion of antigen specific T cells and adoptive transfer of these cells into recipients in order to antigen-specific T cells against tumor (Greenberg, R. & Riddell, S. (1999) 285: 546-51). These methods may also be used to activate T cell responses to infectious agents such as CMV (see below). Ex vivo activation in the presence of anti-CTLA-4 antibodies may be expected to increase the frequency and activity of the adoptively transferred T cells.

b. Infectious diseases

Other methods of the invention are used to treat patients that have been exposed to particular toxins or pathogens. Similar to its application to tumors as discussed above, antibody mediated CTLA-4 blockade can be used alone, or as an adjuvant, in combination with vaccines, to stimulate the immune response to pathogens, toxins, and self-antigens. CTLA-4 blockade has been shown to be effective in the acute phase of infections of *Nippostrongylus brasiliensis* (McCoy, K. *et al.*. (1997) 186(2); 183-187) and *Leishmania donovani* (Murphy, M. *et al.* (1998) *J. Immunol.* 161:4153-4160). Examples of pathogens for which this therapeutic approach may be particularly useful, include pathogens for which there is currently no effective vaccine, or pathogens

for which conventional vaccines are less than completely effective. These include, but are not limited to HIV, *Hepatitis* (A, B, & C), *Influenza*, *Herpes*, *Giardia*, *Malaria*, *Leishmania*, *Staphylococcus Aureus*, *Pseudomonas aeruginosa*. CTLA-4 blockade is particularly useful against established infections by agents such as HIV that present altered antigens over the course of the infections. These novel epitopes are recognized as foreign at the time of anti-human CTLA-4 administration, thus provoking a strong T cell response that is not dampened by negative signals through CTLA-4.

Some examples of pathogenic viruses causing infections treatable by methods of the invention include *hepatitis* (A, B, or C), *herpes* virus (*e.g.*, VZV, HSV-1, HAV-6, HSV-II, and CMV, *Epstein Barr* virus), *adenovirus*, *influenza* virus, *flaviviruses*, *echovirus*, *rhinovirus*, *coxsackie* virus, *cornovirus*, respiratory syncytial virus, *mumps* virus, *rotavirus*, *measles* virus, *rubella* virus, parvovirus, vaccinia virus, HTLV virus, *dengue* virus, *papillomavirus*, *molluscum* virus, *poliovirus*, *rabies* virus, JC virus and *arboviral encephalitis* virus.

Some examples of pathogenic bacteria causing infections treatable by methods of the invention include *chlamydia*, *rickettsial* bacteria, *mycobacteria*, *staphylococci*, *streptococci*, *pneumonococci*, *meningococci* and *conococci*, *klebsiella*, *proteus*, *serratia*, *pseudomonas*, *legionella*, *diphtheria*, *salmonella*, *bacilli*, *cholera*, *tetanus*, *botulism*, *anthrax*, *plague*, *leptospirosis*, and *Lymes* disease bacteria.

Some examples of pathogenic fungi causing infections treatable by methods of the invention include *Candida* (*albicans*, *krusei*, *glabrata*, *tropicalis*, *etc.*), *Cryptococcus neoformans*, *Aspergillus* (*fumigatus*, *niger*, *etc.*), *Genus Mucorales* (Mucor, Absidia, Rhizophus), *Sporothrix schenkii*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis* and *Histoplasma capsulatum*.

Some examples of pathogenic parasites causing infections treatable by methods of the invention include *Entamoeba histolytica*, *Balantidium coli*, *Naegleria fowleri*, *Acanthamoeba sp.*, *Giardia lambia*, *Cryptosporidium sp.*, *Pneumocystis carinii*, *Plasmodium vivax*, *Babesia microti*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondi*, *Nippostrongylus brasiliensis*.

In all of the above methods, a CTLA-4 blockade can be combined with other forms of immunotherapy such as cytokine treatment (*e.g.* interferons, GM-CSF, GCSF, IL-2), or bispecific antibody therapy, which provides for enhanced presentation of tumor antigens (*see, e.g.*, Holliger (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak (1994) *Structure* 2:1121-1123).

c. Promoting beneficial "autoimmune" reactions for the treatment of disease and therapeutic intervention.

The ability of anti-CTLA-4 antibodies to provoke and amplify autoimmune responses has been documented in a number of experimental systems (EAE - Experimental Autoimmune Encephalomyelitis, a murine model for MS (Perrin, P. *et al.* (1996) *J Immunol* 157 (4): 1333-1336); diabetes (Luhder, F. *et al.*. (1998) *supra*). Indeed, induction of anti-tumor responses using tumor cell and peptide vaccines reveals that many anti-tumor responses involve anti-self reactivities (depigmentation observed in anti-CTLA-4 + GM-CSF modified B16 melanoma in van Elsas *et al.*. *supra*; depigmentation in Trp-2 vaccinated mice (Overwijk, W. *et al.* (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96: 2982-2987); autoimmune prostatitis evoked by TRAMP tumor cell vaccines (Hurwitz, A. (2000) *supra*), melanoma peptide antigen vaccination and vitilago observed in human clinical trials (Rosenberg, SA and White, DE (1996) *J Immunother Emphasis Tumor Immunol* 19 (1): 81-4).

Therefore, it is possible to consider using anti-CTLA-4 blockade in conjunction with various self proteins in order to devise vaccination protocols to efficiently generate immune responses against these self proteins for disease treatment. For example, Alzheimers disease involves inappropriate accumulation of Aβ peptide in amyloid deposits in the brain; antibody responses against amyloid are able to clear these amyloid deposits (Schenk *et al.*., (1999) Nature 400: 173-177).

Other self proteins may also be used as targets such as IgE for the treatment of allergy and asthma, and TNF for rhematoid arthritis. Finally, antibody responses to various hormones may be induced by the use of anti-CTLA-4 antibody. Neutralizing antibody responses to reproductive hormones may be used for contraception. Neutralizing antibody response to hormones and other soluble factors that are required for the growth of particular tumors may also be considered as possible vaccination targets.

Analogous methods as described above for the use of anti-CTLA-4 antibody can be used for induction of therapeutic autoimmune responses to treat patients having an inappropriate accumulation of other self-antigens, such as amyloid deposits, including Aβ in Alzheimer's disease, cytokines such as TNFα, and IgE.

2. <u>Inactivating Immune Responses</u>

Disorders caused by immune responses are called hypersensitivity diseasė. Diseases caused by failure of self-tolerance and subsequent immune responses

against self, or autologous, antigens are called autoimmune diseases. Hypersensitivity diseases can also result from uncontrolled or excessive responses against foreign antigens, such as microbes.

Although soluble antibodies to human CTLA-4 have been shown to promote the expansion and activation of T cells (*i.e.*, where CTLA-4 function (*e.g.*, binding to ligand) is inhibited; in this scenario the antibodies are antagonists to CTLA-4 function), increasing the valency of these same antibodies produces the opposite effect (where now, in contrast, the antibodies are acting as agonists of CTLA-4 to suppress the immune response) (*see, e.g.*, Krummel and Allison, 1996, *J. Exp. Med.* 183, 2533-2540). For the purposes of inactivating antigen specific T cell responses, such as those that are the targets of pathogenic autoreactive T cells, the target antigen which is specific for these T cells (ie.antigen and/or MHC/antigen complexes) must be administered with the polyvalent form of anti-CTLA-4 antibody.

a. Inflammation

Inflammation represents the consequence of capillary dilation with accumulation of fluid and migration of phagocytic leukocytes, such as granulocytes and monocytes. Inflammation is important in defending a host against a variety of infections but can also have undesirable consequences in inflammatory disorders, such as anaphylactic shock, arthritis, gout and ischemia-reperfusion. Activated T-cells have an important modulatory role in inflammation, releasing interferon γ and colony stimulating factors that in turn activate phagocytic leukocytes. The activated phagocytic leukocytes are induced to express a number of specific cells surface molecules termed homing receptors, which serve to attach the phagocytes to target endothelial cells. Inflammatory responses can be reduced or eliminated by treatment with the therapeutic agents of the present invention. For example, polyvalent preparations of antibodies against CTLA-4 block activation of activated T-cells, thereby preventing these cells from releasing molecules required for activation of phagocytic cell types

b. Autoimmune Diseases

A further situation in which immune suppression is desirable is in treatment of autoimmune diseases such as insulin-dependent diabetes mellitus, multiple sclerosis, stiff man syndrome, rheumatoid arthritis, myasthenia gravis and lupus erythematosus. In these diseases, the body develops a cellular and/or humoral immune response against one of its own antigens leading to destruction of that antigen, and potentially crippling and/or fatal consequences. Activated T-cells are believed to play a

major role in many autoimmune diseases such as diabetes mellitus. Autoimmune diseases are treated by administering one of the therapeutic agents of the invention that inhibits activation of T cells. Optionally, the autoantigen, or a fragment thereof, against which the autoimmune disease is targeted can be administered shortly before, concurrently with, or shortly after the immunosuppressive agent. In this manner, tolerance can be induced to the autoantigen under cover of the suppressive treatment, thereby obviating the need for continued immunosuppression. *See, e.g.*, Cobbold *et al.*., WO 90/15152 (1990).

c. Graft Versus Host Disease

A related use for the therapeutic agents of the present invention is in modulating the immune response involved in "graft versus host" disease (GVHD). GVHD is a potentially fatal disease that occurs when immunologically competent cells are transferred to an allogeneic recipient. In this situation, the donor's immunocompetent cells may attack tissues in the recipient. Tissues of the skin, gut epithelia and liver are frequent targets and may be destroyed during the course of GVHD. The disease presents an especially severe problem when immune tissue is being transplanted, such as in bone marrow transplantation; but less severe GVHD has also been reported in other cases as well, including heart and liver transplants. The therapeutic agents of the present invention are used to inhibit activation of donor leukocytes, thereby inhibiting their ability to lyse target cells in the host.

d. Transplant Rejection

Over recent years there has been a considerable improvement in the efficiency of surgical techniques for transplanting tissues and organs such as skin, kidney, liver, heart, lung, pancreas and bone marrow. Perhaps the principal outstanding problem is the lack of satisfactory agents for inducing immune-tolerance in the recipient to the transplanted allograft or organ. When allogeneic cells or organs are transplanted into a host (*i.e.*, the donor and donee are different individual from the same species), the host immune system is likely to mount an immune response to foreign antigens in the transplant (host-versus-graft disease) leading to destruction of the transplanted tissue. $CD8^+$ cells, $CD4^+$ cells and monocytes are all involved in the rejection of transplant tissues. The therapeutic agents of the present invention are useful to inhibit T-cell mediated alloantigen-induced immune responses in the donee thereby preventing such cells from participating in the destruction of the transplanted tissue or organ.

B. Methods for detecting/measuring the presence of CTLA-4 in a sample

The invention further provides methods for detecting the presence of human CTLA-4 antigen in a sample, or measuring the amount of human CTLA-4 antigen, comprising contacting the sample, and a control sample, with a human monoclonal antibody, or an antigen binding portion thereof, which specifically binds to human CTLA-4, under conditions that allow for formation of a complex between the antibody or portion thereof and human CTLA-4. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of human CTLA-4 antigen in the sample.

C. Kits

Also within the scope of the invention are kits comprising the compositions (*e.g.*, human sequence antibodies, human antibodies, multispecific and bispecific molecules) of the invention and instructions for use. The kit can further contain a least one additional reagent, or one or more additional human antibodies of the invention (*e.g.*, a human antibody having a complementary activity which binds to an epitope in CTLA-4 antigen distinct from the first human antibody). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

EXAMPLES

Example 1. Generation of Cmu targeted mice

Construction of a CMD targeting vector.

The plasmid pICEmu contains an EcoRI/XhoI fragment of the murine Ig heavy chain locus, spanning the mu gene, that was obtained from a Balb/C genomic lambda phage library (Marcu *et al.*. *Cell* 22: 187, 1980). This genomic fragment was subcloned into the XhoI/EcoRI sites of the plasmid pICEMI9H (Marsh *et al.*; Gene 32, 481-485, 1984). The heavy chain sequences included in pICEmu extend downstream of the EcoRI site located just 3' of the mu intronic enhancer, to the XhoI site located approximately 1 kb downstream of the last transmembrane exon of the mu gene; however, much of the mu switch repeat region has been deleted by passage in E. coli.

The targeting vector was constructed as follows (*see* **Figure 1**). A 1.3 kb HindIII/SmaI fragment was excised from pICEmu and subcloned into HindIII/SmaI

digested pBluescript (Stratagene, La Jolla, CA). This pICEmu fragment extends from the HindIII site located approximately 1 kb 5' of Cmu1 to the SmaI site located within Cmu1. The resulting plasmid was digested with SmaI/SpeI and the approximately 4 kb SmaI/XbaI fragment from pICEmu, extending from the Sma I site in Cmu1 3' to the XbaI site located just downstream of the last Cmu exon, was inserted. The resulting plasmid, pTAR1, was linearized at the SmaI site, and a neo expression cassette inserted. This cassette consists of the neo gene under the transcriptional control of the mouse phosphoglycerate kinase (pgk) promoter (XbaI/TaqI fragment; Adra *et al.*. (1987) Gene 60: 65-74) and containing the pgk polyadenylation site (PvuII/HindIII fragment; Boer *et al.*. (1990) Biochemical Genetics 28: 299-308). This cassette was obtained from the plasmid pKJ1 (described by Tybulewicz *et al.*. (1991) *Cell* 65: 1153-1163) from which the neo cassette was excised as an EcoRI/HindIII fragment and subcloned into EcoRI/HindIII digested pGEM-7Zf (+) to generate pGEM-7 (KJ1). The neo cassette was excised from pGEM-7 (KJ1) by EcoRI/SalI digestion, blunt ended and subcloned into the SmaI site of the plasmid pTAR1, in the opposite orientation of the genomic Cmu sequences. The resulting plasmid was linearized with Not I, and a herpes simplex virus thymidine kinase (tk) cassette was inserted to allow for enrichment of ES clones bearing homologous recombinants, as described by Mansour *et al.*. (1988) *Nature* 336: 348-352. This cassette consists of the coding sequences of the tk gene bracketed by the mouse pgk promoter and polyadenylation site, as described by Tybulewicz *et al.*. (1991) Cell 65: 1153-1163. The resulting CMD targeting vector contains a total of approximately 5.3 kb of homology to the heavy chain locus and is designed to generate a mutant mu gene into which has been inserted a neo expression cassette in the unique SmaI site of the first Cmu exon. The targeting vector was linearized with PvuI, which cuts within plasmid sequences, prior to electroporation into ES cells.

Generation and analysis of targeted ES cells.

AB-1 ES cells (McMahon, A. P. and Bradley, A., (1990) Cell 62: 1073-1085) were grown on mitotically inactive SNL76/7 cell feeder layers (ibid.) essentially as described (Robertson, E. J. (1987) in Teratocarcinomas and Embryonic Stem Cells: a Practical Approach (E. J. Robertson, ed.) Oxford: IRL Press, p. 71-112). The linearized CMD targeting vector was electroporated into AB-1 cells by the methods described Hasty *et al.*. (Hasty, P. R. *et al.*. (1991) *Nature* 350: 243-246). Electroporated cells were plated into 100 mm dishes at a density of 1-2 x 106 cells/dish. After 24 hours, G418 (200 micrograms/ml of active component) and FIAU (5 x 10-7 M) were added to the medium,

and drug-resistant clones were allowed to develop over 8-9 days. Clones were picked, trypsinized, divided into two portions, and further expanded. Half of the cells derived from each clone were then frozen and the other half analyzed for homologous recombination between vector and target sequences.

DNA analysis was carried out by Southern blot hybridization. DNA was isolated from the clones as described Laird *et al.*. (Laird, P. W. *et al.*., (1991) *Nucleic Acids Res.* 19 : 4293). Isolated genomic DNA was digested with SpeI and probed with a 915 bp SacI fragment, probe A (**Figure 1**), which hybridizes to a sequence between the mu intronic enhancer and the mu switch region. Probe A detects a 9.9 kb SpeI fragment from the wild type locus, and a diagnostic 7.6 kb band from a mu locus which has homologously recombined with the CMD targeting vector (the neo expression cassette contains a SpeI site). Of 1132 G418 and FIAU resistant clones screened by Southern blot analysis, 3 displayed the 7.6 kb Spe I band indicative of homologous recombination at the mu locus. These 3 clones were further digested with the enzymes BglI, BstXI, and EcoRI to verify that the vector integrated homologously into the mu gene. When hybridized with probe A, Southern blots of wild type DNA digested with BglI, BstXI, or EcoRI produce fragments of 15.7, 7.3, and 12.5 kb, respectively, whereas the presence of a targeted mu allele is indicated by fragments of 7.7, 6.6, and 14.3 kb, respectively. All 3 positive clones detected by the SpeI digest showed the expected BglI, BstXI, and EcoRI restriction fragments diagnostic of insertion of the neo cassette into the Cmu1 exon.

Generation of mice bearing the mutated mu gene.

The three targeted ES clones, designated number 264, 272, and 408, were thawed and injected into C57BL/6J blastocysts as described by Bradley (Bradley, A. (1987) in Teratocarcinomas and Embryonic Stem Cells: a Practical Approach. (E. J. Robertson, ed.) Oxford: IRL Press, p. 113-151). Injected blastocysts were transferred into the uteri of pseudopregnant females to generate chimeric mice representing a mixture of cells derived from the input ES cells and the host blastocyst. The extent of ES cell contribution to the chimera can be visually estimated by the amount of agouti coat coloration, derived from the ES cell line, on the black C57BL/6J background. Clones 272 and 408 produced only low percentage chimeras (*i.e.* low percentage of agouti pigmentation) but clone 264 produced high percentage male chimeras. These chimeras were bred with C57BL/6J females and agouti offspring were generated, indicative of germline transmission of the ES cell genome. Screening for the targeted mu gene was carried out by Southern blot analysis of BglI digested DNA from tail biopsies (as

described above for analysis of ES cell DNA). Approximately 50% of the agouti offspring showed a hybridizing BglI band of 7.7 kb in addition to the wild type band of 15.7 kb, demonstrating a germline transmission of the targeted mu gene.

Analysis of transgenic mice for functional inactivation of mu gene.

To determine whether the insertion of the neo cassette into Cmu1 has inactivated the Ig heavy chain gene, a clone 264 chimera was bred with a mouse homozygous for the JHD mutation, which inactivates heavy chain expression as a result of deletion of the JH gene segments (Chen *et al.*, (1993) Immunol. 5: 647-656). Four agouti offspring were generated. Serum was obtained from these animals at the age of 1 month and assayed by ELISA for the presence of murine IgM. Two of the four offspring were completely lacking IgM (**Table 1**). Genotyping of the four animals by Southern blot analysis of DNA from tail biopsies by BglI digestion and hybridization with probe A (**Figure 1**), and by StuI digestion and hybridization with a 475 bp EcoRI/StuI fragment (ibid.) demonstrated that the animals which fail to express serum IgM are those in which one allele of the heavy chain locus carries the JHD mutation, the other allele the Cmu1 mutation. Mice heterozygous for the JHD mutation display wild type levels of serum Ig. These data demonstrate that the Cmu1 mutation inactivates expression of the mu gene.

Mouse	Serum IgM (micrograms/ml)	Ig H chain genotype
42	<0.002	CMD/JHD
43	196	+/JHD
44	<0.002	CMD/JHD
45	174	+/JHD
129 x BL6 F1	153	+/+
JHD	<0.002	JHD/JHD

Table 1. Level of serum IgM, detected by ELISA, for mice carrying both the CMD and JHD mutations (CMD/JHD), for mice heterozygous for the JHD mutation (+/JHD), for wild type (129Sv x C57BL/6J)F1 mice (+/+), and for B cell deficient mice homozygous for the JHD mutation (JHD/JHD).

Example 2. Generation of HCo12 transgenic mice

The HCo12 human heavy chain transgene.

The HCo12 transgene was generated by coinjection of the 80 kb insert of pHC2 (Taylor *et al.*, 1994, Int. Immunol., 6: 579-591) and the 25 kb insert of pVx6. The plasmid pVx6 was constructed as described below.

An 8.5 kb HindIII/SalI DNA fragment, comprising the germline human VH1-18 (DP-14) gene together with approximately 2.5 kb of 5' flanking, and 5 kb of 3' flanking genomic sequence was subcloned into the plasmid vector pSP72 (Promega, Madison, WI) to generate the plasmid p343.7.16. A 7 kb BamHI/HindIII DNA fragment, comprising the germline human VH5-51 (DP-73) gene together with approximately 5 kb of 5' flanking and 1 kb of 3' flanking genomic sequence, was cloned into the pBR322 based plasmid cloning vector pGP1f (Taylor *et al.*. 1992, Nucleic Acids Res. 20: 6287-6295), to generate the plasmid p251f. A new cloning vector derived from pGP1f, pGP1k (Seq. ID #1), was digested with EcoRV/BamHI, and ligated to a 10 kb EcoRV/BamHI DNA fragment, comprising the germline human VH3-23 (DP47) gene together with approximately 4 kb of 5' flanking and 5 kb of 3' flanking genomic sequence. The resulting plasmid, p112.2RR.7, was digested with BamHI/SalI and ligated with the 7 kb purified BamHI/SalI insert of p251f. The resulting plasmid, pVx4, was digested with XhoI and ligated with the 8.5 kb XhoI/SalI insert of p343.7.16. A clone was obtained with the VH1-18 gene in the same orientation as the other two V genes. This clone, designated pVx6, was then digested with NotI and the purified 26 kb insert coinjected--together with the purified 80 kb NotI insert of pHC2 at a 1:1 molar ratio--into the pronuclei of one-half day (C57BL/6J x DBA/2J)F2 embryos as described by Hogan *et al.*. (B. Hogan *et al.*., Manipulating the Mouse Embryo, A Laboratory Manual, 2nd edition, 1994, Cold Spring Harbor Laboratory Press, Plainview NY). Three independent lines of transgenic mice comprising sequences from both Vx6 and HC2 were established from mice that developed from the injected embryos. These lines are designated (HCo12)14881, (HCo12)15083, and (HCo12)15087. Each of the three lines were then bred with mice comprising the CMD mutation described in Example 1, the JKD mutation (Chen *et al.*. 1993, EMBO J. 12: 811-820), and the (KCo5)9272 transgene (Fishwild *et al.*. 1996, *Nature* Biotechnology 14: 845-851). The resulting mice express human heavy and kappa light chain transgenes in a background homozygous for disruption of the endogenous mouse heavy and kappa light chain loci.

Example 3. Generation of Human IgG Kappa Anti-Human CTLA-4 Monoclonal Antibodies

Cell based antigen

A DNA segment encoding a fusion protein comprising sequences from the human CTLA-4 and the murine CD3zeta genes was constructed by PCR amplification of

cDNA clones together with bridging synthetic oligonucleotides. The encoded fusion protein contains the following sequences: i. human CTLA-4 encoding amino acids 1-190 (containing the signal peptide, the extracellular domain of human CTLA-4 and the entirety of the presumed transmembrane sequence of human CTLA-4) and ii. murine CD3zeta from amino acid 52 to the carboxy terminus (Weissman *et al.*. (1988) *Science* 239: 1018-1021). The amplified PCR product was cloned into a plasmid vector and the DNA sequence was determined. The cloned insert was then subcloned into the vector pBABE (which contains a gene encoding for puromycin resistance (Morganstern, JP and Land, H Nucl. Acids Res. 18: 3587-96 (1990)) to create pBABE-huCTLA-4/CD3z. pBABE-huCTLA-4/CD3z was transfected into the retroviral packaging line, ψ-2, and a pool of puromycin resistant cells were selected. These cells were co-cultured with the murine T cell hybridoma BW5147 (ATCC #TIB-47). After 2 days of co-culture the non-adherent BW5147 cells were removed and selected for resistance to puromycin. The puromycin resistant cell pool was subcloned by limiting dilution and tested for surface expression of human CTLA-4 by FACS. A clone expressing high levels of human CTLA-4 at the cell surface was selected.

Soluble Antigen

Recombinant CTLA-4 fusion protein comprising the extracellular domain of human CTLA-4 was purchased from R&D Systems (Cat. #325-CT-200). Extracellular CTLA-4 fragment was prepared by proteolytic cleavage of the CTLA-4 fusion protein at a Factor Xa protease cleavage site located after the C-terminus of the CTLA-4 extracellular domain. Fusion protein was treated with Factor Xa at a ratio of 50:1 of fusion protein to Factor Xa, and the CTLA-4 fragment was isolated by passage over protein G-Sepharose and Mono Q HPLC. Fractions were tested for the presence of human CTLA-4 dimer were by SDS-PAGE and by binding to cells expressing mouse B7 molecules (LtkmB7.1: mouse Ltk(-) cells transfected with a mouse B7.1 cDNA clone expression vector). Positive fractions were pooled and dialyzed into PBS buffer.

Transgenic mice

Two different strains of mice were used to generate CTLA-4 reactive monoclonal antibodies. Strain ((CMD)++; (JKD)++; (HCo7)11952+/++; (KCo5)9272+/++), and strain ((CMD)++; (JKD)++; (HCo12)15087+/++; (KCo5)9272+/++). Each of these strains are homozygous for disruptions of the endogenous heavy chain (CMD) and kappa light chain (JKD) loci. Both strains also comprise a human kappa light chain transgene (KCo5), with individual animals either

hemizygous or homozygous for insertion #11952. The two strains differ in the human heavy chain transgene used. Mice were hemizygous or homozygous for either the HCo7 or the HCo12 transgene. The CMD mutation is described above in Example 1. The generation of (HCo12)15087 mice is described in Example 2. The JKD mutation (Chen *et al.*. 1993, *EMBO J.* 12: 811-820) and the (KCo5)9272 (Fishwild *et al.*. 1996, *Nature Biotechnology* 14: 845-851) and (HCo7)11952 mice, are described in U.S. Patent No. 5,770,429 (Lonberg & Kay, 6/23/98).

Immunization

Transgenic mice were initially immunized i.p. with 1 - 3 x 10^7 cells in PBS, or with 10 – 50 ug soluble fusion protein in adjuvant (either complete Freund's or Ribi). Immunized mice were subsequently boosted every 2 to 4 weeks i.p. with 1 - 3 x 10^7 cells in PBS. Animals were kept on protocol for 2 to 5 months. Prior to fusion, animals were boosted i.v. on days –3 and -2 with approximately 10^6 cells, or with 10 – 20 ug soluble antigen (fusion protein or fusion protein and extracellular fragment). Some animals also received fusion protein i.v. on day –4. Successful fusions resulting in CTLA-4 reactive IgG kappa monoclonal antibodies were obtained from mice immunized by a variety of different protocols, including cells only, soluble antigen only, and cell immunizations followed by soluble antigen given i.v. prior to fusion.

Fusions

Spleen cells were fused to mouse myeloma cells (line P3 X63 Ag8.6.53, ATCC CRL 1580, or SP2/0-Ag14, ATCC CRL 1581) by standard procedures (Harlow and Lane, 1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York; Kennett *et al.*. 1980, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analysis.* Plenum, New York; Oi and Hertzenberg, 1980, *Immunoglobulin Producing Hybrid Cell Lines*, in *Selected Methods In Cellular Immunology*, ed. Mishell and Shiigi, pp. 357-372. Freeman, San Francisco; Halk, 1984, *Methods in Enzymology*: *Plant Molecular Biology*, ed. Weissbach and Weissbach, pp. 766-780, Academic Press, Orlando, FL). Cells were cultured in DMEM, 10% FBS, OPI (Sigma O-5003), BME (Gibco 21985-023), 3% Origen Hybridoma Cloning Factor (Igen IG50-0615), and 5% P388d1 (ATCC TIB 63) conditioned media. HAT or HT supplement was added to the medium during initial growth and selection.

Hybridoma Screening

To identify hybridomas secreting human IgG kappa antibodies, ELISA plates (Nunc MaxiSorp) were coated overnight at 4°C with 100 ul/well goat anti-human

Fcgamma specific antibody (Jackson Immuno Research #109-006-098) at 1 ug/ml in PBS. Plates were washed and blocked with 100 ul/well PBS-Tween containing 1% BSA. Fifty ul cell culture supernatant was added followed by a 1 – 2 hour incubation. Plates were washed and then incubated for one hour with 100 ul/well goat anti-Kappa light chain conjugated to alkaline phosphatase or horseradish peroxidase (Sigma #A-3813, or #A-7164). Plates were washed three times in PBS-Tween between each step. An analogous assay was used to identify hybridomas that secrete human antibodies reactive with human CTLA-4. This assay was identical except that the ELISA plates were coated with recombinant CTLA-4 fusion protein instead of goat anti-human Fcgamma antibody.

Characterization of monoclonal antibodies

Seventy two hybridomas that were shown by ELISA to secrete human IgG kappa binding to human CTLA-4 were subcloned. Forty seven of these subclones were tested to determine if the secreted human antibodies bind to CTLA-4 expressing cells, and if the antibodies inhibit soluble CTLA-4 from binding to cells expressing B7. Binding was determined by flow cytometry. To measure inhibition, 50 microliters of each supernatant was incubated with 10^5 LtkmB7.1 cells and 25 ng recombinant CTLA-4 fusion protein. Mean channel fluorescence was then determined by flow cytometry. **Figure 2** shows inhibition of soluble CTLA-4 binding to cells expressing B7.1. Mean channel fluorescence (MCF) of LtkmB7.1 cells stained with recombinant human CTLA-4 fusion protein was determined in the presence of hybridoma supernatant. Hybridomas that secrete blocking antibodies resulted in lower MCF values. BNI3.1 (Cat.#34580D, Pharmingen, San Diego, CA) was used as a positive control mouse monoclonal antibody that blocks CTLA-4/B7 binding.

Approximately 40% of the hybridomas appear to strongly inhibit CTLA-4 binding to the B7 ligand.

Antibodies from clones 10D1.3, 4B6.12, and 11E8, were then assayed by BIAcore (Biacore AB, Uppsala, Sweden) to determine binding kinetics. Purified recombinant CTLA-4 extracellular fragment was coupled to the CM5 sensor chip @ 1200 RU. Binding was measured by adding antibody at concentrations of 0.25, 0.5, 1, 2.5, and 5 ug/ml at a flow rate of 5 ul/min. The binding curves were fit to a Langmuir binding model using BIAevaluation software (Biacore AB, Uppsala, Sweden). Antibodies were purified by protein–A Sepharose chromatography. Determined on and off rates are shown in **Table 2**:

Hybridoma	ka (1/Ms)	kd (1/s)	Ka (1/M)
10D1.3	4.1×10^5	1.0×10^{-4}	4×10^9
4B6.12	5.1×10^5	1.3×10^{-4}	4×10^9
11E8	4.3×10^5	1.8×10^{-4}	2×10^9

Table 2. Kinetics of binding of human IgG kappa antibodies to recombinant CTLA-4 immobilized on a surface.

Serial dilutions of 10 different human IgG kappa anti-human CTLA-4 monoclonal antibodies (3A4, 9A5, 2E2, 2E7, 4B6, 4E10, 5C4, 5G1, 11E8, and 11G1) were added to microtiter wells coated with recombinant CTLA-4 fusion protein. After a 2 hour incubation, biotinylated antibody 11E8 was added to each well at a concentration of 0.1 ug/ml. The samples were incubated for 30 minutes, washed, and bound antibody detected with alkaline phosphatase/streptavidin conjugate. The titrations are shown in **Figure 3.** Antibody 11E8 binding was blocked by itself and 7 of the other human antibodies. However, binding was not blocked by antibodies 3A4 or 9A5. Reciprocal binding experiments showed that 11E8 binding did not block either 3A4 or 9A5 binding to CTLA-4.

DNA sequence

RNA was extracted from approximately 2×10^6 cells of each subcloned hybridoma cell line and used to synthesize cDNA using reagents and protocols from Invitrogen (Micro-FastTrack and cDNA Cycle: Cat. #L1310-01, and #K1520-02, Invitrogen, Carlsbad, CA). Human immunoglobulin heavy and kappa light chain V region fragments were amplified by PCR using pfu polymerase (Stratagene, La Jolla, CA), degenerate FR1 primers and unique constant region primers. The resulting PCR fragments were cloned into the pCR-Blunt vector (Invitrogen, Carlsbad, CA) and the sequence of the insert determined. The preliminary sequences for the heavy and light chain fragment of hybridoma 10D1.3 are shown in **Figure 4.** The determined sequences for the heavy and light chain fragment of hybridoma 10D1.3 are shown in **Figure 5** through **Figure 8.**

Table 3. CDR sequences of light and heavy chains for MAbs 10D1, 4B6, and 1E2.

Chain	HuMAb	CDR1	SEQ ID NO:	CDR2	SEQ ID NO:	CDR3	SEQ ID NO:
Light Chain	10D1	RASQSVGSSYLA	24	GAFSRAT	29	QQYGSSPWT	35
	4B6	RASQSVSSSFLA	25	GASSRAT	30	QQYGSSPWT	35
	1E2	RASQGISSWLA	26	AASSLQS	31	QQYNSYPPT	36
Heavy Chain	10D1	SYTMH	27	FISYDGNNKYYADSVKG	32	TGWLGPFDY	37
	4B6	SYTMH	27	FISYDGSNKHYADSVKG	33	TGWLGPFDY	38
	1E2	SYGMH	28	VIWYDGSNKYYADSVKG	34	APNYIGAFDV	39

Example 4. Use of Partial Antibody Sequences to Express Intact Antibodies

Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complimentarity determining regions (CDR's). For this reason, the amino acid sequences within CDR's are more diverse between individual antibodies than sequences outside of CDR's. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (Jones *et al.*. 1986, *Nature* 321, 522-525). Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences. These germline sequences will differ from mature antibody gene sequences because they will not include completely assembled variable genes, which are formed by V(D)J joining during B cell maturation. Germline gene sequences will also differ from the sequence of a high affinity secondary repertoire antibody at individual nucleotides because of somatic mutations. However, somatic mutations are not distributed evenly across the variable region. For example, somatic mutations are relatively infrequent in the amino-terminal portion of framework region 1 and in the carboxy-terminal portion of framework region 4. Furthermore, many somatic mutations do not significantly alter the binding properties of the antibody. For this reason, it is not necessary to obtain the entire DNA sequence of a particular antibody in order to recreate an intact recombinant antibody having binding properties similar to those of the original antibody (*see* PCT/US99/05535 filed on March 12, 1999, which is herein incorporated by

reference for all purposes). Partial heavy and light chain sequence spanning the CDR regions is typically sufficient for this purpose. The partial sequence is used to determine which germline variable and joining gene segments contributed to the recombined antibody variable genes. The germline sequence is then used to fill in missing portions of the variable region. Heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody. For this reason it is not necessary to use the corresponding germline leader sequence for expression constructs. To add missing sequences, cloned cDNA sequences can be combined with synthetic oligonucleotides by ligation or PCR amplification. Alternatively, the entire variable region can be synthesized as a set of short, overlapping, oligonucleotides and combined by PCR amplification to create an entirely synthetic variable region clone. This process has certain advantages such as elimination or inclusion of particular restriction sites, or optimization of particular codons.

The nucleotide sequences of heavy and light chain transcripts from a hybridomas are used to design an overlapping set of synthetic oligonucleotides to create synthetic V sequences with identical amino acid coding capacities as the natural sequences. The synthetic heavy and kappa light chain sequences can differ from the natural sequences in three ways: strings of repeated nucleotide bases are interrupted to facilitate oligonucleotide synthesis and PCR amplification; optimal translation initiation sites are incorporated according to Kozak's rules (Kozak, 1991, J. Biol. Chem. 266, 19867-19870); and, HindIII sites are engineered upstream of the translation initiation sites.

For both the heavy and light chain variable regions, the optimized coding, and corresponding non-coding, strand sequences are broken down into 30 – 50 nucleotide segments such that the breaks between nucleotides for the coding strand sequence occur at approximately the midpoint of the corresponding non-coding oligonucleotide. Thus, for each chain, the oligonucleotides can be assemble into overlapping double stranded sets that completely span the desired sequence. These oligonucleotides are combined into pools that span segments of 150 – 400 nucleotides. The pools are then used as templates to produce PCR amplification products of 150 – 400 nucleotides. Typically, a single variable region oligonucleotide set will be broken down into two pools which are separately amplified to generate two overlapping PCR products. These overlapping products are then combined by PCR amplification to form the complete variable region. It may also be desirable to include an overlapping fragment of the heavy or light chain

constant region (including the BbsI site of the kappa light chain, or the AgeI site if the gamma heavy chain) in the PCR amplification to generate fragments that can easily be cloned into the expression vector constructs.

The reconstructed heavy and light chain variable regions are then combined with cloned promoter, translation initiation, constant region, 3' untranslated, polyadenylation, and transcription termination, sequences to form expression vector constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a host cell expressing both chains.

Plasmids for use in construction of expression vectors for human IgGk are described below. The plasmids were constructed so that PCR amplified V heavy and V kappa light chain cDNA sequences could be used to reconstruct complete heavy and light chain minigenes. These plasmids can be used to express completely human, or chimeric IgG1k or IgG4k antibodies. Similar plasmids can be constructed for expression of other heavy chain isotypes, or for expression of antibodies comprising lambda light chains.

The kappa light chain plasmid, pCK7-96 (SEQ ID NO:40), includes the kappa constant region and polyadenylation site, such that kappa sequences amplified with 5' primers that include HindIII sites upstream of the initiator methionine can be digested with HindIII and BbsI, and cloned into pCK7-96 digested with HindIII and BbsI to reconstruct a complete light chain coding sequence together with a polyadenylation site. This cassette can be isolated as a HindIII/NotI fragment and ligated to transcription promoter sequences to create a functional minigene for transfection into cells.

The gamma1 heavy chain plasmid, pCG7-96 (SEQ ID NO:41), includes the human gamma1 constant region and polyadenylation site, such that gamma sequences amplified with 5' primers that include HindIII sites upstream of the initiator methionine can be digested with HindIII and AgeI, and cloned into pCG7-96 digested with HindIII and AgeI to reconstruct a complete gamma1 heavy chain coding sequence together with a polyadenylation site. This cassette can be isolated as a HindIII/SalI fragment and ligated to transcription promoter sequences to create a functional minigene for transfection into cells.

The gamma4 heavy chain plasmid, pG4HE (SEQ ID NO:42), includes the human gamma4 constant region and polyadenylation site, such that gamma sequences amplified with 5' primers that include HindIII sites upstream of the initiator methionine can be digested with HindIII and AgeI, and cloned into pG4HE digested with HindIII and

AgeI to reconstruct a complete gamma4 heavy chain coding sequence together with a polyadenylation site. This cassette can be isolated as a HindIII/EcoRI fragment and ligated to transcription promoter sequences to create a functional minigene for transfection into cells.

A number of different promoters (including but not limited to CMV, ubiquitin, SRalpha, and beta-actin) can be used to express the reconstructed heavy and light chain genes. For example the vector pCDNA3.1+ (Invitrogen, Carlsbad, CA), can be cleaved with HindIII and either NotI, XhoI, or EcoRI, for ligation with either the kappa, gamma1, or gamma4 cassettes described above, to form expression vectors that can be directly transfected into mammalian cells.

Example 5. 10D.1 Binding to CTLA-4

A. 10D1 Binding to purified recombinant human CTLA-4

Binding of 10D1 to purified recombinant human CTLA-4 was shown by ELISA using standard methods and procedures (**Figure 9** and **Figure 10**). Microtiter plates coated with purified CTLA-4 were incubated with varying concentration of 10D1, and then developed with goat anti-human IgG $F(ab')_2$ conjugated to alkaline phosphatase. The data demonstrate dose-dependent binding of 10D1 that is well fit to a 4-parameter curve (correlation coefficient is -1.0). The half-maximal binding at 15 ng/ml reflects the high binding capacity of 10D1 to CTLA-4. Saturation of binding was observed at approximately 0.1 μg/ml.

B. 10D.1 Binding to CTLA-4 expressed on the plasma membrane of T-cells

In order to demonstrate binding of 10D1 to CTLA-4 expressed on the plasma membrane of T-cells, the results in **Figure 10** from a flow cytometric assay are shown. The flow cytometric assay was used with a T-cell line transfected to express high levels of human CTLA-4 (designated 58αβCTLA-4/CD3zeta cells). Varying concentrations of fluoresceinated 10D1 (10D1-FITC) were incubated with 58αβCTLA-4 cells. The cell associated fluorescence was determined by flow cytometry. As seen with the purified CTLA4, 10D1 bound to CTLA4-expressing cells in a dose-dependent manner that was well fit to a 4-paramater equation (correlation coefficient is -.999). The half-maximal binding was 190 ng/ml, and saturation was achieved at 2 μg/ml. 10D1 did not bind to any CTLA4-negative cell lines tested, including SKBR-3, BT474 and MCF10A

breast epithelial tumors and L540 Hodgkin's tumor cells, nor did it bind to cells expressing murine CTLA-4. These data indicate the specificity of 10D1 for human CTLA. However, 10D1 was shown to cross-react with macaque CTLA-4 (see below).

C. Cross-reactivity of 10D1 with Normal Human Tissues

In this study, a fluoresceinated form of the test article (10D1-FITC) was used to evaluate binding. The objective of the study was to evaluate potential cross-reactivity of 10D1-FITC with cryosections of normal human tissues. No unanticipated cross-reactivity was observed.

The study was conducted in accordance with the Food and Drug Administration's Good Laboratory Practice (GLP) Regulations (21 CFR Part 58). The human tissue panel included all the tissue on the "suggested list of human tissues to be used for immunohistochemical investigations of cross reactivity' in Annex II of the EC CPMP Guideline III/5271/94, "Production and quality control of monoclonal antibodies" and all the tissues recommended in the 1997 US FDA/CBER "Points to Consider in the Manufacture and Testing of Monclonal Antibody Products for Human Use".

Using an indirect immunoperoxidase method, 10D1-FITC specifically stained positive control, human CTLA4-expressing, 58αβCTLA4CD3zeta cells as well as positive control lymphocytes in human tonsil. 10D1-FITC reactivity was moderate to intense and two concentrations of antibody were examined (10 μg/ml and 2.5 μg/ml). In both positive control 58αβCTLA4CD3zeta and positive control human tonsillar lymphocytes, 10D1-FITC specifically stained discrete, round, granules at membrane and in the cytoplasm immediately below the membrane. Reactivity was observed with occasional follicular, interfollicular, and subepithelial lymphocytes. Less than 1-2% of all tonsillar lymphocytes were reactive with 10D1-FITC.

10D1-FITC did not react with negative control human brain (cerebellum). An isotype-matched negative control antibody (HulgG1-k-FITC) did not specifically bind to either the positive control human CTLA4-expresing 58αβCTLA4CD3zeta or human tonsil; nor did it bind specifically to negative control human brain (cerebellum).

To determine cross-reactivity, 10D1-FITC was applied to a panel of normal human tissues at two concentrations (10 μg/ml and 2.5μg /ml). Specific 10D1-FITC reactivity was observed for lymphocytes in the tonsil (3/3 donors), submucosal lymphoid nodule in the colon (gastrointestinal tract-colon [1/3 donors]), and blood smears (2/3 donors).

Immunoreactive cells were identified as lymphocytes based on typical morphology (round molecular cells with large nucleus: cytoplasm ratio and scant cytoplasm, lack of dendritic processes, 10-15 μm in diameter) and location within the tissues (*e.g.*, typical location within lymphoid tissues). In the tonsils from all three donors (test tissues), lymphocytes, 10D1-FITC specifically stained discrete, round, granules at membrane and in the cytoplasm immediately below the membrane. Reactivity was observed with occasional follicular, interfollicular and subepithelial lymphocytes. Less than 1-2% of all tonsillar lymphcytes were reactive with 10D1-FITC.

In 1/3 donors examined, 10D1-FITC also specifically stained discrete granules in occasional follicular and interfollicular lymphocytes located in submucosal lymphoid nodules in the colon (gastrointestinal tract–colon [large intestine]). Again, discrete membrane granules were stained.

In peripheral blood smears from two of the three donors examined, 10D1-FITC specifically stained discrete granules approximately 1 μm in diameter associated with the membrane of rare lymphocytes. The granules were arranged in a ring or in a curved pattern. Less than 1-2% of all peripheral blood leukocytes were reactive with 10D1-FITC.

Table 4. Cross-Reactivity of MAb 10D1 With Normal Human Tissues

	Test Article 10D1-FITC		Negative Control Antibody HuIgG1-k-FITC			
Tissue	10 µg/ml	2.5 µg/ml	10 µg/ml	2.5 µg/ml	Assay Control *	β_2-microglobulin
Positive Control 58αβCTLA4CD3zeta cells	3-4+	2-4+	Neg	Neg	Neg	Pos
Positive Control Lymphocytes in human tonsil	2-3+	2-3+	Neg	Neg	Neg	Pos
Negative Control Human brain - cerebellum	Neg	Neg	Neg	Neg	Neg	Pos
Adrenal	Neg	Neg	Neg	Neg	Neg	Pos
Blood						Pos
Neutrophils	Neg	Neg	Neg	Neg	Neg	Pos
Lymphocytes	2+ (rare)	Neg	Neg	Neg	Neg	Pos
Eosinophils	Neg	Neg	Neg	Neg	Neg	Pos
Monocytes	Neg	Neg	Neg	Neg	Neg	Pos
Platelets	Neg	Neg	Neg	Neg	Neg	Pos
Blood Vessel (endothelium) Examined in all tissues	Detailed under individual tissues					
Bone Marrow	Neg	Neg	Neg	Neg	Neg	Pos
Brain - Cerebellum	Neg	Neg	Neg	Neg	Neg	Pos
Brain - Cerebrum (cortex)	Neg	Neg	Neg	Neg	Neg	Pos
Breast (mammary gland)	Neg	Neg	Neg	Neg	Neg	Pos
Eye	Neg	Neg	Neg	Neg	Neg	Pos
Gastrointestinal Tract - Colon (large intestine) Submucosal lymphoid nodule (occasional follicular and interfollicular lymphocytes)	2-3+	2-3+	Neg	Neg	Neg	Pos
Gastrointestinal Tract - Colon (large intestine) Other elements	Neg	Neg	Neg	Neg	Neg	Pos
Gastrointestinal Tract - Esophagus	Neg	Neg	Neg	Neg	Neg	Pos
Gastrointestinal Tract - Small Intestine	Neg	Neg	Neg	Neg	Neg	Pos
Gastrointestinal Tract - Stomach	Neg	Neg	Neg	Neg	Neg	Pos
Heart	Neg	Neg	Neg	Neg	Neg	Pos
Kidney (glomerulus, tubule)	Neg	Neg	Neg	Neg	Neg	Pos
Liver	Neg	Neg	Neg	Neg	Neg	Pos
Lung	Neg	Neg	Neg	Neg	Neg	Pos
Lymph Node	Neg	Neg	Neg	Neg	Neg	Pos
Ovary	Neg	Neg	Neg	Neg	Neg	Pos
Fallopian Tube (oviduct)	Neg	Neg	Neg	Neg	Neg	Pos
Pancreas	Neg	Neg	Neg	Neg	Neg	Pos
Parathyroid	Neg	Neg	Neg	Neg	Neg	Pos
Peripheral Nerve	Neg	Neg	Neg	Neg	Neg	Pos
Pituitary	Neg	Neg	Neg	Neg	Neg	Pos
Placenta	Neg	Neg	Neg	Neg	Neg	Pos
Prostate	Neg	Neg	Neg	Neg	Neg	Pos
Salivary Gland	Neg	Neg	Neg	Neg	Neg	Pos
Skin	Neg	Neg	Neg	Neg	Neg	Pos
Spinal Cord	Neg	Neg	Neg	Neg	Neg	Pos
Spleen	Neg	Neg	Neg	Neg	Neg	Pos
Striated (Skeletal) Muscle	Neg	Neg	Neg	Neg	Neg	Pos
Testis	Neg	Neg	Neg	Neg	Neg	Pos
Thymus	Neg	Neg	Neg	Neg	Neg	Pos
Thyroid	Neg	Neg	Neg	Neg	Neg	Pos

	Test Article 10D1-FITC		Negative Control Antibody HulgG1-k-FITC			
Tissue	10 µg/ml	2.5 µg/ml	10 µg/ml	2.5 µg/ml	Assay Control *	β_2-microglobulin
Tonsil Lymphocytes (occasional follicular, interfollicular and subepithelial lymphocytes)	2+	1-2+	Neg	Neg	Neg	Pos
Tonsil Other elements	Neg	Neg	Neg	Neg	Neg	Pos
Ureter	Neg	Neg	Neg	Neg	Neg	Pos
Urinary Bladder	Neg	Neg	Neg	Neg	Neg	Pos
Uterus - Body (endometrium)	Neg	Neg	Neg	Neg	Neg	Pos
Uterus - Cervix	Neg	Neg	Neg	Neg	Neg	Pos
* omission of test antibody						

D. Specific reactivity of 10D.1 with macaque CTLA-4

Specific reactivity with macaque CTLA-4 was demonstrated using T-cells transfected to express the macaque CTLA-4 at high levels (**Table 5**). These data suggest that the CTLA-4 epitope for 10D1 is conserved between macaque and humans, therefore macaque is a good model to evaluate in vivo safety of anti-CTLA4 HuMAb 10D1.

Table 5

Species	reactivity of isotype control (MFI)	reactivity of 10D1 (MFI)
human CTLA4	3	662
macaque CTLA4	4	606
murine CTLA4 (negative control)	5	5

MAb 10D1 (10 µg/ml) was incubated with cell lines expressing recombinant CTLA-4 from various species, and detected by FITC-anti human IgG. The cell-associated fluorescence was determined by FACScan and reported as mean fluorescence intensity (MFI). These data show that MAb 10D1 reacts well with macaque and human CTLA-4, but not with murine CTLA-4.

Example 6. 10D1 Blocking of CTLA-4 to B7 Ligands

In order to show that 10D1 binding to CTLA-4 blocks the interaction of CTLA-4 with CTLA-4 ligands, B7.1 and B7.2, competition assays were performed by flow cytometry (**Figure 11** and **Figure 12**). As shown in **Figure 11**, FITC-labeled human B7.2-Ig fusion protein was incubated with 58αβCTLA4 T-cells and various concentrations of 10D1 MAb. In **Figure 12**, FITC-labeled CTLA4-Ig fusion protein was incubated with murine B7.1 transfected cells and various concentrations of 10D1 MAb.

The competition assays demonstrate the ability of 10D1 to efficiently inhibit CTLA4-B7 interactions at low concentrations (1-10 µg/ml). The effective

concentration would likely be much lower under physiological conditions, which would have far lower concentrations of CTLA-4 and B7 molecules. Similar data was obtained using biotinylated reagents in ELISA assays.

These in vitro studies demonstrate that MAb 10D1 binds human CTLA-4 with high affinity and specificity and that binding of 10D1 abrogates interaction between B7 co-stimulatory molecules and CTLA-4. These data for 10D1 are consistent with the in vitro activity profiles for anti-murine CTLA-4 antibodies that have demonstrated efficacy in murine tumor models.

Example 6. Epitope Mapping of 10D.1

Competitive ELISAs were done with biotin labeled and unlabeled antibodies to determine CTLA-4 epitope specificity. Four anti-CTLA-4 epitope binding groups were identified among the human antibodies, and an additional two epitopes were defined by the commercial murine monclonal antibodies BNI3 (Pharmingen, San Diego, Ca), and 8H5 (Ancell Corp. Bayport, Mn). **Figures** 3, and 13A-13G show results of competitive binding assays that demonstrate differential competition among the antibodies for binding to CTLA-4. These results are summarized in **Table 6.**

Antibodies in anti-CTLA-4 epitope binding groups 4a and 4b have similar binding characteristics, and additionally are strong blockers of CTLA-4–Ig binding to cell surface expressed B7.1 (**Table 6**). For example, **Figure 3** shows results with biotin labeled 11E8 antibody and 10 unlabeled antibodies (3A4, 9A5, 2E2, 2E7, 4B6, 4E10, 5C4, 5G1, 11E8 and 11G1). Antibody 11E8 binding was blocked by itself and 7 of the other human antibodies in epitope groups 4a and 4b. However, binding of 11E8 was not blocked by antibodies 3A4 or 9A5(epitope groups 1 and 2). Reciprocal binding experiments showed that 11E8 binding did not block either 9A5 or 3A4 binding to CTLA-4 (**Figure 13A** and **13B**). Similar results are shown for epitope group 4a antibodies 10D1 and murine antibody 147 (Fig 13D and 13F). Antibodies in epitope group 4b (**Figure 13E**) are similar to group 4a antibodies with the exception that the epitope 4b antibodies compete with epitope group 2 antibodies in reciprocal binding experiments (**Figure 13B**). Human antibodies that belong to epitope groups 3, 4a and 4b are effective blockers of CTLA-4/B7.1 binding (**Figure 3**, and **Table 6**).

Table 5. CTLA-4 MABs: Epitope and CTLA-4/B7.1 Blocking Properties

Epitope	Monoclonal Antibody	Competition for CTLA-4 Binding	Blocks binding of CTLA-4-Ig to B7.1 on Ltk mB7.1
1	9A5	No competition from groups 3, 4a, 4b, 5, and 6 Weak Competition form group 2	No
2	3A4 1E2	One way competition from groups 1,4b, 5 and 6 No competition with 4a. Weak competition form group 3	No
3	5A8	Competes with 4a and 4b. Some competition with 2. No competition form 1 and 5	Yes
4a	10D1 147* 11E8 11G1 4E10 5C4 3F10	Cross competes with all members of 4b. Competition from 6 (non-reciprocal) No competition with 1, 2, and 5. Weak competition with 3.	Yes
4b	4B6 4A1 2E2 2E7 2G1	Cross competes with all members of 4a Competes with 2 Weak competition with 3. No competition with 1, and 5. Competition from 6 (non-reciprocal)	Yes
5	BNI3**	Competes with 6, no competition with groups 1 to 4	Yes
6	8H5***	Competes with 5, no competition with groups 1 to 4 Competition with group 3 not tested	Yes

* Murine monoclonal antibody
** Available from Pharmingen, BNI3 Catalog # 34580 D, San Diego CA.
*** Available from Ancell, ANC 152.2/8H5 Catalog # 359-020, Ancell Corp. Bayport, Mn.

Example 7. 10D1 binds to human activated T cells

The ability of 10D1 antibody to bind to CTLA-4 expressed by normal human T cells was investigated by flow cytometric analysis of resting and activated T cells (**Figure 14**). Freshly isolated human peripheral blood mononuclear cells at 2 x 10^6/ml were incubated in the presence or absence of 2 ug/ml of the T-cell mitogen, phytohemagglutinin (PHA). After four days incubation, the cells were washed and stained with the following antibodies: 1) no antibody; 2) HuIgG1-FITC, a human IgG1 anti EGF receptor antibody; 3) 10D1-FITC, human IgG1 antiCTLA-4 antibody; and 4) 147-FITC –

mouse anti-human CTLA-4 antibody. After incubation for 1 hr., cells were washed and stained with rabbit anti-FITC IgG followed by goat anti-rabbit-PE. Analysis was performed on lymphocytes gated by forward versus side scatter. As shown in **Figure 14**, resting lymphocytes do not bind 10D1 antibody, while PHA-activated T cells express low levels of CTLA-4 at the cell surface

Example 8. 10D1 does not mediate complement-dependent or antibody-dependent lysis of activated T-cells

The ability of MAb 10D1 to mediate complement-dependent cellular cytotoxicity (CDCC) or antibody-dependent cellular cytotoxicity (ADCC) of CTLA-4 expressing cells was investigated.

For CDCC experiments, rabbit serum was used as a source of compliment, in order to provide optimal conditions for CDCC. Rabbit complement has been shown to be more effective in mediating CDCC with human IgG_1 than human complement (Jurianz, Maslak *et al.*. 1999). PHA-stimulated T-cells were labeled with ^{51}Cr and incubated with various concentrations of anti-CTLA4 MAb 10D1 or anti-CD3 MAb with or without rabbit serum as a source of complement. After a 1 hour incubation, the ^{51}Cr released by dying cells was determined using a gamma counter. Target cells incubated with 2% SDS served as 100% lysis controls. The anti-CTLA-4 MAb 10D1 did not mediate CDCC of the activated T-cells (**Figure 15**). Under the same conditions, the murine $IgG2_a$ anti-CD3 MAb led to significant CDCC. Both murine $IgG2_a$ and human IgG_1 efficiently fix rabbit complement; therefore these differences most likely reflect the greatly reduced expression of CTLA-4 as compared to CD3 on activated T-cells.

Similarly, no ADCC activity was observed for MAb 10D1 using autologous mononuclear cells as effector cells (**Figure 16**). PHA-stimulated T-cells were labeled with ^{51}Cr and incubated with various concentrations of anti-CTLA4 MAb 10D1 or anti-CD3 MAb and fresh autologous mononuclear cells. The effector to target cell ratio was 100:1. After a 4 hour incubation, the ^{51}Cr released by dying cells was determined using a gamma counter. Target cells incubated with 2% SDS served as 100% lysis controls. Although the anti-CD3 MAb is a murine $IgG2_a$, which can mediate efficient ADCC with human effector cells, only low levels of ADCC were observed. These data are consistent with the requirement of high levels of antigen expression on the surface of target cells for efficient ADCC. Since MAb 10D1 is a human IgG_1, an isotype generally capable of mediating CDCC and ADCC, the lack of these activities is likely due

to the very low expression of CTLA-4 on activated T-cells. Furthermore, the observation of increased numbers of activated T-cells in the primate toxicology studies (see below) is consistent with the lack of ADCC and CDCC activity of activated T-cells by MAb 10D1 *in vivo*.

Example 9. 10D1 preclinical toxicity studies in cynomolgus monkeys

Two independent toxicology studies of 10D1 antibody and macaques were performed. A total of eight monkeys were analyzed. Four monkeys (two males and two females) tolerated three bolus i.v. doses of 3 mg/Kg human anti-CTLA4, and four monkeys (two males and two females) tolerated three bolus i.v. doses of 10 mg/Kg human anti-CTLA4 without significant clinical, immunotoxicology, or histopathological findings.

A. 10D1 primate toxicology study (3.0 mg/Kg)

To investigate the effects of 10D1 *in vivo*, a primate toxicology study was performed with two macaques. In a multiple dose toxicity study of MAb 10D1, this antibody was administered via intravenous injection of macaques. The objective of this study was to determine the tolerability of MAb 10D1 in two monkeys given at a dose and schedule compatible with efficacious treatment in a murine tumor regression model and proposed dose in human clinical studies. Two female cynomolgus monkeys (Macaca fascicilaris) were treated with three intravenous bolus doses of 3.0 mg/Kg 10D1 on days 1, 4,and 7 to evaluate safety and T-cell activation in these animals. The animals were observed for any adverse reactions, weight loss/gain, and morbidity and mortality up to 14 days post administration of the first dose. Seven days after the last dose the animals were sacrificed and necropsied to examine their organs individually.. Blood samples were collected before each dose and before necropsy for examination of T-cell populations and expression of activation markers by flow cytometry. Plasma was also collected from blood samples to determine 10D1 antibody levels and anti-10D1 antibody responses by ELISA.

The animals tolerated three doses of antibody 10D1 without any clinical symptoms during the treatment course. The weight of these animals did not change significantly. No gross findings were documented on 47 organs/tissues examined at necropsy for either animal.

Histopathology studies were performed at Redfield laboratories, Redfield, AR. The results from these studies indicated that multiple doses of MAb 10D1 did not produce acute toxicity in any of the organs and tissues examined.

Pharmacokinetic analysis revealed the presence of significant levels (up to 97.3 μg/ml) of 10D1 MAb in the plasma of both monkeys (*see* **Table 7**). Plasma levels of 10D1 were determined by a competition assay with FITC-10D1 using flow cytometry and 58αβCTLA-4 T-cells.

Table 7. 10D1 plasma levels

Time point	Monkey #1	Monkey #2
Pre-1st dose	0.0 (μg/ml plasma)	0.0 (μg/ml plasma)
Day 4, pre-2nd dose	17.4 (μg/ml plasma)	43.6 (μg/ml plasma)
Day 7, pre-3rd dose	83.6 (μg/ml plasma)	97.3 (μg/ml plasma)
Day 14	90.2 (μg/ml plasma)	70.9 (μg/ml plasma)

Evaluation of the anti-10D1-antibody response was performed by ELISA. No significant anti-10D1 response was observed in either animal during the course of study (**Figure 17**). Microtiter plates were coated with 10D1 MAb (for IgM assay) or 10D1 $F(ab')_2$ (for IgG assay). Dilutions of plasma samples from various time points were incubated with the plates, and anti-10D1 antibodies were detected with either anti-IgM or IgG Fc-specific alkaline phosphatase reagents. IgM anti-10D1 antibodies appear to have developed by day 14, however, the titers are very low. IgM anti-10D1 ant5ibodies appear to have developed by day 14, however, the titers are very low. These data demonstrate that the monkeys did not develop anti-10D1 antibody responses after 3 doses of the antibody.

These data demonstrate that the animals did not develop a significant antibody response against MAb 10D1 during the course of this study.

Immunotoxicology was investigated by flow cytometric analysis of lymphocyte populations during the course of the study. The lymphocyte subsets examined included CD3 as a marker for total T-cells and CD20 as a marker for total B-cells. T-cells, were further subdivided for expression of CD4 (helper T-cell marker) and CD8 (cytotoxic T-cell marker), as well as for activtion markers CD25, CD29, CD69 and HLA-DR. No remarkable changes in T-cell populations or expression of activation markers was noted. The results are summarized in **Table 8** below.

Table 8. Flow cytometric analysis of lymphocyte populations

Time point	Monkey #1	Monkey #2
Pre-1st dose	%CD3 = 61, %CD20 = 16 %CD4 = 43, %CD8 = 50 %CD25 ≤ 1, %CD29 = 41 %CD69 = < 1, %HLA-DR = 4	%CD3 = 54, %CD20 = 22 %CD4 = 59, %CD8 = 36 %CD25 ≤ 1, %CD29 = 29 %CD69 ≤ 1, %HLA-DR = 1
Day 4, pre-2nd dose	%CD3 = 58, %CD20 = 13 %CD4 = 38, %CD8 = 52 %CD25 ≤ 1, %CD29 = 52 %CD69 ≤ 1, %HLA-DR = 2	%CD3 = 56, %CD20 = 16 %CD4 = 62, %CD8 = 37 %CD25 ≤1, %CD29 = 36 %CD69 ≤ 1, %HLA-DR ≤1
Day 7, pre-3rd dose	%CD3 = 59, %CD20 = 15 %CD4 = 47, %CD8 = 59 %CD25 = 2, %CD29 = 44 %CD69 = 1, %HLA-DR = 4	%CD3 = 51, %CD20 = 17 %CD4 = 51, %CD8 = 39 %CD25 = 1, %CD29 = 39 %CD69 = 1, %HLA-DR = 2
Day 14	%CD3 = 64, %CD20 = 14 %CD4 = 49, %CD8 = 44 %CD25 = 1, %CD29 = 44 %CD69 ≤ 1, %HLA-DR = 15	%CD3 = 59, %CD20 = 20 %CD4 = 60, %CD8 = 35 %CD25 ≤ 1, %CD29 = 34 %CD69 ≤ 1, %HLA-DR = 1

Heparinized blood samples were analyzed fresh by flow cytometry using FITC- or PE-labeled anti-lymphocyte reagents. %CD3 and %CD20 are based on a lymphocyte gate. The additional T-cell markers and activation markers are all based on CD3-positive cells. These data indicate that multiple doses of MAb 10D1 does not have a significant effect on B and T-cell populations or T-cell activation markers.

B. 10D1 primate toxicology study (3.0 and 10.0 mg/Kg)

Six cynomolgus monkeys (four males and two females), experimentally non-naïve and weighing 2.4 to 3.8 kg at the outset of the study, were assigned to treatment groups as shown in **Table 9** below.

Table 9

Group No.	Number of Males/Females	Dose Level (mg/kg)	Dose Vol. (ml/kg)	Dose Solution Conc. mg/ml)
1	2/0	3	0.6	5.0
2	2/2	10	2.0	5.0

Each animal received a dose of human anti-CTLA4 (5 mg/ml concentration) by intravenous injection (*i.e.*, "slow-push" bolus injection) every three days for one week (*i.e.*, on Days 1, 4 and 7). Detailed clinical observations were conducted at least twice daily ("cageside observations"), and a thorough physical examination was performed on each animal prior to the study and on Day 12. Body weights were measured weekly (prestudy and Days 7 and 14), and ophthalmoscopic examination was conducted on all animals prior to the study and on Day 12. Blood samples for evaluation of serum chemistry, hematology and coagulation parameters were collected from all animals prestudy and on Day 14. Additional samples for selected hematology parameters (total and differential white blood cells only) were collected prior

to dosing on each dosing day (Days 1, 4, and 7). Urine samples for standard urinalysis were obtained by drainage from specially designed cage-pans prior to dosing and on Day 13. Blood samples were also collected prior to each dose (Days 1, 4 and 7) and prior to termination (Day 14) for various analyses conducted by Medarex. These included analysis of test article concentration (pharmacokinetics), determination of the presence of antibodies to the test article, and flow cytometry analysis. All animals were euthanized on Day 14, at which time, a complete gross necropsy was conducted, major organs were weighed, and a standard complete set of tissues was collected from each animal and processed for examination by light microscopy.

Intravenous administration of human anti-CTLA4 at dose levels of 3 mg/kg and 10 mg/kg given every three days for a total of three doses was very well tolerated by cynomolgus monkeys. There were no clinical signs of toxicity from the cageside observations and physical examinations, and no effects on body weight, ocular examination findings, clinical pathology parameters, gross necropsy findings, organ weights or tissue histomorphology.

The results of the analysis of test article concentration in serum samples (*i.e.*, trough levels measured in samples obtained prior to dosing on Days 4 and 7, and prior to necropsy on Day 14) indicated dose-dependent exposure to the test article. On Day 7, predose mean concentrations were approximately 84 and 240 μg/ml for the 3- and 10-mg/kg dose groups, respectively.

A potential for accumulation of the test article in serum with the every-three-day dosing schedule in monkeys was evident from the difference between the Day 4 and Day 7 trough levels (*i.e.*, means concentrations on Day 7 were approximately twice as high as on Day 4), as well as from the high residual levels on Day 14 (one week after the last dose), which were similar to the Day 7 trough levels. Evidence of antibody formation against the test article was detected in two of the six study animals (one from Group 1 and another from Group 2). In the former case, it appeared that the antibody response might have affected the clearance of the test article from circulation. Flow cytometric analysis of lymphocyte subsets revealed a modest increase in total CD3-positive cells between Days 1 and Day 14, which correlated with an increase in CD3/CD4-positive cells, and a respective decrease in CD3/CD8-positive cells (Group 2 only). The percentage of CD3 cells expressing CD29 and HLA-DR moderately increased over the course of the study, which was consistent with previous findings that anti-CTLA4 antibodies can enhance antigen-specific T-cells.

In conclusion, apart from the minor changes in circulating lymphocyte subpopulations, the highest dose level tested in this study (*i.e.*, three doses of 10 mg/kg given at three-day intervals) was an absolute no-effect dose level in cynomolgus monkeys.

Example 10. A Phase I human clinical trial of MAb 10D1 in prostate cancer (MDXCTLA4-01) and melanoma (MDXCTLA4-02)

MDXCTLA4-01 is an open-label study of anti-cytotoxic T-lymphocyte-associated antigen-4 (anti-CTLA-4) monoclonal antibody 10D1 (MAb 10D1) in patients with progressive, metastatic, hormone-refractory prostate cancer. Treatment is a single dose of MAb 10D1 that is administered intravenously, as an infusion, at a dosage of 3.0 mg/Kg.

The objectives of this trial are to determine if i. administration of MAb 10D1 causes nonspecific T-cell activation, ii. to establish a safety/tolerability profile for MAb 10D1 in these patients and, iii. to determine the pharmacokinetic profile of MAb 10D1 and assess the development of a host immune response to MAb 10D1. In addition the study will attempt to identify preliminary evidence of efficacy. The study is a multicenter, open-label study of a single dose of MAb 10D1 in 14 subjects. The study consists of four phases: Screening, Infusion, Post-infusion, and Follow-up (*see* **Table 10** below).

Table 10

Phase	Screen	Infusion	Post-Infusion											Follow-up
Time	days -14 to 0	-30 to 130 min	145 min	160 min	190 min	250 min	370 min	24 hrs	48 hrs	72 hrs	day 7	day 14	day 21	day 28	monthly

Patients with histologic diagnosis of primary adenocarcinoma of the prostate, and progressive metastatic carcinoma of the prostate after androgen deprivation and at least one systemic non-hormonal manipulation, are being screened for participation in this study. Subjects must have progressive measurable disease, progressive PSA, PSA >5 ng/ml, testosterone <50 ng/dl, primary gonadal androgen suppression, life expectancy >12 weeks, and Karnofsky Performance Status ≥ 60%.

Subjects undergo physical examination, ECG, chest radiography, diagnostic imaging, and blood sampling for hematological, biochemical, and immune function assessments, and have vital signs monitored. Monthly telephone interviews are

used to collect and record information on a subset of adverse events, including autoimmune adverse events after disease progression, until six months after treatment. PSA (decline, duration of decline, progression, time to progression) and disease response (complete, partial, stable, progressive)are monitored. Plasma concentrations of MAb 10D1 are being assessed immediately prior to, during, and up to two months after, infusion.

Data from four prostate cancer subjects that have been treated are shown in **Table 11.** No adverse events have been recorded. For all of the subjects treated, MAb 10D1 appears to be well tolerated.

Because of the importance of monitoring the immune status of patients in the trial and the specific goal of monitoring generalized effects on T cell activation by anti-CTLA-4 antibody, the entry criteria in this study included minimum levels of CD4 and CD8 T cells of ≥ 500/ml and ≥ 500/ml respectively. However, it was observed during the initial accrual in the study that prostate cancer patients have significantly reduced T cell numbers although CD4 and CD8 T cells are clearly present. Many patients were initially rejected based on the above entry criteria (*see* **Table 11**). The apparent reduced T cell counts observed is a previously undocumented observation in prostate cancer patients that may have relevance in treatments involving cancer vaccination in these patients. Subsequent to these observations, the entry criteria were amended to include patients having CD4 and CD8 count of ≥ 300/ml and ≥ 200/ml respectively.

In order to evaluate whether administration of MAb 10D1 can induce undesirable non-specific T cell activation, peripheral blood lymphocytes from the prostate cancer subjects were analyzed by flow cytometry for each of the following markers: CD4, CD8, CD25, CD44, CD69 and HLA-DR. Blood samples were taken at time points indicated in **Table 10.** No significant change in the frequency of any of these markers was observed during the course of the treatment for each of the prostate cancer subjects treated thus far. An example of this analysis is shown in **Table 12** which shows the frequency of CD4, CD25,CD69-positive cells and CD8, CD25,CD69-positive cells at times prior to, during, and subsequent to MAb 10D1 administration in two of the subjects. These data demonstrate that MAb 10D1 does not result in non-specific T cell activation.

Table 12. Flow cytometric analysis of T cell activation markers in prostate cancer subjects treated with 3.0 mg/Kg MAb 10D1.

Patient Number	Time Point	CD(4+25+69) %	CD (8+25+69) %
3	Screen	1.7	0.8
3	-30MIN (Pre-Infusion)	2.6	0.8
3	40 MIN	2.5	0.7
3	130 MIN	1.9	0.9
3	145 MIN	1.7	0.5
3	160 MIN	1.7	1
3	190 MIN	1.5	1.5
3	250 MIN	2.1	1.2
3	370 MIN	1.3	0.9
3	24 HR	1.6	1.6
3	48 HR	2.7	3
3	72 HR	0.9	0.5
3	Day 7	0.9	0.1
3	Day 14	0.4	0.5
3	Day 21	2.3	1.9
4	Screen	1.4	0.8
4	-30 MIN (Pre-Infusion)	0.5	0.3
4	40 MIN	0.3	0.1
4	130 MIN	0.3	0.1
4	145 MIN	0.4	0.2
4	160 MIN	0.2	0.2
4	190 MIN	0.8	0.3
4	250 MIN	0.1	0
4	370 MIN	0.3	0.1
4	24 HR	0.2	0.3
4	48 HR	0.4	0.6
4	72 HR	0.8	0.3
4	Day 7	1	0.7
4	Day 14	1.1	0.8

A second clinical trial (MDXCTLA4-02) using MAb 10D1 in subjects with Stage IV malignant melanoma has also been initiated. A single dose of MAb 10D1 will be administered intravenously, as an infusion, at a dosage of 3.0 mg/Kg. This study also consists of four phases (Screening, Infusion, Post-Infusion and Follow-up) as described in **Table 9**, above.

The goals of this study are as those regarding the above-described study in prostate cancers as well as to specifically establish a safety/tolerability profile for MAb 10D1 in patients with Stage IV malignant melanoma. One patient has been treated in this study (see **Table 13**). As in the prostate cancer study, MAb 10D1 appears to be well

tolerated. Flow cytometric analysis of T cell activation markers in this subject, analogous to that performed for the prostate tumor trial,also showed no evidence of non-specific T cell activation.

AGGAGAATGAATAAATAAAGTGAATCTTTGCACCTGTGGTTTCTCTCTTTCCTCAATTTAATAATTATTATCTGT
TGTTTACCAACTACTCAATTTCTCTTATAAGGGACTAAATATGTAGTCATCCTAAGGCGCATAACCATTTATAAA
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CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC
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pCG7-96 (SEQ ID NO:41)

GAACTCGAGCAGCTGAAGCTTTCTGGGGCAGGCCAGGCCTGACCTTGGCTTTGGGGCAGGGAGGGGGCTAAGGTG
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pG4HE (SEQ ID NO:42)

GAACTCGAGCAGCTGAAGCTTTCTGGGGCAGGCCGGGCCTGACTTTGGCTGGGGGCAGGGAGGGGGCTAAGGTGA
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CATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGC
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CATAACCTTATGTATCATACACATACGATTTAGGTGACACTATA

10D1 VH

CAGGTGCAGC TGGTGGAGTC TGGGGGAGGC GTGGTCCAGC CTGGGAGGTC 50
CCTGAGACTC TCCTGTGCAG CCTCTGGATT CACCTTCAGT AGCTATACTA 100
TGCACTGGGT CCGCCAGGCT CCAGGCAAGG GGCTGGAGTG GGTGACATTT 150
ATATCATATG ATGGAAACAA TAAATACTAC GCAGACTCCG TGAAGGGCCG 200
ATTCACCATC TCCAGAGACA ATTCCAAGAA CACGCTGTAT CTGCAAATGA 250
ACAGCCTGAG AGCTGAGGAC ACGGCTATAT ATTACTGTGC GAGGACCGGC 300
TGGCTGGGGC CCTTTGACTA CTGGGGCCAG GGAACCCTGG TCACCGTCTC 350
CTCAG

10D1 VK

GAAATTGTGT TGACGCAGTC TCCAGGCACC CTGTCTTTGT CTCCAGGGGA 50
AAGAGCCACC CTCTCCTGCA GGGCCAGTCA GAGTGTTGGC AGCAGCTACT 100
TAGCCTGGTA CCAGCAGAAA CCTGGCCAGG CTCCCAGGCT CCTCATCTAT 150
GGTGCATTCA GCAGGGCCAC TGGCATCCCA GACAGGTTCA GTGGCAGTGG 200
GTCTGGGACA GACTTCACTC TCACCATCAG CAGACTGGAG CCTGAAGATT 250
TTGCAGTGTA TTACTGTCAG CAGTATGGTA GCTCACCGTG GACGTTCGGC 300
CAAGGGACCA AGGTGGAAAT CAAAC 325

4B6 VH

CAGGTGCAGC TGGTGGAGTC TGGGGGAGGC GTGGTCCAGC CTGGGAGGTC 50
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TGCACTGGGT CCGCCAGGCT CCAGGCAAGG GGCTGGAGTG GGTGACATTT 150
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TGGCTGGGGC CCTTTGACTA CTGGGGCCAG GGAACCCTGG TCACCGTCTC 350
CTCAG

4B6 VK

GAAATTGTGT TGACGCAGTC TCCAGGCACC CTGTCTTTGT CTCCAGGGGA 50
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TAGCCTGGTA CCAGCAGAAA CCTGGCCAGG CTCCCAGGCT CCTCATCTAT 150
GGTGCATCCA GCAGGGCCAC TGGCATCCCA GACAGGTTCA GTGGCAGTGG 200
GTCTGGGACA GACTTCACTC TCACCATCAG CAGACTGGAG CCTGAAGATT 250
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CAAGGGACCA AGGTGGAAAT CAAAC 325

1E2 VH

CAGGTGCAGC TGGTGGAGTC TGGGGGAGGC GTGGTCCAGC CTGGGAGGTC 50
CCTGAGACTC TCCTGTGCAG CGTCTGGATT CACCTTCAGT AGCTATGGCA 100
TGCACTGGGT CCGCCAGGCT CCAGGCAAGG GGCTGGAGTG GGTGGCAGTT 150

ATATGGTATG ATGGAAGTAA TAAATACTAT GCAGACTCCG TGAAGGGCCG 200
ATTCACCATC TCCAGAGACA ATTCCAAGAA CACGCTGTAT CTGCAAATGA 250
ACAGCCTGAG AGCCGAGGAC ACGGCTGTGT TTTACTGTGC GAGAGCTCCC 300
AATTATATTG GTGCTTTTGA TGTCTGGGGC CAAGGGACAA TGGTCACCGT 350
CTCTTCAG

1E2 VK

GACATCCAGA TGACCCAGTC TCCATCCTCA CTGTCTGCAT CTGTAGGAGA 50
CAGAGTCACC ATCACTTGTC GGGCGAGTCA GGGTATTAGC AGCTGGTTAG 100
CCTGGTATCA GCAGAAACCA GAGAAAGCCC CTAAGTCCCT GATCTATGCT 150
GCATCCAGTT TGCAAAGTGG GGTCCCATCA AGGTTCAGCG GCAGTGGATC 200
TGGGACAGAT TTCACTCTCA CCATCAGCAG CCTGCAGCCT GAAGATTTTG 250
CAACTTATTA CTGCCAACAG TATAATAGTT ACCCTCCGAC GTTCGGCCAA 300
GGGACCAAGG TGGAAATCAA AC 322

WHAT IS CLAIMED IS:

1. A human sequence antibody that specifically binds to human CTLA-4 ,wherein the antibody binds to human CTLA-4 with an equilibrium association constant (K_a) of at least $10^8 M^{-1}$.

2. A therapeutically-effective human sequence antibody that specifically binds to human CTLA-4.

3. The therapeutically-effective human sequence antibody of claim 2, wherein the antibody binds CTLA-4 on the cell surface of normal human T cells.

4. The therapeutically-effective human sequence antibody of claim 2, wherein the antibody is well-tolerated in a patient.

5. The therapeutically-effective human sequence antibody of claim 2, wherein the T cell subpopulations marked by CD antigens CD4, CD8, CD25, and CD69 remain stable during and subsequent to the administration of the antibody.

6. An isolated human sequence antibody that specifically binds to human CTLA-4 which is substantially free of non-immunoglobulin associated human proteins.

7. A composition of polyclonal antibodies comprising a plurality of antibodies according to claim 1.

8. The composition of polyclonal antibodies of claim 7 comprising at least about 10 different antibodies according to claim 1.

9. The composition of polyclonal antibodies of claim 7 comprising at least about 100 different antibodies according to claim 1.

10. The composition of polyclonal antibodies of claim 7 comprising at least about 1000 different antibodies according to claim 1.

11. The human sequence antibody of claim 1, wherein the human antibody blocks binding of human CTLA-4 to B7 ligands.

12. The human sequence antibody of claim 1, wherein the human sequence antibody does not block binding of human CTLA-4 to B7 ligands.

13. The human sequence antibody of claim 1, wherein the antibody binds to human CTLA-4 with an equilibrium association constant (K_a) of at least 10^9M^{-1}.

14. The human sequence antibody of claim 1, wherein the antibody blocks binding of human CTLA-4 to B7 ligands by at least about 20%.

15. The human sequence antibody of claim 1, wherein the antibody blocks binding of human CTLA-4 to B7 ligands by at least about 30%.

16. The human sequence antibody of claim 1, wherein the antibody blocks binding of human CTLA-4 to B7 ligands by at least about 40%.

17. The human sequence antibody of claim 1, wherein the antibody blocks binding of human CTLA-4 to B7 ligands by at least about 50%.

18. The human sequence antibody of claim 1, wherein the antibody heavy chain is IgG or IgM.

19. The human sequence antibody of claim 18, wherein the IgG antibody heavy chain is IgG_1, IgG_2, IgG_3 or IgG_4.

20. The human sequence antibody of claim 1, wherein the antibody light chain is a kappa light chain.

21. The human sequence antibody of claim 1, wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids that comprise nucleotide sequences in their variable regions as set forth in SEQ ID NO:16 and SEQ ID NO:6, respectively.

22. The human sequence antibody of claim 1, wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids that comprise nucleotide sequences in their variable regions as set forth in SEQ ID NO:18 and SEQ ID NO:8, respectively.

23. The human sequence antibody of claim 1, wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids that comprise nucleotide sequences in their variable regions as set forth in SEQ ID NO:22 and SEQ ID NO:12, respectively.

24. A human sequence antibody of claim 1, wherein the human sequence antibody is encoded by heavy chain and light chain variable region amino acid sequences as set for the in SEQ ID NO:17 and SEQ ID NO:7, respectively.

25. A human sequence antibody of claim 1, wherein the human sequence antibody is encoded by heavy chain and light chain variable region amino acid sequences as set for the in SEQ ID NO:19 and SEQ ID NO:9, respectively.

26. A human sequence antibody of claim 1, wherein the human sequence antibody is encoded by heavy chain and light chain variable region amino acid sequences as set for the in SEQ ID NO:23 and SEQ ID NO:13, respectively.

27. The human sequence antibody of claim 1, wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain

nucleic acids comprising variable heavy and light chain sequences from V gene segments VH 3-30.3 and VK A-27, respectively.

28. The human sequence antibody of claim 1, wherein the human sequence antibody is encoded by human IgG heavy chain and human kappa light chain nucleic acids comprising variable heavy and light chain sequences from V gene segments VH 3-33 and VK L-15, respectively.

29. The human sequence antibody of claim 1, comprising heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGNNKYYADSVKG (SEQ ID NO:32) and TGWLGPFDY (SEQ ID NO:37), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVGSSYLA (SEQ ID NO:24), GAFSRAT (SEQ ID NO:29), and QQYGSSPWT (SEQ ID NO:35), respectively.

30. The human sequence antibody of claim 1, comprising heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGSNKHYADSVKG (SEQ ID NO:33) and TGWLGPFDY (SEQ ID NO:38), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVSSSFLA (SEQ ID NO:25), GASSRAT (SEQ ID NO:30), and QQYGSSPWT (SEQ ID NO:35), respectively.

31. The human sequence antibody of claim 1, comprising heavy chain CDR1, CDR2, and CDR3 sequences, SYGMH (SEQ ID NO:28), VIWYDGSNKYYADSVKG (SEQ ID NO:34) and APNYIGAFDV (SEQ ID NO:39), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQGISSWLA (SEQ ID NO:26), AASSLQS (SEQ ID NO:31), and QQYNSYPPT (SEQ ID NO:36), respectively.

32. The human sequence antibody of claim 1, wherein said human sequence antibody is produced by a transgenic non-human animal.

33. The human sequence antibody of claim 32, wherein said transgenic non-human animal is a mouse.

34. The human sequence antibody of claim 1, wherein the human sequence antibody is a F_{ab} fragment.

35. A polyvalent complex comprising at least two human sequence antibodies each of which specifically binds to human CTLA-4.

36. The polyvalent complex of claim 35, wherein the two different antibodies are linked to each other covalently.

37. The polyvalent complex of claim 35, wherein the two different antibodies are linked to each other non-covalently.

38. A nucleic acid encoding a heavy chain of a human sequence antibody.

39. The nucleic acid of claim comprising the nucleotide sequence as set forth in SEQ ID NO:1.

40. A transgenic non-human animal having a genome comprising a human sequence heavy chain transgene and a human sequence light chain transgene, which animal has been immunized with a human CTLA-4, or a fragment or an analog thereof, whereby the animal expresses human sequence antibodies to the human CTLA-4.

41. The transgenic non-human animal of claim 40, wherein the transgenic non-human animal is a transgenic mouse.

42. The transgenic mouse of claim 41 comprising HCo7 or HCo12.

43. A cell line comprising a B cell obtained from a transgenic non-human animal having a genome comprising a human sequence heavy chain transgene and a human sequence light chain transgene, wherein the hybridoma produces a human sequence antibody that specifically binds to human CTLA-4.

44. The cell line of claim 43, wherein the cell line is a hybridoma.

45. A hybridoma secreting a human sequence antibody that specifically binds human CTLA-4 or binding fragment thereof, wherein the antibody is selected from the group consisting of:

a human sequence antibody comprising heavy chain heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGNNKYYADSVKG (SEQ ID NO:32) and TGWLGPFDY (SEQ ID NO:37), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVGSSYLA (SEQ ID NO:24), GAFSRAT (SEQ ID NO:29), and QQYGSSPWT (SEQ ID NO:35), respectively, and heavy chain and light chain variable region amino acid sequences as set forth in SEQ ID NO:17 and SEQ ID NO:7, respectively,

a human sequence antibody comprising heavy chain CDR1, CDR2, and CDR3 sequences, SYTMH (SEQ ID NO:27), FISYDGSNKHYADSVKG (SEQ ID NO:33) and TGWLGPFDY (SEQ ID NO:38), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQSVSSSFLA (SEQ ID NO:25), GASSRAT (SEQ ID NO:30), and QQYGSSPWT (SEQ ID NO:35), respectively, and heavy chain and light chain variable region amino acid sequences as set forth in SEQ ID NO:19 and SEQ ID NO:9, respectively, and

a human sequence antibody of claim 1, comprising heavy chain CDR1, CDR2, and CDR3 sequences, SYGMH (SEQ ID NO:28), VIWYDGSNKYYADSVKG (SEQ ID NO:34) and APNYIGAFDV (SEQ ID NO:39), respectively, and light chain CDR1, CDR2, and CDR3 sequences, RASQGISSWLA (SEQ ID NO:26), AASSLQS (SEQ ID NO:31), and QQYNSYPPT (SEQ ID NO:36), respectively, and heavy chain and light chain

variable region amino acid sequences as set forth in SEQ ID NO:23 and SEQ ID NO:13, respectively.

46. A pharmaceutical composition comprising a human sequence antibody of claim and a pharmaceutically acceptable carrier.

47. The pharmaceutical composition of claim 46, further comprising an agent effective to induce an immune response against a target antigen.

48. The pharmaceutical composition of claim 46, further comprising a chemotherapeutic agent.

49. The pharmaceutical composition of claim 46, further comprising an antibody to an immunosuppressive molecule.

50. Use of a pharmaceutical composition according to claim 46 for preparing a medicament for inducing, augmenting or prolonging an immune response to an antigen in a patient, wherein the pharmaceutical composition blocks binding of human CTLA-4 to human B7 ligands.

51. The use of claim 50, wherein the antigen is a tumor antigen or an antigen from a pathogen.

52. The use of claim 51, wherein the patient is also to be treated with a bispecific antibody, said bispecific antibody comprising an antibody sequence having an affinity for an antigen from a tumor or a pathogen.

53. The use of claim 51, wherein the pathogen is a virus, a bacterium, a fungus or a parasite.

54. The use of claim 53, wherein the pathogen is HIV.

55. The use of claim 50, wherein the patient is further to be treated by administering the antigen, or a fragment or an analog thereof, to the patient, whereby the antigen in combination with the human sequence antibody induces, augments or prolongs the immune response.

56. The use of claim 55, wherein the antigen is a tumor antigen or an antigen from a pathogen.

57. The use of claim 56, wherein the tumor antigen is telomerase.

58. The use of claim 55, wherein the antigen is a component of an amyloid formation in the patient.

59. The use of claim 55, wherein the patient is suffering from Alzheimer's disease and the antigen is AB peptide.

60. The use of claim 51, further comprising administering a cytokine to the patient.

61. Use of a polyvalent preparation comprising at least two human sequence antibodies to human CTLA-4 linked to each other for preparing a medicament for suppressing an immune response in a patient.

62. Use of a polyclonal preparation comprising at least two human sequence antibodies to human CTLA-4 for preparing a medicament for suppressing an immune response in a patient.

63. Use of a pharmaceutical composition of claim 47 for preparing a medicament for treating an autoimmune disease in a subject caused or exacerbated by increased activity of T cells.

64. Use of a pharmaceutical composition of claim 47 to the subject for preparing a medicament for treating cancer in a subject.

65. The use of claim 64, wherein the cancer is prostate cancer, melanoma, or epithelial cancer.

66. The use of claim 64, wherein the pharmaceutical composition further comprises an agent effective to induce an immune response against a target antigen.

67. The use of claim 66, wherein the vaccine is a tumor cell vaccine, a GM-CSF-modified tumor cell vaccine, or an antigen-loaded dendritic cell vaccine.