METHODS FOR TREATING RHEUMATIC DISEASES USING A SOLUBLE CTLA4 MOLECULE

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

FIELD OF THE INVENTION

The present invention relates generally to the field of rheumatic diseases. In particular, the invention relates to methods and compositions for treating rheumatic diseases, such as rheumatoid arthritis, by administering to a subject an effective amount of soluble CTLA4 mutant molecules.

BACKGROUND OF THE INVENTION

No cure currently exists for rheumatic diseases. Rather, therapeutic agents are used to treat the symptoms. Typically, the therapeutic agents are administered over long periods of time and the therapeutic value is often diminished by adverse side effects.

Rheumatic diseases encompass a group of diseases that affect the musculo-skeletal and connective tissues of the body. These diseases are characterized by chronic inflammation that often leads to permanent tissue damage, deformity, atrophy and disability. Rheumatic diseases affect the joints, bone, soft tissue, or spinal cord (Mathies, H. 1983 *Rheuma*) and are classified as inflammatory rheumatism, degenerative rheumatism, extra-

articular rheumatism, or collagen diseases. Some rheumatic diseases are known to be autoimmune diseases caused by a subject's altered immune response.

Rheumatoid arthritis is a progressive rheumatic disease, affecting approximately 2% of the adult population of developed countries (Utsinger, P. D., et al., 1985 *Rheumatoid Arthritis*, p. 140). This disease is characterized by persistent inflammatory synovitis that causes destruction of cartilage and bone erosion, leading to structural deformities in the peripheral joints. The symptoms associated with rheumatoid arthritis include joint swelling, joint tenderness, inflammation, morning stiffness, and pain, especially upon flexing. Subjects having advanced stages of arthritis suffer from structural damage, including joint destruction with bone erosion (in: "Principals of Internal Medicine, Harrison, 13th edition, pages 1648-1655). In addition, patients can present other clinical symptoms of various organic lesions, including lesions of the skin, kidney, heart, lung, central nervous system, and eyes due to vasculitis related to the autoimmune process.

Other symptoms that correlate with rheumatoid arthritis include elevated erythrocyte sedimentation rates, and elevated levels of serum C-reactive protein (CRP) and/or soluble IL-2 receptor (IL-2r). The erythrocyte sedimentation rate is increased in nearly all patients with active rheumatoid arthritis. The level of serum C-reactive protein is also elevated and correlates with disease activity and the likelihood of progressive joint damage. Additionally, the level of soluble IL-2r, a product of activated T-cells, is elevated in blood serum and synovial fluid of patients with active rheumatoid arthritis (see: "Principals of Internal Medicine, Harrison, 13th edition, page 1650).

Rheumatoid arthritis is believed to be a T-cell-mediated autoimmune disease, involving antigen-nonspecific intercellular interactions between T-lymphocytes and antigen-presenting cells. In general, the magnitude of the T-cell response is determined by the co-stimulatory response elicited by the interaction between T-cell surface molecules and their ligands (Mueller, et al., 1989 *Ann. Rev. Immunol.* 7:445-480). Key co-stimulatory signals are provided by the interaction between T-cell surface receptors, CD28 and CTLA4, and their ligands, such as B7-related molecules CD80 (i.e., B7-1) and CD86

(i.e., B7-2), on antigen presenting cells (Linsley, P. and Ledbetter, J. 1993 *Ann. Rev. Immunol.* 11:191-212).

T-cell activation in the absence of co-stimulation results in anergic T-cell response (Schwartz, R. H., 1992 *Cell* 71:1065-1068) wherein the immune system becomes nonresponsive to stimulation.

Since rheumatoid arthritis is thought to be a T-cell-mediated immune system disease, one strategy to develop new agents to treat rheumatoid arthritis is to identify molecules that block co-stimulatory signals between T-lymphocytes and antigen presenting cells, by blocking the interaction between endogenous CD28 or CTLA4 and B7. Potential molecules include soluble CTLA4 molecules that are modified to bind to B7 with higher avidity than wildtype CTLA4 (the sequence of which is shown in Figure 23) or CD28, thereby blocking the co-stimulatory signals.

Soluble forms of CD28 and CTLA4 have been constructed by fusing variable (V)-like extracellular domains of CD28 and CTLA4 to immunoglobulin (Ig) constant domains resulting in CD28Ig and CTLA4Ig. A nucleotide and amino acid sequence of CTLA4Ig is shown in Figure 24 with the protein beginning with methionine at position +1 or alanine at position –1 and ending with lysine at position +357. CTLA4Ig binds both CD80-positive and CD86-postive cells more strongly than CD28Ig (Linsley, P., et al., 1994 *Immunity* 1:793-80). Many T-cell-dependent immune responses have been found to be blocked by CTLA4Ig both *in vitro and in vivo*. (Linsley, P., et al., 1991b, *supra*; Linsley, P., et al., 1992a *Science* 257:792-795; Linsley, P., et al., 1992b *J. Exp. Med.* 176:1595-1604; Lenschow, D.J., et al. 1992 *Science* 257:789-792; Tan, P., et al., 1992 *J. Exp. Med.* 177:165-173; Turka, L.A., 1992 *Proc. Natl. Acad. Sci. USA* 89:11102-11105).

To alter binding affinity to natural ligands, such as B7, soluble CTLA4Ig fusion molecules were modified by mutation of amino acids in the CTLA4 portion of the molecules. Regions of CTLA4 that, when mutated, alter the binding affinity or avidity for B7 ligands include the complementarity determining region 1 (CDR-1 as described in

U.S. Patents 6,090,914, 5,773,253, 5,844,095; in copending U.S. Patent Application Serial Number 60/214,065; and by Peach et al, 1994. J. Exp. Med., 180:2049-2058) and complementarity determining region 3 (CDR-3)-like regions (CDR-3 is the conserved region of the CTLA4 extracellular domain as described in U.S. Patents U.S. Patents 6,090,914, 5,773,253 and 5,844,095; in copending U.S. Patent Application Serial Number 60/214,065; and by Peach, R.J., et al *J Exp Med* 1994 180:2049-2058. The CDR-3-like region encompasses the CDR-3 region and extends, by several amino acids, upstream and/or downstream of the CDR-3 motif). The CDR-3-like region includes a hexapeptide motif MYPPPY that is highly conserved in all CD28 and CTLA4 family members. Alanine scanning mutagenesis through the hexapeptide motif in CTLA4, and at selected residues in CD28Ig, reduced or abolished binding to CD80 (Peach, R.J., et al *J Exp Med* 1994 180:2049-2058).

Further modifications were made to soluble CTLA4Ig molecules by interchanging homologous regions of CTLA4 and CD28. These chimeric CTLA4/CD28 homologue mutant molecules identified the MYPPPY hexapeptide motif common to CTLA4 and CD28, as well as certain non-conserved amino acid residues in the CDR-1- and CDR-3-like regions of CTLA4, as regions responsible for increasing the binding avidity of CTLA4 with CD80 (Peach, R. J., *et al.*, 1994 *J Exp Med* 180:2049-2058).

Soluble CTLA4 molecules, such as CTLA4Ig, CTLA4 mutant molecules or chimeric CTLA4/CD28 homologue mutants as described *supra*, introduce a new group of therapeutic drugs to treat rheumatic diseases.

Present treatments for rheumatic diseases, such as rheumatoid arthritis, include administering nonspecific cytotoxic immunosuppressive drugs, such as methotrexate, cyclophosphamide, azathioprine, cyclosporin A, and tumor necrosis factor-alpha (TNFα) blockers or antagonists. These immunosuppressive drugs suppress the entire immune system of the subject, and long-term use increases the risk of infection. Moreover, these drugs merely slow down the progress of the rheumatoid arthritis, which resumes at an accelerated pace after the therapy is discontinued. Additionally, prolonged therapy with

these nonspecific drugs produces toxic side effects, including a tendency towards development of certain malignancies, kidney failure, bone marrow suppression, pulmonary fibrosis, malignancy, diabetes, and liver function disorders. These drugs also gradually cease being effective after about 2-5 years (Kelley's Textbook of Rheumatology, 6th Edition, pages 1001-1022).

Alternatively, therapeutic agents that are non-specific immunosuppressive and anti-inflammatory drugs have been used to obtain symptomatic relief. These drugs are dose-dependent and do not protect from disease progression. These drugs include steroid compounds, such as prednisone and methylprednisolone. Steroids also have significant toxic side effects associated with their long-term use. (Kelley's Textbook of Rheumatology, 6th Edition, pages 829-833).

Thus, current treatments for rheumatoid arthritis are of limited efficacy, involve significant toxic side effects, and cannot be used continuously for prolonged periods of time.

Accordingly, there exists a need for treatments that are effective and more potent for treating rheumatic diseases, such as rheumatoid arthritis, and avoids the disadvantages of conventional methods and agents, by targeting a pathophysiological mechanism of auto-immunity.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for treating immune system diseases, by administering to a subject soluble CTLA4 molecules, which bind to B7 molecules on B7-positive cells, thereby inhibiting endogenous B7 molecules from binding CTLA4 and/or CD28 on T-cells. Soluble CTLA4 molecules used in the methods of the invention include CTLA4Ig and soluble CTLA4 mutant molecule L104EA29YIg.

The present invention also provides methods for inhibiting T-cell function but not T-cell depletion in a human by contacting B7-positive cells in the human with a soluble CTLA4. Examples of soluble CTLA4 include CTLA4Ig and soluble CTLA4 mutant molecule such as L104EA29YIg.

The present invention also provides methods for treating (e.g. reducing symptoms) rheumatic diseases, such as rheumatoid arthritis, by administering to a subject diagnosed with rheumatoid arthritis, soluble CTLA4 molecules such as CTLA4Ig and/or soluble CTLA4 mutant molecule L104EA29YIg. The CTLA4 mutant molecule L104EA29YIg e.g. beginning with methionine at position +1 or alanine at position –1 and ending with lysine at position +357 as shown in Figure 19 is preferred for use in the methods of the invention.

The present invention also provides methods for reducing pathophysiological changes associated with rheumatic disease, such as structural damage, by administering to a subject diagnosed with rheumatoid arthritis, soluble CTLA4 molecules.

The present invention also provides a pharmaceutical composition for treating immune system diseases, such as rheumatic diseases, comprising a pharmaceutically acceptable carrier and a biologically effective agent such as soluble CTLA4 molecules.

Kits comprising pharmaceutical compositions therapeutic for immune system disease are also encompassed by the invention. In one embodiment, a kit comprising one or more of the pharmaceutical compositions of the invention is used to treat an immune system disease e.g. rheumatoid arthritis. For example, the pharmaceutical composition comprises an effective amount of soluble CTLA4 mutant molecules that bind to B7 molecules on B7-positive cells, thereby blocking the B7 molecules from binding CTLA4 and/or CD28 on T-cells. Further, the kit may contain one or more immunosuppressive agents used in conjunction with the pharmaceutical compositions of the invention. Potential immunosuppressive agents include, but are not limited to, corticosteroids, nonsteroidal antiinflammatory drugs (e.g. Cox-2 inhibitors), cyclosporin prednisone, azathioprine,

methotrexate, TNFα blockers or antagonists, infliximab, any biological agent targeting an inflammatory cytokine, hydroxychloroquine, sulphasalazopryine, gold salts, etanercept, and anakinra.

The present invention also provides methods for reducing the erythrocyte sedimentation rate that is associated with rheumatoid arthritis.

Additionally, the present invention provides methods for reducing the levels of certain components of blood serum which are associated with rheumatoid arthritis, including C-reactive protein, soluble ICAM-1, soluble E-selectin and/or soluble IL-2r.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A: Demographic data of patient cohorts. Demographic data including gender, race, and disease duration as described in Example 3, *infra*.

Figure 1B: Demographic data of patient cohorts. Demographic data including gender, age, weight, and disease activity evaluated by the patient and by the physician as described in Example 3, *infra*.

Figure 1C: Demographic data of patient cohorts as described in Example 3, *infra*. Demographic data including disease activity, erythrocyte sedimentation rate (ESR), physical function (disability evaluated by health questionnaire), and C-reactive protein (CRP).

Figure 1D: Demographic data of patient cohorts as described in Example 3, *infra*. Demographic data including joint swelling, joint tenderness, morning stiffness, and pain.

Figure 1E: Demographic data of patient cohorts as described in Example 3, *infra*. Demographic data including prior treatments.

Figure 2: Summary of discontinuations at day 85 by reason as described in Example 3, *infra*.

Figure 3A: ACR responses at Day 85 as described in Example 3, *infra*: ACR-20, -50, and –70 responses.

Figure 3B: ACR-20 responses at Day 85, including placebo response, as described in Example 3, *infra*: ACR-20 response with 95% confidence limits.

Figure 3C: ACR-20 responses at Day 85 as described in Example 3, *infra*: Difference in ACR-20 response with respect to 95% confidence intervals.

Figure 4A: Basic (20% improvement) clinical responses in swollen and tender joint count in percentage of patients at Day 85 as described in Example 3, *infra*: basic clinical response, ACR-20.

Figure 4B: Clinical responses (in percentage improvement) in swollen and tender joint count in percentage of patients at Day 85 as described in Example 3, *infra*: change in clinical response in percentage improvement.

Figure 5A: Pain response (by Likert scale by mean unit change from baseline) in percentage of patients at Day 85 as described in Example 3, *infra*: pain score changes from baseline.

Figure 5B: Patient global disease changes (by Likert scale by mean unit change from baseline) in percentage of patients at Day 85 as described in Example 3, *infra*: patient global disease activity changes.

Figure 5C: Physician global disease changes (by Likert scale by mean unit change from baseline) in percentage of patients at Day 85 as described in Example 3, *infra*: physician global disease activity changes.

Figure 5D: Pain (by Likert scale by mean unit change from baseline) in percentage of patients at Day 85 as described in Example 3, *infra*: pain changes from baseline.

Figure 6A: Patient global assessment of disease activity change from baseline by range of 2 units at Day 85 as described in Example 3, *infra*; disease activity improvement..

Figure 6B: Physician global assessment of disease activity change from baseline by range of 2 units at Day 85 as described in Example 3, *infra*; disease activity improvement.

Figure 7A: Percent reduction in C-reactive protein (CRP) levels at Day 85 as described in Example 3, *infra*: percentage reduction in CRP levels from baseline.

Figure 7B: Difference in reduction in C-reactive protein (CRP) levels at Day 85 as described in Example 3, *infra*: percent reduction difference in CRP levels with 95% confidence intervals.

Figure 7C: Mean reduction in C-reactive protein (CRP) levels at Day 85 as described in Example 3, *infra*: mean change from baseline.

Figure 8: Reduction in soluble IL-2 receptor levels mean change from baseline at Day 85 as described in Example 3, *infra*.

Figure 9A: The effect of CTLA4Ig on tender joints over time as described in Example 3, *infra*: median difference from baseline.

Figure 9B: The effect of CTLA4Ig on tender joints over time as described in Example 3, *infra*: mean difference from baseline.

Figure 10A: The effect of CTLA4Ig on swollen joints over time as described in Example 3, *infra*: median difference from baseline.

Figure 10B: The effect of CTLA4Ig on swollen joints over time as described in Example 3, *infra*: mean difference from baseline.

Figure 11: The effect of CTLA4Ig on pain assessment mean difference from baseline over time as described in Example 3, *infra*.

Figure 12A: The effect of CTLA4Ig on patient assessment of disease activity mean difference from baseline over time as described in Example 3, *infra*.

Figure 12B: The effect of CTLA4Ig on physician assessment of disease activity mean difference from baseline over time as described in Example 3, *infra*.

Figure 13A: The effect of L104EA29YIg on tender joints over time as described in Example 3, *infra*: median difference from baseline.

Figure 13B: The effect of L104EA29YIg on tender joints over time as described in Example 3, *infra*: mean change from baseline.

Figure 14A: The effect of L104EA29YIg on swollen joints over time as described in Example 3, *infra*: median difference from baseline.

Figure 14B: The effect of L104EA29YIg on swollen joints over time as described in Example 3, *infra*: mean change from baseline.

Figure 15: The effect of L104EA29YIg on pain assessment over time as described in Example 3, *infra*: mean change from baseline over time.

Figure 16A: The effect of L104EA29YIg on patient assessment of disease activity mean difference from baseline over time as described in Example 3, *infra*.

Figure 16B: The effect of L104EA29YIg on physician assessment of disease activity mean difference from baseline over time as described in Example 3, *infra*.

Figure 17: Percent improvement in patient disability assessed by Health Assessment Questionnaire (HAQ) compared to the baseline at Day 85 with CTLA4Ig and L104EA29YIg treatment as described in Example 3, *infra*.

Figure 18: Nucleotide and amino acid sequence of L104EIg as described in Example 1, *infra*.

Figure 19: Nucleotide and amino acid sequence of L104EA29YIg as described in Example 1, *infra*.

Figure 20: Nucleotide and amino acid sequence of L104EA29LIg as described in Example 1, *infra*.

Figure 21: Nucleotide and amino acid sequence of L104EA29TIg as described in Example 1, *infra*.

Figure 22: Nucleotide and amino acid sequence of L104EA29WIg as described in Example 1, *infra*.

Figure 23: Nucleotide and amino acid sequence of CTLA4 receptor.

Figure 24: Nucleotide and amino acid sequence of CTLA4Ig.

Figure 25: SDS gel (FIG. 25A) for CTLA4Ig (lane 1), L104EIg (lane 2), and L104EA29YIg (lane 3A); and size exclusion chromatographs of CTLA4Ig (FIG. 25B) and L104EA29YIg (FIG. 25C).

Figures 26 (left and right depictions): A ribbon diagram of the CTLA4 extracellular Ig V-like fold generated from the solution structure determined by NMR spectroscopy. FIG. 26 (right depiction) shows an expanded view of the CDR-1 (S25-R33) region and the MYPPPY region indicating the location and side-chain orientation of the avidity enhancing mutations, L104 and A29.

Figures 27A & 27B: FACS assays showing binding of L104EA29YIg, L104EIg, and CTLA4Ig to human CD80- or CD86-transfected CHO cells as described in Example 2, *infra*.

Figures 28A & 28B: Graphs showing inhibition of proliferation of CD80-positive and CD86-positive CHO cells as described in Example 2, *infra*.

Figures 29A & 29B: Graphs showing that L104EA29YIg is more effective than CTLA4Ig at inhibiting proliferation of primary and secondary allostimulated T cells as described in Example 2, *infra*.

Figures 30A-C: Graphs illustrating that L104EA29YIg is more effective than CTLA4Ig at inhibiting IL-2 (FIG. 30A), IL-4 (FIG. 30B), and gamma (γ)-interferon (FIG. 30C) cytokine production of allostimulated human T cells as described in Example 2, *infra*.

Figure 31: A graph demonstrating that L104EA29YIg is more effective than CTLA4Ig at inhibiting proliferation of phytohemaglutinin- (PHA) stimulated monkey T cells as described in Example 2, *infra*.

Figure 32: A graph showing the equilibrium binding analysis of L104EA29YIg, L104EIg, and wild-type CTLA4Ig to CD86Ig.

Figures 33A & B: Reduction in soluble ICAM-1 and soluble E-selectin levels mean change from baseline at Day 85 as described in Example 3, *infra*.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in this application, the following words or phrases have the meanings specified.

As used herein, "ligand" refers to a molecule that specifically recognizes and binds another molecule, for example, a ligand for CTLA4 is a B7 molecule.

As used herein "wild type CTLA4" or "non-mutated CTLA4" has the amino acid sequence of naturally occurring, full length CTLA4 as shown in Figure 23 (also as described U.S. Patent Nos. 5,434,131, 5,844,095, 5,851,795), or any portion or derivative thereof, that recognizes and binds a B7 or interferes with a B7 so that it blocks binding to CD28 and/or CTLA4 (e.g., endogenous CD28 and/or CTLA4). In particular embodiments, the extracellular domain of wild type CTLA4 begins with methionine at position +1 and ends at aspartic acid at position +124, or the extracellular domain of wild type CTLA4 begins with alanine at position -1 and ends at aspartic acid at position +124. Wild type CTLA4 is a cell surface protein, having an N-terminal extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic domain. The extracellular domain binds to target molecules, such as a B7 molecule. In a cell, the naturally occurring, wild type CTLA4 protein is translated as an immature polypeptide, which includes a signal peptide at the N-terminal end. The immature polypeptide undergoes post-translational processing, which includes cleavage and removal of the signal peptide to generate a CTLA4 cleavage product having a newly generated N-terminal end that differs from the N-terminal end in the immature form. One skilled in the art will appreciate that additional post-translational processing may occur, which removes one or more of the amino acids from the newly generated N-terminal end of the CTLA4 cleavage product. Alternatively, the signal peptide may not be removed completely, generating molecules

that begin before the common starting amino acid methionine. Thus, the mature CTLA4 protein may start at methionine at position +1 or alanine at position –1. The mature form of the CTLA4 molecule includes the extracellular domain or any portion thereof, which binds to B7.

As used herein, a "CTLA4 mutant molecule" means wildtype CTLA4 as shown in Figure 23 or any portion or derivative thereof, that has a mutation or multiple mutations (preferably in the extracellular domain of wildtype CTLA4). A CTLA4 mutant molecule has a sequence that it is similar but not identical to the sequence of wild type CTLA4 molecule, but still binds a B7. The mutations may include one or more amino acid residues substituted with an amino acid having conservative (e.g., substitute a leucine with an isoleucine) or non-conservative (e.g., substitute a glycine with a tryptophan) structure or chemical properties, amino acid deletions, additions, frameshifts, or truncations. CTLA4 mutant molecules may include a non-CTLA4 molecule therein or attached thereto. The mutant molecules may be soluble (i.e., circulating) or bound to a cell surface. Additional CTLA4 mutant molecules include those described in U.S. Patent Application Serial Numbers 09/865,321, 60/214,065 and 60/287,576; in U.S. Patent Numbers 6,090,914 5,844,095 and 5,773,253; and as described by Peach, R. J., *et al.*, in *J Exp Med* 180:2049-2058 (1994)). CTLA4 mutant molecules can be made synthetically or recombinantly.

"CTLA4Ig" is a soluble fusion protein comprising an extracellular domain of wildtype CTLA4 joined to an Ig tail, or a portion thereof that binds a B7. A particular embodiment comprises the extracellular domain of wild type CTLA4 (as shown in Figure 23) starting at methionine at position +1 and ending at aspartic acid at position +124; or starting at alanine at position -1 to aspartic acid at position +124; a junction amino acid residue glutamine at position +125; and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357 (DNA encoding CTLA4Ig was deposited on May 31, 1991 with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 under the provisions of the Budapest Treaty, and has been accorded ATCC accession number ATCC 68629; Linsley, P., et al., 1994 *Immunity* 1:793-80). CTLA4Ig-24, a Chinese Hamster Ovary (CHO) cell line

expressing CTLA4Ig was deposited on May 31, 1991 with ATCC identification number CRL-10762). The soluble CTLA4Ig molecules used in the methods and/or kits of the invention may or may not include a signal (leader) peptide sequence. Typically, in the methods and/or kits of the invention, the molecules do not include a signal peptide sequence.

"L104EA29YIg" is a fusion protein that is a soluble CTLA4 mutant molecule comprising an extracellular domain of wildtype CTLA4 with amino acid changes A29Y (a tyrosine amino acid residue substituting for an alanine at position 29) and L104E (a glutamic acid amino acid residue substituting for a leucine at position +104), or a portion thereof that binds a B7 molecule, joined to an Ig tail (included in Figure 19; DNA encoding L104EA29YIg was deposited on June 20, 2000 with ATCC number PTA-2104; copending in U.S. Patent Application Serial Numbers 09/579,927, 60/287,576 and 60/214,065, incorporated by reference herein). The soluble L104EA29YIg molecules used in the methods and/or kits of the invention may or may not include a signal (leader) peptide sequence. Typically, in the methods and/or kits of the invention, the molecules do not include a signal peptide sequence.

As used herein, "soluble" refers to any molecule, or fragments and derivatives thereof, not bound or attached to a cell, i.e., circulating. For example, CTLA4, B7 or CD28 can be made soluble by attaching an immunoglobulin (Ig) moiety to the extracellular domain of CTLA4, B7 or CD28, respectively. Alternatively, a molecule such as CTLA4 can be rendered soluble by removing its transmembrane domain. Typically, the soluble molecules used in the methods of the invention do not include a signal (or leader) sequence.

As used herein, "soluble CTLA4 molecules" means non-cell-surface-bound (i.e. circulating) CTLA4 molecules or any functional portion of a CTLA4 molecule that binds B7 including, but not limited to: CTLA4Ig fusion proteins (e.g. ATCC 68629), wherein the extracellular domain of CTLA4 is fused to an immunoglobulin (Ig) moiety rendering the fusion molecule soluble, or fragments and derivatives thereof; proteins with the

extracellular domain of CTLA4 fused or joined with a portion of a biologically active or chemically active protein such as the papillomavirus E7 gene product (CTLA4-E7), melanoma-associated antigen p97 (CTLA4-p97) or HIV env protein (CTLA4-env gp120), or fragments and derivatives thereof; hybrid (chimeric) fusion proteins such as CD28/CTLA4Ig, or fragments and derivatives thereof; CTLA4 molecules with the transmembrane domain removed to render the protein soluble (Oaks, M. K., et al., 2000 *Cellular Immunology* 201:144-153), or fragments and derivatives thereof. "Soluble CTLA4 molecules" also include fragments, portions or derivatives thereof, and soluble CTLA4 mutant molecules, having CTLA4 binding activity. The soluble CTLA4 molecules used in the methods of the invention may or may not include a signal (leader) peptide sequence. Typically, in the methods of the invention, the molecules do not include a signal peptide sequence.

As used herein "the extracellular domain of CTLA4" is the portion of CTLA4 that recognizes and binds CTLA4 ligands, such as B7 molecules. For example, an extracellular domain of CTLA4 comprises methionine at position +1 to aspartic acid at position +124 (Figure 23). Alternatively, an extracellular domain of CTLA4 comprises alanine at position –1 to aspartic acid at position +124 (Figure 23). The extracellular domain includes fragments or derivatives of CTLA4 that bind a B7 molecule. The extracellular domain of CTLA4 as shown in Figure 23 may also include mutations that change the binding avidity of the CTLA4 molecule for a B7 molecule.

As used herein, the term "mutation" means a change in the nucleotide or amino acid sequence of a wildtype molecule, for example, a change in the DNA and/or amino acid sequences of the wild-type CTLA4 extracellular domain. A mutation in DNA may change a codon leading to a change in the amino acid sequence. A DNA change may include substitutions, deletions, insertions, alternative splicing, or truncations. An amino acid change may include substitutions, deletions, insertions, additions, truncations, or processing or cleavage errors of the protein. Alternatively, mutations in a nucleotide sequence may result in a silent mutation in the amino acid sequence as is well understood in the art. In that regard, certain nucleotide codons encode the same amino acid.

Examples include nucleotide codons CGU, CGG, CGC, and CGA encoding the amino acid, arginine (R); or codons GAU, and GAC encoding the amino acid, aspartic acid (D). Thus, a protein can be encoded by one or more nucleic acid molecules that differ in their specific nucleotide sequence, but still encode protein molecules having identical sequences. The amino acid coding sequence is as follows:

Amino Acid	Symbol	One Letter Symbol	Codons
Alanine	Ala	A	GCU, GCC, GCA, GCG
Cysteine	Cys	C	UGU, UGC
Aspartic Acid	Asp	D	GAU, GAC
Glutamic Acid	Glu	E	GAA, GAG
Phenylalanine	Phe	F	UUU, UUC
Glycine	Gly	G	GGU, GGC, GGA, GGG
Histidine	His	H	CAU, CAC
Isoleucine	Ile	I	AUU, AUC, AUA
Lysine	Lys	K	AAA, AAG
Leucine	Leu	L	UUA, UUG, CUU, CUC, CUA, CUG
Methionine	Met	M	AUG
Asparagine	Asn	N	AAU, AAC
Proline	Pro	P	CCU, CCC, CCA, CCG
Glutamine	Gln	Q	CAA, CAG
Arginine	Arg	R	CGU, CGC, CGA, CGG, AGA, AGG
Serine	Ser	S	UCU, UCC, UCA, UCG, AGU, AGC
Threonine	Thr	T	ACU, ACC, ACA, ACG
Valine	Val	V	GUU, GUC, GUA, GUG
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAU, UAC

The mutant molecule may have one or more mutations.

As used herein, a "non-CTLA4 protein sequence" or "non-CTLA4 molecule" means any protein molecule that does not bind B7 and does not interfere with the binding of CTLA4 to its target. An example includes, but is not limited to, an immunoglobulin (Ig) constant region or portion thereof. Preferably, the Ig constant region is a human or monkey Ig constant region, e.g., human C(gamma)1, including the hinge, CH2 and CH3 regions. The Ig constant region can be mutated to reduce its effector functions (U.S. Patents 5,637,481, 5,844,095 and 5,434,131).

As used herein, a "fragment" or "portion" is any part or segment of a CTLA4 molecule, preferably the extracellular domain of CTLA4 or a part or segment thereof, that recognizes and binds its target, e.g., a B7 molecule.

As used herein, "B7" refers to the B7 family of molecules including, but not limited to, B7-1 (CD80), B7-2 (CD86) and B7-3 that may recognize and bind CTLA4 and/or CD28.

As used herein, "B7-positive cells" are any cells with one or more types of B7 molecules expressed on the cell surface.

As used herein, a "derivative" is a molecule that shares sequence homology and activity of its parent molecule. For example, a derivative of CTLA4 includes a soluble CTLA4 molecule having an amino acid sequence at least 70% similar to the extracellular domain of wildtype CTLA4, and which recognizes and binds B7 e.g. CTLA4Ig or soluble CTLA4 mutant molecule L104EA29YIg.

As used herein, to "block" or "inhibit" a receptor, signal or molecule means to interfere with the activation of the receptor, signal or molecule, as detected by an art-recognized test. For example, blockage of a cell-mediated immune response can be detected by determining reduction of Rheumatic Disease associated symptoms. Blockage or inhibition may be partial or total.

As used herein, "blocking B7 interaction" means to interfere with the binding of B7 to its ligands, such as CD28 and/or CTLA4, thereby obstructing T-cell and B7-positive cell interactions. Examples of agents that block B7 interactions include, but are not limited to, molecules such as an antibody (or portion or derivative thereof) that recognizes and binds to the any of CTLA4, CD28 or B7 molecules (e.g. B7-1, B7-2); a soluble form (or portion or derivative thereof) of the molecules such as soluble CTLA4; a peptide fragment or other small molecule designed to interfere with the cell signal through the CTLA4/CD28/B7-mediated interaction. In a preferred embodiment, the blocking agent is a soluble CTLA4 molecule, such as CTLA4Ig (ATCC 68629) or L104EA29YIg (ATCC PTA-2104), a soluble CD28 molecule such as CD28Ig (ATCC 68628), a soluble B7 molecule such as B7Ig (ATCC 68627), an anti-B7 monoclonal antibody (e.g. ATCC HB-253, ATCC CRL-2223, ATCC CRL-2226, ATCC HB-301, ATCC HB-11341 and monoclonal antibodies as described in by Anderson et al in U.S. Patent 6,113,898 or Yokochi et al., 1982. J. Immun., 128(2)823-827), an anti-CTLA4 monoclonal antibody (e.g. ATCC HB-304, and monoclonal antibodies as described in references 82-83) and/or an anti-CD28 monoclonal antibody (e.g. ATCC HB 11944 and mAb 9.3 as described by Hansen (Hansen et al., 1980. Immunogenetics 10: 247-260) or Martin (Martin et al., 1984. J. Clin. Immun., 4(1):18-22)).

As used herein, "immune system disease" means any disease mediated by T-cell interactions with B7-positive cells including, but not limited to, autoimmune diseases, graft related disorders and immunoproliferative diseases. Examples of immune system diseases include graft versus host disease (GVHD) (e.g., such as may result from bone marrow transplantation, or in the induction of tolerance), immune disorders associated with graft transplantation rejection, chronic rejection, and tissue or cell allo- or xenografts, including solid organs, skin, islets, muscles, hepatocytes, neurons. Examples of immunoproliferative diseases include, but are not limited to, psoriasis, T-cell lymphoma, T-cell acute lymphoblastic leukemia, testicular angiocentric T-cell lymphoma, benign lymphocytic angiitis, lupus (e.g. lupus erythematosus, lupus nephritis), Hashimoto's thyroiditis, primary myxedema, Graves' disease, pernicious anemia, autoimmune atrophic gastritis, Addison's disease, diabetes (e.g. insulin dependent diabetes mellitis, type I diabetes

mellitis, type II diabetes mellitis), good pasture's syndrome, myasthenia gravis, pemphigus, Crohn's disease, sympathetic ophthalmia, autoimmune uveitis, multiple sclerosis, autoimmune hemolytic anemia, idiopathic thrombocytopenia, primary biliary cirrhosis, chronic action hepatitis, ulceratis colitis, Sjogren's syndrome, rheumatic diseases (e.g. rheumatoid arthritis), polymyositis, scleroderma, and mixed connective tissue disease.

As used herein, "rheumatic diseases" means any disease that affects the joints, bone, soft tissue, or spinal cord (Mathies, H. 1983 *Rheuma*) and comprises inflammatory rheumatism, degenerative rheumatism, extra-articular rheumatism, and collagen diseases. Additionally, rheumatic diseases include, but are not limited to, chronic polyarthritis, psoriasis arthropathica, ankylosing spondylitis, rheumatoid arthritis, panarteriitis nodosa, systemic lupus erythematosus, progressive systemic scleroderma, periarthritis humeroscapularis, arthritis uratica, chondorcalcinosis, dermatomyositis, muscular rheumatism, myositis, and myogelosis. Some rheumatic diseases are known to be autoimmune diseases caused by a subject's altered immune response.

As used herein, "gene therapy" is a process to treat a disease by genetic manipulation so that a sequence of nucleic acid is transferred into a cell, the cell then expressing any genetic product encoded by the nucleic acid. For example, as is well known by those skilled in the art, nucleic acid transfer may be performed by inserting an expression vector containing the nucleic acid of interest into cells *ex vivo* or *in vitro* by a variety of methods including, for example, calcium phosphate precipitation, diethyaminoethyl dextran, polyethylene glycol (PEG), electroporation, direct injection, lipofection or viral infection (Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press 1989); Kriegler M. *Gene Transfer ad Expression: A Laboratory Manual* (W. H. Freeman and Co, New York, N.Y., 1993) and Wu, *Methods in Enzymology* (Academic Press, New York, 1993), each of which is incorporated herein by reference). Alternatively, nucleic acid sequences of interest may be transferred into a cell *in vivo* in a variety of vectors and by a variety of methods including, for example, direct administration of the nucleic acid into a subject (Williams *et al*, 1991 *PNAS* 88:2726-2730), or insertion of the nucleic acid into a viral vector and infection of the subject with

the virus (Battleman *et al*, 1993 *J Neurosci* 13:94-951; Carroll *et al*, 1993 *J Cell Biochem* 17E:241; Lebkowski *et al*, U.S. Patent 5,354,678; Davison and Elliott, Molecular Virology: A Practical Approach (IRL Press, New York, 1993)). Other methods used for *in vivo* transfer include encapsulation of the nucleic acid into liposomes, and direct transfer of the liposomes, or liposomes combined with a hemagglutinating Sendai virus, to a subject (U.S. Patent 5,824,655, incorporated by reference herein). The transfected or infected cells express the protein products encoded by the nucleic acid in order to ameliorate a disease or the symptoms of a disease.

In order that the invention herein described may be more fully understood the following description is set forth.

COMPOSITIONS AND METHODS OF THE INVENTION

The present invention provides compositions and methods for treating immune system diseases, such as rheumatic diseases, by administering to a subject an effective amount of a ligand that binds B7 for example, soluble CTLA4 molecules (such as CTLA4Ig and/or L104EA29YIg) and mAbs that recognize and bind B7. An effective amount is defined as the amount of soluble CTLA4 molecules that, when bound to B7 molecules on B7-positive cells, inhibit B7 molecules from binding endogenous ligands such as CTLA4 and CD28.

In a preferred embodiment, the immune disease is a rheumatic disease. Rheumatic diseases are any diseases which are characterized by (i) inflammation or degeneration of musculo-skeletal or connective tissue structures of the body, particularly the joints, and including muscles, tendons, cartilage, synovial and fibrous tissues, (ii) accompanied by joint swelling, joint tenderness, inflammation, morning stiffness, and/or pain, or impairment of locomotion or function of those structures and, in some cases, (iii) often accompanied by serological evidence of rheumatoid factor and other inflammatory surrogate markers.

Rheumatic diseases include, but are not limited to, rheumatoid arthritis. The symptoms of rheumatoid arthritis include joint swelling, joint tenderness, inflammation, morning stiffness, and pain leading to physical disability. Subjects afflicted with the advanced stages of arthritis suffer from symptoms of structural damage and debilitating pain. Other organs also can be impaired by the autoimmune mechanism.

Compositions

The present invention provides compositions for treating immune diseases, such as rheumatic diseases, comprising soluble CTLA4 molecules. Further, the present invention provides compositions comprising a biological agent that inhibits T-cell function but not T-cell depletion in a human by contacting B7-positive cells in the human with a soluble CTLA4. Examples of soluble CTLA4 include CTLA4Ig and soluble CTLA4 mutant molecule e.g. L104EA29YIg.

CTLA4 molecules, with mutant or wildtype sequences, may be rendered soluble by deleting the CTLA4 transmembrane segment (Oaks, M. K., et al., 2000 *Cellular Immunology* 201:144-153).

Alternatively, soluble CTLA4 molecules, with mutant or wildtype sequences, may be fusion proteins, wherein the CTLA4 molecules are fused to non-CTLA4 moieties such as immunoglobulin (Ig) molecules that render the CTLA4 molecules soluble. For example, a CTLA4 fusion protein may include the extracellular domain of CTLA4 fused to an immunoglobulin constant domain, resulting in the CTLA4Ig molecule (Figure 24) (Linsley, P. S., et al., 1994 *Immunity* 1:793-80).

For clinical protocols, it is preferred that the immunoglobulin region does not elicit a detrimental immune response in a subject. The preferred moiety is the immunoglobulin constant region, including the human or monkey immunoglobulin constant regions. One example of a suitable immunoglobulin region is human Cγ1, including the hinge, CH2

and CH3 regions which can mediate effector functions such as binding to Fc receptors, mediating complement-dependent cytotoxicity (CDC), or mediate antibody-dependent cell-mediated cytotoxicity (ADCC). The immunoglobulin moiety may have one or more mutations therein, (e.g., in the CH2 domain, to reduce effector functions such as CDC or ADCC) where the mutation modulates the binding capability of the immunoglobulin to its ligand, by increasing or decreasing the binding capability of the immunoglobulin to Fc receptors. For example, mutations in the immunoglobulin may include changes in any or all its cysteine residues within the hinge domain, for example, the cysteines at positions +130, +136, and +139 are substituted with serine (Figure 24). The immunoglobulin molecule may also include the proline at position +148 substituted with a serine, as shown in Figure 24. Further, the mutations in the immunoglobulin moiety may include having the leucine at position +144 substituted with phenylalanine, leucine at position +145 substituted with glutamic acid, or glycine at position +147 substituted with alanine.

Additional non-CTLA4 moieties for use in the soluble CTLA4 molecules or soluble CTLA4 mutant molecules include, but are not limited to, p97 molecule, env gp120 molecule, E7 molecule, and ova molecule (Dash, B. et al. 1994 *J. Gen. Virol.* 75 (Pt 6):1389-97; Ikeda, T., et al. 1994 *Gene* 138(1-2):193-6; Falk, K., et al. 1993 *Cell. Immunol.* 150(2):447-52; Fujisaka, K. et al. 1994 *Virology* 204(2):789-93). Other molecules are also possible (Gerard, C. et al. 1994 *Neuroscience* 62(3):721; Byrn, R. et al. 1989 63(10):4370; Smith, D. et al. 1987 *Science* 238:1704; Lasky, L. 1996 *Science* 233:209).

The soluble CTLA4 molecule of the invention can include a signal peptide sequence linked to the N-terminal end of the extracellular domain of the CTLA4 portion of the molecule. The signal peptide can be any sequence that will permit secretion of the molecule, including the signal peptide from oncostatin M (Malik, et al., (1989) Molec. Cell. Biol. 9: 2847-2853), or CD5 (Jones, N. H. et al., (1986) Nature 323:346-349), or the signal peptide from any extracellular protein.

The soluble CTLA4 molecule of the invention can include the oncostatin M signal peptide linked at the N-terminal end of the extracellular domain of CTLA4, and the human immunoglobulin molecule (e.g., hinge, CH2 and CH3) linked to the C-terminal end of the extracellular domain (wildtype or mutated) of CTLA4. This molecule includes the oncostatin M signal peptide encompassing an amino acid sequence having methionine at position –26 through alanine at position –1, the CTLA4 portion encompassing an amino acid sequence having methionine at position +1 through aspartic acid at position +124, a junction amino acid residue glutamine at position +125, and the immunoglobulin portion encompassing an amino acid sequence having glutamic acid at position +126 through lysine at position +357.

Specifically, the soluble CTLA4 mutant molecules of the invention, comprising the mutated CTLA4 sequences described *infra*, are fusion molecules comprising human IgCγ1 moieties fused to the mutated CTLA4 fragments.

In one embodiment, the soluble CTLA4 mutant molecules comprise IgCγ1 fused to a CTLA4 fragment comprising a single-site mutation in the extracellular domain. The extracellular domain of CTLA4 comprises methionine at position +1 through aspartic acid at position +124 (e.g., Figure 23). The extracellular portion of the CTLA4 can comprise alanine at position –1 through aspartic acid at position +124 (e.g., Figure 23). Examples of single-site mutations include the following wherein the leucine at position +104 is changed to any other amino acid:

Single-site mutant:	**Codon change:**
L104EIg	Glutamic acid GAG
L104SIg	Serine AGT
L104TIg	Threonine ACG
L104AIg	Alanine GCG
L104WIg	Tryptophan TGG
L104QIg	Glutamine CAG

L104KIg	Lysine AAG
L104RIg	Arginine CGG
L104GIg	Glycine GGG

Further, the invention provides mutant molecules having the extracellular domain of CTLA4 with two mutations, fused to an Ig Cγ1 moiety. Examples include the following wherein the leucine at position +104 is changed to another amino acid (e.g. glutamic acid) and the glycine at position +105, the serine at position +25, the threonine at position +30 or the alanine at position +29 is changed to any other amino acid:

Double-site mutants:	**Codon change:**
L104EG105FIg	Phenylalanine TTC
L104EG105WIg	Tryptophan TGG
L104EG105LIg	Leucine CTT
L104ES25RIg	Arginine CGG
L104ET30GIg	Glycine GGG
L104ET30NIg	Asparagine AAT
L104EA29YIg	Tyrosine TAT
L104EA29LIg	Leucine TTG
L104EA29TIg	Threonine ACT
L104EA29WIg	Tryptophan TGG

Further still, the invention provides mutant molecules having the extracellular domain of CTLA4 comprising three mutations, fused to an Ig Cγ1 moiety. Examples include the following wherein the leucine at position +104 is changed to another amino acid (e.g. glutamic acid), the alanine at position +29 is changed to another amino acid (e.g. tyrosine) and the serine at position +25 is changed to another amino acid:

Triple-site Mutants:	**Codon changes:**
L104EA29YS25KIg	Lysine AAA

L104EA29YS25KIg	Lysine AAG
L104EA29YS25NIg	Asparagine AAC
L104EA29YS25RIg	Arginine CGG

Soluble CTLA4 mutant molecules may have a junction amino acid residue which is located between the CTLA4 portion and the Ig portion of the molecule. The junction amino acid can be any amino acid, including glutamine. The junction amino acid can be introduced by molecular or chemical synthesis methods known in the art.

The present invention provides CTLA4 mutant molecules including a signal peptide sequence linked to the N-terminal end of the extracellular domain of the CTLA4 portion of the mutant molecule. The signal peptide can be any sequence that will permit secretion of the mutant molecule, including the signal peptide from oncostatin M (Malik, et al., 1989 *Molec. Cell. Biol.* 9: 2847-2853), or CD5 (Jones, N. H. et al., 1986 *Nature* 323:346-349), or the signal peptide from any extracellular protein.

The invention provides soluble CTLA4 mutant molecules comprising a single-site mutation in the extracellular domain of CTLA4 such as L104EIg (as included in Figure 18) or L104SIg, wherein L104EIg and L104SIg are mutated in their CTLA4 sequences so that leucine at position +104 is substituted with glutamic acid or serine, respectively. The single-site mutant molecules further include CTLA4 portions encompassing methionine at position +1 through aspartic acid at position +124, a junction amino acid residue glutamine at position +125, and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357. The immunoglobulin portion of the mutant molecule may also be mutated so that the cysteines at positions +130, +136, and +139 are substituted with serine, and the proline at position +148 is substituted with serine. Alternatively, the single-site soluble CTLA4 mutant molecule may have a CTLA4 portion encompassing alanine at position -1 through aspartic acid at position +124.

The invention provides soluble CTLA4 mutant molecules comprising a double-site

mutation in the extracellular domain of CTLA4, such as L104EA29YIg, L104EA29LIg, L104EA29TIg or L104EA29WIg, wherein leucine at position +104 is substituted with a glutamic acid and alanine at position +29 is changed to tyrosine, leucine, threonine and tryptophan, respectively. The sequences for L104EA29YIg, L104EA29LIg, L104EA29TIg and L104EA29WIg, starting at methionine at position +1 and ending with lysine at position +357, plus a signal (leader) peptide sequence are included in the sequences as shown in Figures 19-22 respectively. The double-site mutant molecules further comprise CTLA4 portions encompassing methionine at position +1 through aspartic acid at position +124, a junction amino acid residue glutamine at position +125, and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357. The immunoglobulin portion of the mutant molecule may also be mutated, so that the cysteines at positions +130, +136, and +139 are substituted with serine, and the proline at position +148 is substituted with serine. Alternatively, these mutant molecules can have a CTLA4 portion encompassing alanine at position -1 through aspartic acid at position +124.

The invention provides soluble CTLA4 mutant molecules comprising a double-site mutation in the extracellular domain of CTLA4, such as L104EG105FIg, L104EG105WIg and L104EG105LIg, wherein leucine at position +104 is substituted with a glutamic acid and glycine at position +105 is substituted with phenylalanine, tryptophan and leucine, respectively. The double-site mutant molecules further comprise CTLA4 portions encompassing methionine at position +1 through aspartic acid at position +124, a junction amino acid residue glutamine at position +125, and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357. The immunoglobulin portion of the may also be mutated, so that the cysteines at positions +130, +136, and +139 are substituted with serine, and the proline at position +148 is substituted with serine. Alternatively, these mutant molecules can have a CTLA4 portion encompassing alanine at position -1 through aspartic acid at position +124.

The invention provides L104ES25RIg which is a double-site mutant molecule including a CTLA4 portion encompassing methionine at position +1 through aspartic acid at position

+124, a junction amino acid residue glutamine at position +125, and the immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357. The portion having the extracellular domain of CTLA4 is mutated so that serine at position +25 is substituted with arginine, and leucine at position +104 is substituted with glutamic acid. Alternatively, L104ES25RIg can have a CTLA4 portion encompassing alanine at position -1 through aspartic acid at position +124.

The invention provides soluble CTLA4 mutant molecules comprising a double-site mutation in the extracellular domain of CTLA4, such as L104ET30GIg and L104ET30NIg, wherein leucine at position +104 is substituted with a glutamic acid and threonine at position +30 is substituted with glycine and asparagine, respectively. The double-site mutant molecules further comprise CTLA4 portions encompassing methionine at position +1 through aspartic acid at position +124, a junction amino acid residue glutamine at position +125, and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357. The immunoglobulin portion of the mutant molecule may also be mutated, so that the cysteines at positions +130, +136, and +139 are substituted with serine, and the proline at position +148 is substituted with serine. Alternatively, these mutant molecules can have a CTLA4 portion encompassing alanine at position -1 through aspartic acid at position +124.

The invention provides soluble CTLA4 mutant molecules comprising a triple-site mutation in the extracellular domain of CTLA4, such as L104EA29YS25KIg, L104EA29YS25NIg, L104EA29YS25RIg, wherein leucine at position +104 is substituted with a glutamic acid, alanine at position +29 is changed to tyrosine and serine at position +25 is changed to lysine, asparagine and arginine, respectively. The triple-site mutant molecules further comprise CTLA4 portions encompassing methionine at position +1 through aspartic acid at position +124, a junction amino acid residue glutamine at position +125, and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357. The immunoglobulin portion of the mutant molecule may also be mutated, so that the cysteines at positions +130, +136, and +139 are substituted with serine, and the proline at position +148 is substituted with serine.

Alternatively, these mutant molecules can have a CTLA4 portion encompassing alanine at position -1 through aspartic acid at position +124.

Additional embodiments of soluble CTLA4 mutant molecules include chimeric CTLA4/CD28 homologue mutant molecules that bind a B7 (Peach, R. J., *et al.*, 1994 *J Exp Med* 180:2049-2058). Examples of these chimeric CTLA4/CD28 mutant molecules include HS1, HS2, HS3, HS4, HS5, HS6, HS4A, HS4B, HS7, HS8, HS9, HS10, HS11, HS12, HS13 and HS14 (U.S. patent number 5,773,253)

Preferred embodiments of the invention are soluble CTLA4 molecules such as CTLA4Ig (as shown in Figure 24, starting at methionine at position +1 and ending at lysine at position +357) and soluble CTLA4 mutant L104EA29YIg (as shown in Figure 19, starting at methionine at position +1 and ending at lysine at position +357). The invention further provides nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequences corresponding to the soluble CTLA4 molecules of the invention. In one embodiment, the nucleic acid molecule is a DNA (e.g., cDNA) or a hybrid thereof. DNA encoding CTLA4Ig (Figure 24) was deposited on May 31, 1991with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 and has been accorded ATCC accession number ATCC 68629. DNA encoding L104EA29YIg (sequence included in Figure 19) was deposited on June 19, 2000 with ATCC and has been accorded ATCC accession number PTA-2104. Alternatively, the nucleic acid molecules are RNA or a hybrid thereof.

Additionally, the invention provides a vector, which comprises the nucleotide sequences of the invention. Examples of expression vectors for include, but are not limited to, vectors for mammalian host cells (e.g., BPV-1, pHyg, pRSV, pSV2, pTK2 (Maniatis); pIRES (Clontech); pRc/CMV2, pRc/RSV, pSFV1 (Life Technologies); pVPakc Vectors, pCMV vectors, pSG5 vectors (Stratagene)), retroviral vectors (e.g., pFB vectors (Stratagene)), pCDNA-3 (Invitrogen) or modified forms thereof, adenoviral vectors; adeno-associated virus vectors, baculovirus vectors, yeast vectors (e.g., pESC vectors (Stratagene)).

A host vector system is also provided. The host vector system comprises the vector of the invention in a suitable host cell. Examples of suitable host cells include, but are not limited to, prokaryotic and eukaryotic cells. In accordance with the practice of the invention, eukaryotic cells are also suitable host cells. Examples of eukaryotic cells include any animal cell, whether primary or immortalized, yeast (e.g., Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Pichia pastoris), and plant cells. Myeloma, COS and CHO cells are examples of animal cells that may be used as hosts. Particular CHO cells include, but are not limited to, DG44 (Chasin, et la., 1986 Som. Cell. Molec. Genet. 12:555-556; Kolkekar 1997 Biochemistry 36:10901-10909), CHO-K1 (ATCC No. CCL-61), CHO-K1 Tet-On cell line (Clontech), CHO designated ECACC 85050302 (CAMR, Salisbury, Wiltshire, UK), CHO clone 13 (GEIMG, Genova, IT), CHO clone B (GEIMG, Genova, IT), CHO-K1/SF designated ECACC 93061607 (CAMR, Salisbury, Wiltshire, UK), and RR-CHOK1 designated ECACC 92052129 (CAMR, Salisbury, Wiltshire, UK). Exemplary plant cells include tobacco (whole plants, cell culture, or callus), corn, soybean, and rice cells. Corn, soybean, and rice seeds are also acceptable.

The CTLA4 mutant molecules of the invention may be isolated as naturally-occurring polypeptides, or from any source whether natural, synthetic, semi-synthetic or recombinant. Accordingly, the CTLA4 mutant polypeptide molecules may be isolated as naturally-occurring proteins from any species, particularly mammalian, including bovine, ovine, porcine, murine, equine, and preferably human. Alternatively, the CTLA4 mutant polypeptide molecules may be isolated as recombinant polypeptides that are expressed in prokaryote or eukaryote host cells, or isolated as a chemically synthesized polypeptide.

A skilled artisan can readily employ standard isolation methods to obtain isolated CTLA4 mutant molecules. The nature and degree of isolation will depend on the source and the intended use of the isolated molecules.

CTLA4 mutant molecules and fragments or derivatives thereof, can be produced by recombinant methods. Accordingly, an isolated nucleotide sequence encoding wild-type

CTLA4 molecules may be manipulated to introduce mutations, resulting in nucleotide sequences that encode the CTLA4 mutant polypeptide molecules. For example, the nucleotide sequences encoding the CTLA4 mutant molecules may be generated by site-directed mutagenesis methods, using primers and PCR amplification. The primers can include specific sequences designed to introduce desired mutations. Alternatively, the primers can be designed to include randomized or semi-randomized sequences to introduce random mutations. Standard recombinant methods (*Molecular Cloning; A Laboratory Manual*, 2nd edition, Sambrook, Fritch, and Maniatis 1989, Cold Spring Harbor Press) and PCR technology (U. S. Patent No. 4,603,102) can be employed for generating and isolating CTLA4 mutant polynucleotides encoding CTLA4 mutant polypeptides.

The invention includes pharmaceutical compositions for use in the treatment of immune system diseases comprising pharmaceutically effective amounts of soluble CTLA4 molecules. In certain embodiments, the immune system diseases are mediated by CD28/CTLA4/B7 interactions. The soluble CTLA4 molecules are preferably soluble CTLA4 molecules with wildtype sequence and/or soluble CTLA4 molecules having one or more mutations in the extracellular domain of CTLA4. The pharmaceutical composition can include soluble CTLA4 protein molecules and/or nucleic acid molecules, and/or vectors encoding the molecules. In preferred embodiments, the soluble CTLA4 molecules have the amino acid sequence of the extracellular domain of CTLA4 as shown in either Figures 24 or 19 (CTLA4Ig or L104EA29Y, respectively). Even more preferably, the soluble CTLA4 mutant molecule is L104EA29YIg as disclosed herein. The compositions may additionally include other therapeutic agents, including, but not limited to, drug toxins, enzymes, antibodies, or conjugates.

As is standard practice in the art, pharmaceutical compositions, comprising the molecules of the invention admixed with an acceptable carrier or adjuvant which is known to those of skill of the art, are provided. The pharmaceutical compositions preferably include suitable carriers and adjuvants which include any material which when combined with the molecule of the invention (e.g., a soluble CTLA4 molecule, such as, CTLA4Ig or

L104EA29Y) retains the molecule's activity and is non-reactive with the subject's immune system. These carriers and adjuvants include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, phosphate buffered saline solution, water, emulsions (e.g. oil/water emulsion), salts or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances and polyethylene glycol. Other carriers may also include sterile solutions; tablets, including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar (e.g. sucrose, glucose, maltose), certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods. Such compositions may also be formulated within various lipid compositions, such as, for example, liposomes as well as in various polymeric compositions, such as polymer microspheres.

Methods

The invention provides methods for regulating functional CTLA4- and CD28- positive cell interactions with B7-positive cells. The methods comprise contacting the B7-positive cells with a soluble CTLA4 molecule of the invention so as to form soluble CTLA4/B7 complexes, the complexes interfering with reaction of an endogenous CTLA4 and/or CD28 molecule with a B7 molecule.

The present invention also provides methods for inhibiting T-cell function but not T-cell depletion in a human by contacting B7-positive cells in the human with a soluble CTLA4. Examples of soluble CTLA4 include CTLA4Ig and soluble CTLA4 mutant molecule e.g. L104EA29YIg.

The present invention further provides methods for treating immune system diseases such as rheumatic diseases. The methods comprise administering a therapeutic composition, comprising soluble CTLA4 molecules of the invention, to a subject in an amount effective to relieve at least one of the symptoms associated with immune system diseases. Additionally, the invention may provide long-term therapy for immune system diseases by blocking the T-cell/B7-positive cell interactions, thereby blocking T-cell stimulation by co-stimulatory signals such as B7 binding to CD28, leading to induction of T-cell anergy or tolerance. Immune system diseases include, but are not limited to, autoimmune diseases, immunoproliferative diseases, and graft-related disorders. Examples of graft-related diseases include graft versus host disease (GVHD) (e.g., such as may result from bone marrow transplantation, or in the induction of tolerance), immune disorders associated with graft transplantation rejection, chronic rejection, and tissue or cell allo- or xenografts, including solid organs, skin, islets, muscles, hepatocytes, neurons. Examples of immunoproliferative diseases include, but are not limited to, psoriasis; T cell lymphoma; T cell acute lymphoblastic leukemia; testicular angiocentric T cell lymphoma; benign lymphocytic angiitis; and autoimmune diseases such as lupus (e.g., lupus erythematosus, lupus nephritis), Hashimoto's thyroiditis, primary myxedema, Graves' disease, pernicious anemia, autoimmune atrophic gastritis, Addison's disease, diabetes (e.g. insulin dependent diabetes mellitis, type I diabetes mellitis, type II diabetes mellitis), good pasture's syndrome, myasthenia gravis, pemphigus, Crohn's disease, sympathetic ophthalmia, autoimmune uveitis, multiple sclerosis, autoimmune hemolytic anemia, idiopathic thrombocytopenia, primary biliary cirrhosis, chronic action hepatitis, ulceratis colitis, Sjogren's syndrome, rheumatic diseases (e.g., rheumatoid arthritis), polymyositis, scleroderma, and mixed connective tissue disease.

The soluble CTLA4 molecules of the invention exhibit inhibitory properties *in vivo*. Under conditions where T-cell/B7-positive cell interactions, for example T cell/B cell interactions, are occurring as a result of contact between T cells and B7-positive cells, binding of introduced CTLA4 molecules to react to B7-positive cells, for example B cells, may interfere, i.e., inhibit, the T cell/ B7-positive cell interactions resulting in regulation of immune responses.

The invention provides methods for downregulating immune responses. Down regulation of an immune response by the soluble CTLA4 molecules of the invention may be by way of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response. The soluble CTLA4 molecules of the invention may inhibit the functions of activated T cells, such as T lymphocyte proliferation and cytokine secretion, by suppressing T cell responses or by inducing specific tolerance in T cells, or both. Further, the soluble CTLA4 molecules of this invention, interfering with the CTLA4/CD28/B7 pathway may inhibit T-cell proliferation and/or cytokine secretion, and thus result in reduced tissue destruction and induction of T-cell unresponsiveness or anergy.

A preferred embodiment of the invention comprises use of the soluble CTLA4 mutant molecule L104EA29YIg to regulate functional CTLA4- and CD28- positive cell interactions with B7-positive cells, to treat immune system diseases such as rheumatic diseases and/or to downregulate immune responses. The L104EA29YIg of the invention is a soluble CTLA4 mutant molecule comprising at least the two amino acid changes, the leucine (L) to glutamic acid (E) at position +104 and the alanine (A) to tyrosine (Y) change at position +29. The L104EA29YIg molecule may encompass further mutations beyond the two specified herein.

A preferred embodiment includes methods for treating a rheumatic disease, such as rheumatoid arthritis, by administering an effective amount of soluble CTLA4 molecules to a subject. Administration of an effective amount of the therapeutic composition, thereby relieving the subject of at least one of the symptoms associated with the disease, including reducing: joint swelling, joint tenderness, inflammation, morning stiffness, and pain, and structural damage subsequently decreasing the physical disability. The methods of the invention also may be used to reduce at least one symptom associated with rheumatoid arthritis, including reducing erythrocyte sedimentation rates, serum levels of C-reactive protein, soluble ICAM-1, soluble E-selectin and/or soluble IL-2r.

The amount of symptom relief provided by the present invention can be measured using any of the accepted criteria established to measure and document symptom relief in a clinical setting. Acceptable criteria for measuring symptom relief may include scores based on the criteria established by the American College of Rheumatology (e.g., ACR 20), the four measures of symptom relief (in: "CDER Guideline for the Clinical Evaluation of Anti-Inflammatory and Antirheumatic Drugs—FDA 1988), and the Health Assessment Questionnaire (HAQ) (Fries, J. F., et al., 1982 *J. of Rheumatology* 9:789-793). For a general description of these criteria, see "Guidance for Industry: Clinical Development Programs for Drugs, Devices, and Biological products for the Treatment of Rheumatoid Arthritis (RA)", February 1999.

The subjects treated by the present invention include mammalian subjects, including, human, monkey, ape, dog, cat, cow, horse, rabbit, mouse and rat.

The present invention provides various methods, local or systemic, for administering the soluble CTLA4 molecule. The methods include intravenous, intramuscular, intraperitoneal, oral, inhalation and subcutaneous methods, as well as implantable pump, continuous infusion, gene therapy, liposomes, suppositories, topical contact, vesicles, capsules and injection methods. The therapeutic agent, compounded with a carrier, is commonly lyophilized for storage and is reconstituted with water or a buffered solution with a neutral pH (about pH 7-8, e.g., pH 7.5) prior to administration.

As is standard practice in the art, the compositions of the invention may be administered to the subject in any pharmaceutically acceptable form.

In accordance with the practice of the invention, the methods comprise administering to a subject the soluble CTLA4 molecules of the invention to regulate CD28- and/or CTLA4-positive cell interactions with B7-positive cells. The B7-positive cells are contacted with an effective amount of the soluble CTLA4 molecules of the invention, or fragments or derivatives thereof, so as to form soluble CTLA4/B7 complexes. The complexes interfere with interaction between endogenous CTLA4 and CD28 molecules with B7 family

molecules.

The soluble CTLA4 molecules may be administered to a subject in an amount and for a time (e.g. length of time and/or multiple times) sufficient to block endogenous B7 molecules from binding their respective ligands, in the subject. Blockage of endogenous B7/ligand binding thereby inhibiting interactions between B7-positive cells with CD28- and/or CTLA4-positive cells.

Dosage of a therapeutic agent is dependant upon many factors including, but not limited to, the type of tissue affected, the type of autoimmune disease being treated, the severity of the disease, a subject's health and response to the treatment with the agents. Accordingly, dosages of the agents can vary depending on each subject and the mode of administration. The soluble CTLA4 molecules may be administered in an amount between 0.1 to 20.0 mg/kg weight of the patient/day, preferably between 0.5 to 10.0 mg/kg/day.

The invention also encompasses the use of the compositions of the invention together with other pharmaceutical agents to treat immune system diseases. For example, rheumatic diseases may be treated with molecules of the invention in conjunction with, but not limited to, immunosuppressants such as corticosteroids, cyclosporin (Mathiesen 1989 *Cancer Lett.* 44(2):151-156), prednisone, azathioprine, methotrexate (R. Handschumacher, in: "Drugs Used for Immunosuppression" pages 1264-1276), TNFα blockers or antagonists (New England Journal of Medicine, vol. 340: 253-259, 1999; The Lancet vol. 354: 1932-39, 1999, Annals of Internal Medicine, vol. 130: 478-486), or any other biological agent targeting any inflammatory cytokine, nonsteroidal antiinflammatory drugs/Cox-2 inhibitors, hydroxychloroquine, sulphasalazopryine, gold salts, etanercept, infliximab, rapamycin, mycophenolate mofetil, azathioprine, tacrolismus, basiliximab, cytoxan, interferon beta-1a, interferon beta-1b, glatiramer acetate, mitoxantrone hydrochloride, anakinra and/or other biologics.

The soluble CTLA4 molecules (preferably, L104EA29YIg) can also be used in combination with one or more of the following agents to regulate an immune response: soluble gp39 (also known as CD40 ligand (CD40L), CD154, T-BAM, TRAP), soluble CD29, soluble CD40, soluble CD80 (e.g. ATCC 68627), soluble CD86, soluble CD28 (e.g. 68628), soluble CD56, soluble Thy-1, soluble CD3, soluble TCR, soluble VLA-4, soluble VCAM-1, soluble LECAM-1, soluble ELAM-1, soluble CD44, antibodies reactive with gp39 (e.g. ATCC HB-10916, ATCC HB-12055 and ATCC HB-12056), antibodies reactive with CD40 (e.g. ATCC HB-9110), antibodies reactive with B7 (e.g. ATCC HB-253, ATCC CRL-2223, ATCC CRL-2226, ATCC HB-301, ATCC HB-11341, etc), antibodies reactive with CD28 (e.g. ATCC HB-11944 or mAb 9.3 as described by Martin et al (J. Clin. Immun. 4(1):18-22, 1980), antibodies reactive with LFA-1 (e.g. ATCC HB-9579 and ATCC TIB-213), antibodies reactive with LFA-2, antibodies reactive with IL-2, antibodies reactive with IL-12, antibodies reactive with IFN-gamma, antibodies reactive with CD2, antibodies reactive with CD48, antibodies reactive with any ICAM (e.g., ICAM-1 (ATCC CRL-2252), ICAM-2 and ICAM-3), antibodies reactive with CTLA4 (e.g. ATCC HB-304),, antibodies reactive with Thy-1, antibodies reactive with CD56, antibodies reactive with CD3, antibodies reactive with CD29, antibodies reactive with TCR, antibodies reactive with VLA-4, antibodies reactive with VCAM-1, antibodies reactive with LECAM-1, antibodies reactive with ELAM-1, antibodies reactive with CD44. In certain embodiments, monoclonal antibodies are preferred. In other embodiments, antibody fragments are preferred. As persons skilled in the art will readily understand, the combination can include the soluble CTLA4 molecules of the invention and one other immunosuppressive agent, the soluble CTLA4 molecules with two other immunosuppressive agents, the soluble CTLA4 molecules with three other immunosuppressive agents, etc. The determination of the optimal combination and dosages can be determined and optimized using methods well known in the art.

Some specific combinations include the following: L104EA29YIg and CD80 monoclonal antibodies (mAbs); L104EA29YIg and CD86 mAbs; L104EA29YIg, CD80 mAbs, and CD86 mAbs; L104EA29YIg and gp39 mAbs; L104EA29YIg and CD40

mAbs; L104EA29YIg and CD28 mAbs; L104EA29YIg, CD80 and CD86 mAbs, and gp39 mAbs; L104EA29YIg, CD80 and CD86 mAbs and CD40 mAbs; and L104EA29YIg, anti-LFA1 mAb, and anti-gp39 mAb. A specific example of a gp39 mAb is MR1. Other combinations will be readily appreciated and understood by persons skilled in the art.

The soluble CTLA4 molecules of the invention, for example L104EA29YIg, may be administered as the sole active ingredient or together with other drugs in immunomodulating regimens or other anti-inflammatory agents e.g. for the treatment or prevention of allo- or xenograft acute or chronic rejection or inflammatory or autoimmune disorders, or to induce tolerance. For example, it may be used in combination with a calcineurin inhibitor, e.g. cyclosporin A or FK506; an immunosuppressive macrolide, e.g. rapamycine or a derivative thereof; e.g. 40-O-(2-hydroxy)ethyl-rapamycin, a lymphocyte homing agent, e.g. FTY720 or an analog thereof; corticosteroids; cyclophosphamide; azathioprene; methotrexate; leflunomide or an analog thereof; mizoribine; mycophenolic acid; mycophenolate mofetil; 15-deoxyspergualine or an analog thereof; immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., MHC, CD2, CD3, CD4, CD 11a/CD18, CD7, CD25, CD 27, B7, CD40, CD45, CD58, CD 137, ICOS, CD150 (SLAM), OX40, 4-1BB or their ligands; or other immunomodulatory compounds, e.g. CTLA4/CD28-Ig, or other adhesion molecule inhibitors, e.g. mAbs or low molecular weight inhibitors including LFA-1 antagonists, Selectin antagonists and VLA-4 antagonists. The compound is particularly useful in combination with a compound that interferes with CD40 and its ligand, e.g. antibodies to CD40 and antibodies to CD40-L.

Where the soluble CTLA4 mutant molecules of the invention are administered in conjunction with other immunosuppressive/immunomodulatory or anti-inflammatory therapy, e.g. as hereinabove specified, dosages of the co-administered immunosuppressant, immunomodulatory or anti-inflammatory compound will of course vary depending on the type of co-drug employed, e.g. whether it is a steroid or a cyclosporin, on the specific drug employed, on the condition being treated and so forth.

In accordance with the foregoing the present invention provides in a yet further aspect methods as defined above comprising co-administration, e.g. concomitantly or in sequence, of a therapeutically effective amount of soluble CTLA4 molecules of the invention, e.g. CTLA4Ig and/or L104EA29YIg, in free form or in pharmaceutically acceptable salt form, and a second drug substance, said second drug substance being an immunosuppressant, immunomodulatory or anti-inflammatory drug, e.g. as indicated above. Further provided are therapeutic combinations, e.g. a kit, e.g. for use in any method as defined above, comprising a soluble CTLA4 molecule, in free form or in pharmaceutically acceptable salt form, to be used concomitantly or in sequence with at least one pharmaceutical composition comprising an immunosuppressant, immunomodulatory or anti-inflammatory drug. The kit may comprise instructions for its administration.

The invention also provides methods for producing the soluble CTLA4 mutant molecules molecules of the invention. Expression of soluble CTLA4 mutant molecules can be in prokaryotic cells or eukaryotic cells.

Prokaryotes most frequently are represented by various strains of bacteria. The bacteria may be a gram positive or a gram negative. Typically, gram-negative bacteria such as E. coli are preferred. Other microbial strains may also be used. Sequences encoding soluble CTLA4 mutant molecules can be inserted into a vector designed for expressing foreign sequences in prokaryotic cells such as E. coli. These vectors can include commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, including such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang, et al., (1977) Nature 198:1056), the tryptophan (trp) promoter system (Goeddel, et al., (1980) Nucleic Acids Res. 8:4057) and the lambda derived P_L promoter and N-gene ribosome binding site (Shimatake, et al., (1981) Nature 292:128).

Such expression vectors will also include origins of replication and selectable markers, such as a beta-lactamase or neomycin phosphotransferase gene conferring resistance to antibiotics, so that the vectors can replicate in bacteria and cells carrying the plasmids can be selected for when grown in the presence of antibiotics, such as ampicillin or kanamycin.

The expression plasmid can be introduced into prokaryotic cells via a variety of standard methods, including but not limited to $CaCl_2$-shock (Cohen, (1972) Proc. Natl. Acad. Sci. USA 69:2110, and Sambrook et al. (eds.), "Molecular Cloning: A Laboratory Manual", 2nd Edition, Cold Spring Harbor Press, (1989)) and electroporation.

In accordance with the practice of the invention, eukaryotic cells are also suitable host cells. Examples of eukaryotic cells include any animal cell, whether primary or immortalized, yeast (e.g., Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Pichia pastoris), and plant cells. Myeloma, COS and CHO cells are examples of animal cells that may be used as hosts. Particular CHO cells include, but are not limited to, DG44 (Chasin, et la., 1986 Som. Cell. Molec. Genet. 12:555-556; Kolkekar 1997 Biochemistry 36:10901-10909), CHO-K1 (ATCC No. CCL-61), CHO-K1 Tet-On cell line (Clontech), CHO designated ECACC 85050302 (CAMR, Salisbury, Wiltshire, UK), CHO clone 13 (GEIMG, Genova, IT), CHO clone B (GEIMG, Genova, IT), CHO-K1/SF designated ECACC 93061607 (CAMR, Salisbury, Wiltshire, UK), and RR-CHOK1 designated ECACC 92052129 (CAMR, Salisbury, Wiltshire, UK). Exemplary plant cells include tobacco (whole plants, cell culture, or callus), corn, soybean, and rice cells. Corn, soybean, and rice seeds are also acceptable.

Nucleic acid sequences encoding the CTLA4 mutant molecules can also be inserted into a vector designed for expressing foreign sequences in an eukaryotic host. The regulatory elements of the vector can vary according to the particular eukaryotic host.

Commonly used eukaryotic control sequences for use in expression vectors include promoters and control sequences compatible with mammalian cells such as, for example,

CMV promoter (CDM8 vector) and avian sarcoma virus (ASV) (πLN vector). Other commonly used promoters include the early and late promoters from Simian Virus 40 (SV40) (Fiers, et al., (1973) Nature 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, and bovine papilloma virus. An inducible promoter, such as hMTII (Karin, et al., (1982) Nature 299:797-802) may also be used.

Vectors for expressing CTLA4 mutant molecules in eukaryotes may also carry sequences called enhancer regions. These are important in optimizing gene expression and are found either upstream or downstream of the promoter region.

Examples of expression vectors for eukaryotic host cells include, but are not limited to, vectors for mammalian host cells (e.g., BPV-1, pHyg, pRSV, pSV2, pTK2 (Maniatis); pIRES (Clontech); pRc/CMV2, pRc/RSV, pSFV1 (Life Technologies); pVPakc Vectors, pCMV vectors, pSG5 vectors (Stratagene)), retroviral vectors (e.g., pFB vectors (Stratagene)), pCDNA-3 (Invitrogen) or modified forms thereof, adenoviral vectors; Adeno-associated virus vectors, baculovirus vectors, yeast vectors (e.g., pESC vectors (Stratagene)).

Nucleic acid sequences encoding CTLA4 mutant molecules can integrate into the genome of the eukaryotic host cell and replicate as the host genome replicates. Alternatively, the vector carrying CTLA4 mutant molecules can contain origins of replication allowing for extrachromosomal replication.

For expressing the nucleic acid sequences in Saccharomyces cerevisiae, the origin of replication from the endogenous yeast plasmid, the 2μ circle can be used. (Broach, (1983) Meth. Enz. 101:307). Alternatively, sequences from the yeast genome capable of promoting autonomous replication can be used (see, for example, Stinchcomb et al., (1979) Nature 282:39); Tschemper et al., (1980) Gene 10:157; and Clarke et al., (1983) Meth. Enz. 101:300).

Transcriptional control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al., (1968) J. Adv. Enzyme Reg. 7:149; Holland et al., (1978) Biochemistry 17:4900). Additional promoters known in the art include the CMV promoter provided in the CDM8 vector (Toyama and Okayama, (1990) FEBS 268:217-221); the promoter for 3-phosphoglycerate kinase (Hitzeman et al., (1980) J. Biol. Chem. 255:2073), and those for other glycolytic enzymes.

Other promoters are inducible because they can be regulated by environmental stimuli or by the growth medium of the cells. These inducible promoters include those from the genes for heat shock proteins, alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, enzymes associated with nitrogen catabolism, and enzymes responsible for maltose and galactose utilization.

Regulatory sequences may also be placed at the 3' end of the coding sequences. These sequences may act to stabilize messenger RNA. Such terminators are found in the 3' untranslated region following the coding sequences in several yeast-derived and mammalian genes.

Exemplary vectors for plants and plant cells include, but are not limited to, Agrobacterium T_i plasmids, cauliflower mosaic virus (CaMV), and tomato golden mosaic virus (TGMV).

General aspects of mammalian cell host system transformations have been described by Axel (U.S. Patent No. 4,399,216 issued Aug. 16, 1983). Mammalian cells can be transformed by methods including but not limited to, transfection in the presence of calcium phosphate, microinjection, electroporation, or via transduction with viral vectors.

Methods for introducing foreign DNA sequences into plant and yeast genomes include (1) mechanical methods, such as microinjection of DNA into single cells or protoplasts, vortexing cells with glass beads in the presence of DNA, or shooting DNA-coated tungsten or gold spheres into cells or protoplasts; (2) introducing DNA by making cell

membranes permeable to macromolecules through polyethylene glycol treatment or subjection to high voltage electrical pulses (electroporation); or (3) the use of liposomes (containing cDNA) which fuse to cell membranes.

Once the CTLA4 mutant molecules of the inventions are expressed, they can be harvested by methods well known in the art such as cell lysis (e.g. sonication, lysozyme and/or detergents) and protein recovery performed using standard protein purification means, e.g., affinity chromatography or ion-exchange chromatography, to yield substantially pure product (R. Scopes in: "Protein Purification, Principles and Practice", Third Edition, Springer-Verlag (1994); Sambrook et al. (eds.), "Molecular Cloning: A Laboratory Manual", 2nd Edition, Cold Spring Harbor Press, (1989)). Expression of CTLA4 mutant molecules can be detected by methods known in the art. For example, the mutant molecules can be detected by Coomassie staining SDS-PAGE gels and immunoblotting using antibodies that bind CTLA4.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE 1

The following provides a description of the methods used to generate the nucleotide sequences encoding the CTLA4 molecules of the invention.

A CTLA4Ig encoding plasmid was first constructed, and shown to express CTLA4Ig molecules as described in U.S. Patent Nos. 5,434,131, 5,885,579 and 5,851,795. Then single-site mutant molecules (e.g., L104EIg) were generated from the CTLA4Ig encoding sequence, expressed and tested for binding kinetics for various B7 molecules. The L104EIg nucleotide sequence (as included in the sequence shown in Figure 18) was used as a template to generate the double-site CTLA4 mutant sequences (as included in the sequences shown in Figures 19-22) which were expressed as proteins and tested for

binding kinetics. The double-site CTLA4 mutant sequences include: L104EA29YIg, L104EA29LIg, L104EA29TIg, and L104EA29WIg. Triple-site mutants were also generated.

CTLA4Ig Construction

A genetic construct encoding CTLA4Ig comprising the extracellular domain of CTLA4 and an IgCgamma1 domain was constructed as described in U.S. Patents 5,434,131, 5,844,095 and 5,851,795, the contents of which are incorporated by reference herein. The extracellular domain of the CTLA4 gene was cloned by PCR using synthetic oligonucleotides corresponding to the published sequence (Dariavach et al., Eur. Journ. Immunol. 18:1901-1905 (1988)).

Because a signal peptide for CTLA4 was not identified in the CTLA4 gene, the N-terminus of the predicted sequence of CTLA4 was fused to the signal peptide of oncostatin M (Malik et al., Mol. and Cell. Biol. 9:2847 (1989)) in two steps using overlapping oligonucleotides. For the first step, the oligonucleotide, CTCAGTCTGGTCCTTGCACTCCTGTTTCCAAGCATGGCGAGCATGGCAATGCA CGTGGCCCAGCC (which encoded the C terminal 15 amino acids from the oncostatin M signal peptide fused to the N terminal 7 amino acids of CTLA4) was used as forward primer, and TTTGGGCTCCTGATCAGAATCTGGGCACGGTTG (encoding amino acid residues 119-125 of the amino acid sequence encoding CTLA4 receptor and containing a Bcl I restriction enzyme site) as reverse primer. The template for this step was cDNA synthesized from 1 micro g of total RNA from H38 cells (an HTLV II infected T-cell leukemic cell line provided by Drs. Salahudin and Gallo, NCI, Bethesda, MD). A portion of the PCR product from the first step was reamplified, using an overlapping forward primer, encoding the N terminal portion of the oncostatin M signal peptide and containing a Hind III restriction endonuclease site, CTAGCCACTGAAGCTTCACCAATGGGTGTACTGCTCACACAGAGGACGCTGC TCAGTCTGGTCCTTGCACTC and the same reverse primer. The product of the PCR reaction was digested with Hind III and Bcl I and ligated together with a Bcl 1/Xba I

cleaved cDNA fragment encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of IgC(gamma)1 into the Hind III/Xba I cleaved expression vector, CDM8 or Hind III/Xba I cleaved expression vector piLN (also known as πLN).

DNA encoding the amino acid sequence corresponding to CTLA4Ig has been deposited with the ATCC under the Budapest Treaty on May 31, 1991, and has been accorded ATCC accession number 68629.

CTLA4Ig Codon Based Mutagenesis:

A mutagenesis and screening strategy was developed to identify mutant CTLA4Ig molecules that had slower rates of dissociation ("off" rates) from CD80 and/or CD86 molecules i.e. improved binding ability. In this embodiment, mutations were carried out in and/or about the residues in the CDR-1, CDR-2 (also known as the C' strand) and/or CDR-3 regions of the extracellular domain of CTLA4 (as described in U.S. Patents U.S. Patents 6,090,914, 5,773,253 and 5,844,095; in copending U.S. Patent Application Serial Number 60/214,065; and by Peach, R.J., et al *J Exp Med* 1994 180:2049-2058. A CDR-like region encompasses the each CDR region and extends, by several amino acids, upstream and/or downstream of the CDR motif). These sites were chosen based on studies of chimeric CD28/CTLA4 fusion proteins (Peach et al., J. Exp. Med., 1994, 180:2049-2058), and on a model predicting which amino acid residue side chains would be solvent exposed, and a lack of amino acid residue identity or homology at certain positions between CD28 and CTLA4. Also, any residue which is spatially in close proximity (5 to 20 Angstrom Units) to the identified residues is considered part of the present invention.

To synthesize and screen soluble CTLA4 mutant molecules with altered affinities for a B7 molecule (e.g. CD80, CD86), a two-step strategy was adopted. The experiments entailed first generating a library of mutations at a specific codon of an extracellular portion of CTLA4 and then screening these by BIAcore analysis to identify mutants with altered reactivity to B7. The Biacore assay system (Pharmacia, Piscataway, N.J.) uses a

surface plasmon resonance detector system that essentially involves covalent binding of either CD80Ig or CD86Ig to a dextran-coated sensor chip which is located in a detector. The test molecule can then be injected into the chamber containing the sensor chip and the amount of complementary protein that binds can be assessed based on the change in molecular mass which is physically associated with the dextran-coated side of the sensor chip; the change in molecular mass can be measured by the detector system.

Specifically, single-site mutant nucleotide sequences were generated using non-mutated (e.g., wild-type) DNA encoding CTLA4Ig (U.S. Patent Nos: 5,434,131, 5,844,095; 5,851,795; and 5,885,796; ATCC Accession No. 68629) as a template. Mutagenic oligonucleotide PCR primers were designed for random mutagenesis of a specific codon by allowing any base at positions 1 and 2 of the codon, but only guanine or thymine at position 3 (XXG/T or also noted as NNG/T). In this manner, a specific codon encoding an amino acid could be randomly mutated to code for each of the 20 amino acids. In that regard, XXG/T mutagenesis yields 32 potential codons encoding each of the 20 amino acids. PCR products encoding mutations in close proximity to the CDR3-like loop of CTLA4Ig (MYPPPY), were digested with SacI/XbaI and subcloned into similarly cut CTLA4Ig (as included in Figure 24) πLN expression vector. This method was used to generate the single-site CTLA4 mutant molecule L104EIg (as included in Figure 18).

For mutagenesis in proximity to the CDR-1-like loop of CTLA4Ig, a silent NheI restriction site was first introduced 5' to this loop, by PCR primer-directed mutagenesis. PCR products were digested with NheI/XbaI and subcloned into similarly cut CTLA4Ig or L104EIg expression vectors. This method was used to generate the double-site CTLA4 mutant molecule L104EA29YIg (as included in Figure 19). In particular, the nucleic acid molecule encoding the single-site CTLA4 mutant molecule, L104EIg, was used as a template to generate the double-site CTLA4 mutant molecule, L104EA29YIg.

The double-site mutant nucleotide sequences encoding CTLA4 mutant molecules, such as L104EA29YIg (deposited on June 19, 2000 with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 and accorded

ATCC accession number PTA-2104), were generated by repeating the mutagenesis procedure described above using L104EIg as a template. This method was used to generate numerous double-site mutants nucleotide sequences such as those encoding CTLA4 molecules L104EA29YIg (as included in the sequence shown in Figure 19), L104EA29LIg (as included in the sequence shown in Figure 20), L104EA29TIg (as included in the sequence shown in Figure 21), and L104EA29WIg (as included in the sequence shown in Figure 22). Triple-site mutants, such as those encoding L104EA29YS25KIg, L104EA29YS25NIg and L104EA29YS25RIg, were also generated

The soluble CTLA4 molecules were expressed from the nucleotide sequences and used in the phase II clinical studies described in Example 3, *infra*.

As those skilled-in-the-art will appreciate, replication of nucleic acid sequences, especially by PCR amplification, easily introduces base changes into DNA strands. However, nucleotide changes do not necessarily translate into amino acid changes as some codons redundantly encode the same amino acid. Any changes of nucleotide from the original or wildtype sequence, silent (i.e. causing no change in the translated amino acid) or otherwise, while not explicitly described herein, are encompassed within the scope of the invention.

EXAMPLE 2

The following example provides a description of the screening methods used to identify the single- and double-site mutant CTLA polypeptides, expressed from the constructs described in Example 1, that exhibited a higher binding avidity for B7 molecules, compared to non-mutated CTLA4Ig molecules.

Current *in vitro* and *in vivo* studies indicate that CTLA4Ig by itself is unable to completely block the priming of antigen specific activated T cells. *In vitro* studies with CTLA4Ig and either monoclonal antibody specific for CD80 or CD86 measuring inhibition of T cell proliferation indicate that anti-CD80 monoclonal antibody did not

augment CTLA4Ig inhibition. However, anti-CD86 monoclonal antibody did augment the inhibition, indicating that CTLA4Ig was not as effective at blocking CD86 interactions. These data support earlier findings by Linsley et al. (Immunity, (1994), 1:793-801) showing inhibition of CD80-mediated cellular responses required approximately 100 fold lower CTLA4Ig concentrations than for CD86-mediated responses. Based on these findings, it was surmised that soluble CTLA4 mutant molecules having a higher avidity for CD86 than wild type CTLA4 should be better able to block the priming of antigen specific activated cells than CTLA4Ig.

To this end, the soluble CTLA4 mutant molecules described in Example 1 above were screened using a novel screening procedure to identify several mutations in the extracellular domain of CTLA4 that improve binding avidity for CD80 and CD86. This screening strategy provided an effective method to directly identify mutants with apparently slower "off" rates without the need for protein purification or quantitation since "off" rate determination is concentration independent (O'Shannessy et al., (1993) Anal. Biochem., 212:457-468).

COS cells were transfected with individual miniprep purified plasmid DNA and propagated for several days. Three day conditioned culture media was applied to BIAcore biosensor chips (Pharmacia Biotech AB, Uppsala, Sweden) coated with soluble CD80Ig or CD86Ig. The specific binding and dissociation of mutant proteins was measured by surface plasmon resonance (O'Shannessy, D. J., et al., 1997 *Anal. Biochem.* 212:457-468). All experiments were run on BIAcore™ or BIAcore™ 2000 biosensors at 25°C. Ligands were immobilized on research grade NCM5 sensor chips (Pharmacia) using standard N-ethyl-N'-(dimethylaminopropyl) carbodiimidN-hydroxysuccinimide coupling (Johnsson, B., et al. (1991) Anal. Biochem. 198: 268-277; Khilko, S.N., et al.(1993) J. Biol. Chem 268:5425-15434).

Screening Method

COS cells grown in 24 well tissue culture plates were transiently transfected with mutant CTLA4Ig. Culture media containing secreted soluble mutant CTLA4Ig was collected 3 days later.

Conditioned COS cell culture media was allowed to flow over BIAcore biosensor chips derivitized with CD86Ig or CD80Ig (as described in Greene et al., 1996 J. Biol. Chem. 271:26762-26771), and mutant molecules were identified with off-rates slower than that observed for wild type CTLA4Ig. The DNAs corresponding to selected media samples were sequenced and more DNA prepared to perform larger scale COS cell transient transfection, from which CTLA4Ig mutant protein was prepared following protein A purification of culture media.

BIAcore analysis conditions and equilibrium binding data analysis were performed as described in J. Greene et al. 1996 *J. Biol. Chem.* 271:26762-26771 and in U.S. Patent Application Serial Nos. 09/579,927, and 60/214,065 which are herein incorporated by reference.

BIAcore Data Analysis

Senosorgram baselines were normalized to zero response units (RU) prior to analysis. Samples were run over mock-derivatized flow cells to determine background RU values due to bulk refractive index differences between solutions. Equilibrium dissociation constants (K_d) were calculated from plots of R_{eq} versus C, where R_{eq} is the steady-state response minus the response on a mock-derivatized chip, and C is the molar concentration of analyte. Binding curves were analyzed using commercial nonlinear curve-fitting software (Prism, GraphPAD Software).

Experimental data were first fit to a model for a single ligand binding to a single receptor (1-site model, i.e., a simple langmuir system, A+B↔AB), and equilibrium association

constants (K_d=[A]•[B]\[AB]) were calculated from the equation $R=R_{max}•C/(K_d+C)$. Subsequently, data were fit to the simplest two-site model of ligand binding (i.e., to a receptor having two non-interacting independent binding sites as described by the equation $R=R_{max1}•C\(K_{d1}+C)+R_{max2}•C\(K_{d2}+C)$.

The goodness-of-fits of these two models were analyzed visually by comparison with experimental data and statistically by an F test of the sums-of-squares. The simpler one-site model was chosen as the best fit, unless the two-site model fit significantly better ($p<0.1$).

Association and disassociation analyses were performed using BIA evaluation 2.1 Software (Pharmacia). Association rate constants k_{on} were calculated in two ways, assuming both homogenous single-site interactions and parallel two-site interactions. For single-site interactions, k_{on} values were calculated according to the equation $R_t=R_{eq}(1-\exp^{-ks(t-t_0)})$, where R_t is a response at a given time, t; R_{eq} is the steady-state response; t_0 is the time at the start of the injection; and $k_s=dR/dt=k_{on}•Ck_{off}$, where C is a concentration of analyte, calculated in terms of monomeric binding sites. For two-site interactions k_{on} values were calculated according to the equation $R_t=R_{eq1}(1-\exp^{-ks1(t-t_0)})+R_{eq2}(1-\exp^{ks2(t-t_0)})$. For each model, the values of k_{on} were determined from the calculated slope (to about 70% maximal association) of plots of k_s versus C.

Dissociation data were analyzed according to one site (AB=A+B) or two site (AiBj=Ai+Bj) models, and rate constants (k_{off}) were calculated from best fit curves. The binding site model was used except when the residuals were greater than machine background (2-10RU, according to machine), in which case the two-binding site model was employed. Half-times of receptor occupancy were calculated using the relationship $t_{1/2}=0.693/k_{off}$.

Flow Cytometry:

Murine mAb L307.4 (anti-CD80) was purchased from Becton Dickinson (San Jose, California) and IT2.2 (anti-B7-0 [also known as CD86]), from Pharmingen (San Diego, California). For immunostaining, CD80-positive and/or CD86-positive CHO cells were removed from their culture vessels by incubation in phosphate-buffered saline (PBS) containing 10mM EDTA. CHO cells (1-10 x 10^5) were first incubated with mAbs or immunoglobulin fusion proteins in DMEM containing 10% fetal bovine serum (FBS), then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse or anti-human immunoglobulin second step reagents (Tago, Burlingame, California). Cells were given a final wash and analyzed on a FACScan (Becton Dickinson).

SDS-PAGE and Size Exclusion Chromatography

SDS-PAGE was performed on Tris/glycine 4-20% acrylamide gels (Novex, San Diego, CA). Analytical gels were stained with Coomassie Blue, and images of wet gels were obtained by digital scanning. CTLA4Ig (25 μg) and L104EA29YIg (25 μg) were analyzed by size exclusion chromatography using a TSK-GEL G300 SW_{XL} column (7.8 x 300mm, Tosohaas, Montgomeryville, PA) equilibrated in phosphate buffered saline containing 0.02% NAN_3 at a flow rate of 1.0 ml/min.

$CTLA4X_{C120S}$ and $L104EA29YX_{C120S}$:

Single chain $CTLA4X_{C120S}$ was prepared as previously described (Linsley et al., (1995) J. Biol. Chem., 270:15417-15424). Briefly, an oncostatin M CTLA4 (OMCTLA4) expression plasmid was used as a template, the forward primer, GAGGTGATAAAGCTTCACCAATGGGTGTACTGCTCACACAG was chosen to match sequences in the vector; and the reverse primer, GTGGTGTATTGGTCTAGATCAATCAGAATCTGGGCACGGTTC corresponded to the last seven amino acids (i.e. amino acids 118-124) in the extracellular domain of CTLA4, and contained a restriction enzyme site, and a stop codon (TGA). The reverse

primer specified a C120S (cysteine to serine at position 120) mutation. In particular, the nucleotide sequence GCA (nucleotides 34-36) of the reverse primer shown above is replaced with one of the following nucleotide sequences: AGA, GGA, TGA, CGA, ACT, or GCT. As persons skilled in the art will understand, the nucleotide sequence GCA is a reversed complementary sequence of the codon TGC for cysteine. Similarly, the nucleotide sequences AGA, GGA, TGA, CGA, ACT, or GCT are the reversed complementary sequences of the codons for serine. Polymerase chain reaction products were digested with *Hind*III/*Xba*I and directionally subcloned into the expression vector πLN (Bristol-Myers Squibb Company, Princeton, NJ). L104EA29YX$_{C120S}$ was prepared in an identical manner. Each construct was verified by DNA sequencing.

Identification and Biochemical Characterization of High Avidity Mutants

Twenty four amino acids were chosen for mutagenesis and the resulting ~2300 mutant proteins assayed for CD86Ig binding by surface plasmon resonance (SPR; as described, supra). The predominant effects of mutagenesis at each site are summarized in Table II, *infra*. Random mutagenesis of some amino acids in the CDR-1 region (S25-R33) apparently did not alter ligand binding. Mutagenesis of E31 and R33 and residues M97-Y102 apparently resulted in reduced ligand binding. Mutagenesis of residues, S25, A29, and T30, K93, L96, Y103, L104, and G105, resulted in proteins with slow "on" and/or slow "off" rates. These results confirm previous findings that residues in the CDR-1 (S25-R33) region, and residues in or near M97-Y102 influence ligand binding (Peach et al., (1994) J. Exp. Med., 180:2049-2058).

Mutagenesis of sites S25, T30, K93, L96, Y103, and G105 resulted in the identification of some mutant proteins that had slower "off" rates from CD86Ig. However, in these instances, the slow "off" rate was compromised by a slow "on" rate that resulted in mutant proteins with an overall avidity for CD86Ig that was apparently similar to that seen with wild type CTLA4Ig. In addition, mutagenesis of K93 resulted in significant aggregation that may have been responsible for the kinetic changes observed.

Random mutagenesis of L104 followed by COS cell transfection and screening by SPR of culture media samples over immobilized CD86Ig yielded six media samples containing mutant proteins with approximately 2-fold slower "off" rates than wild type CTLA4Ig. When the corresponding cDNA of these mutants were sequenced, each was found to encode a leucine to glutamic acid mutation (L104E). Apparently, substitution of leucine 104 to aspartic acid (L104D) did not affect CD86Ig binding.

Mutagenesis was then repeated at each site listed in Table II, this time using L104E as the PCR template instead of wild type CTLA4Ig, as described above. SPR analysis, again using immobilized CD86Ig, identified six culture media samples from mutagenesis of alanine 29 with proteins having approximately 4-fold slower "off" rates than wild type CTLA4Ig. The two slowest were tyrosine substitutions (L104EA29Y), two were leucine (L104EA29L), one was tryptophan (L104EA29W), and one was threonine (L104EA29T). Apparently, no slow "off" rate mutants were identified when alanine 29 was randomly mutated, alone, in wild type CTLA4Ig.

The relative molecular mass and state of aggregation of purified L104E and L104EA29YIg was assessed by SDS-PAGE and size exclusion chromatography. L104EA29YIg (~1 μg; lane 3) and L104EIg (~1 μg; lane 2) apparently had the same electrophoretic mobility as CTLA4Ig (~1 μg; lane 1) under reducing (~50kDa; +ßME; plus 2-mercaptoethanol) and non-reducing (~100kDa; -ßME) conditions (FIG. 25A). Size exclusion chromatography demonstrated that L104EA29YIg (FIG. 25C) apparently had the same mobility as dimeric CTLA4Ig (FIG. 25B). The major peaks represent protein dimer while the faster eluting minor peak in FIG. 25B represents higher molecular weight aggregates. Approximately 5.0% of CTLA4Ig was present as higher molecular weight aggregates but there was no evidence of aggregation of L104EA29YIg or L104EIg. Therefore, the stronger binding to CD86Ig seen with L104EIg and L104EA29YIg could not be attributed to aggregation induced by mutagenesis.

Equilibrium and Kinetic Binding Analysis

Equilibrium and kinetic binding analysis was performed on protein A purified CTLA4Ig, L104EIg, and L104EA29YIg using surface plasmon resonance (SPR). The results are shown in Table I, *infra*. Observed equilibrium dissociation constants (K_d; Table I) were calculated from binding curves generated over a range of concentrations (5.0-200 nM). L104EA29YIg binds more strongly to CD86Ig than does L104EIg or CTLA4Ig. The lower K_d of L104EA29YIg (3.21 nM) than L104EIg (6.06 nM) or CTLA4Ig (13.9 nM) indicates higher binding avidity of L104EA29YIg to CD86Ig. The lower K_d of L104EA29YIg (3.66 nM) than L104EIg (4.47 nM) or CTLA4Ig (6.51 nM) indicates higher binding avidity of L104EA29YIg to CD80Ig.

Kinetic binding analysis revealed that the comparative "on" rates for CTLA4Ig, L104EIg, and L104EA29YIg binding to CD80 were similar, as were the "on" rates for CD86Ig (Table I). However, "off" rates for these molecules were not equivalent (Table I). Compared to CTLA4Ig, L104EA29YIg had approximately 2-fold slower "off" rate from CD80Ig, and approximately 4-fold slower "off" rate from CD86Ig. L104E had "off" rates intermediate between L104EA29YIg and CTLA4Ig. Since the introduction of these mutations did not significantly affect "on" rates, the increase in avidity for CD80Ig and CD86Ig observed with L104EA29YIg was likely primarily due to a decrease in "off" rates.

To determine whether the increase in avidity of L104EA29YIg for CD86Ig and CD80Ig was due to the mutations affecting the way each monomer associated as a dimer, or whether there were avidity enhancing structural changes introduced into each monomer, single chain constructs of CTLA4 and L104EA29Y extracellular domains were prepared following mutagenesis of cysteine 120 to serine as described supra, and by Linsley et al., (1995) J. Biol. Chem., 270:15417-15424 (84). The purified proteins CTLA4X_{C120S} and L104EA29YX_{C120S} were shown to be monomeric by gel permeation chromatography (Linsley et al., (1995), supra), before their ligand binding properties were analyzed by SPR. Results showed that binding affinity of both monomeric proteins for CD86Ig was

approximately 35-80-fold less than that seen for their respective dimers (Table I). This supports previously published data establishing that dimerization of CTLA4 was required for high avidity ligand binding (Greene et al., (1996) J. Biol. Chem., 271:26762-26771).

L104EA29YX$_{C120S}$ bound with approximately 2-fold higher affinity than CTLA4X$_{C120S}$ to both CD80Ig and CD86Ig. The increased affinity was due to approximately 3-fold slower rate of dissociation from both ligands. Therefore, stronger ligand binding by L104EA29Y was most likely due to avidity enhancing structural changes that had been introduced into each monomeric chain rather than alterations in which the molecule dimerized.

Location and Structural Analysis of Avidity Enhancing Mutations

The solution structure of the extracellular IgV-like domain of CTLA4 has recently been determined by NMR spectroscopy (Metzler et al., (1997) Nature Struct. Biol., 4:527-531). This allowed accurate location of leucine 104 and alanine 29 in the three dimensional fold (FIG. 26 left and right depictions). Leucine 104 is situated near the highly conserved MYPPPY amino acid sequence. Alanine 29 is situated near the C-terminal end of the CDR-1 (S25-R33) region, which is spatially adjacent to the MYPPPY region. While there is significant interaction between residues at the base of these two regions, there is apparently no direct interaction between L104 and A29 although they both comprise part of a contiguous hydrophobic core in the protein. The structural consequences of the two avidity enhancing mutants were assessed by modeling. The A29Y mutation can be easily accommodated in the cleft between the CDR-1 (S25-R33) region and the MYPPPY region, and may serve to stabilize the conformation of the MYPPPY region. In wild type CTLA4, L104 forms extensive hydrophobic interactions with L96 and V94 near the MYPPPY region. It is highly unlikely that the glutamic acid mutation adopts a conformation similar to that of L104 for two reasons. First, there is insufficient space to accommodate the longer glutamic acid side chain in the structure without significant perturbation to the CDR-1 (S25-R33 region). Second, the energetic costs of burying the negative charge of the glutamic acid side chain in the hydrophobic

region would be large. Instead, modeling studies predict that the glutamic acid side chain flips out on to the surface where its charge can be stabilized by solvation. Such a conformational change can easily be accommodated by G105, with minimal distortion to other residues in the regions.

Binding of High Avidity Mutants to CHO Cells Expressing CD80 or CD86

FACS analysis (Fig. 27) of CTLA4Ig and mutant molecules binding to stably transfected CD80+ and CD86+CHO cells was performed as described herein. CD80-positive and CD86-positive CHO cells were incubated with increasing concentrations of CTLA4Ig, L104EA29YIg, or L104EIg, and then washed. Bound immunoglobulin fusion protein was detected using fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin.

As shown in Figure 27, CD80-positive or CD86-positive CHO cells ($1.5x10^5$) were incubated with the indicated concentrations of CTLA4Ig (closed squares), L104EA29YIg (circles), or L104EIg (triangles) for 2 hr. at 23°C, washed, and incubated with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin antibody. Binding on a total of 5,000 viable cells was analyzed (single determination) on a FACScan, and mean fluorescence intensity (MFI) was determined from data histograms using PC-LYSYS. Data were corrected for background fluorescence measured on cells incubated with second step reagent only (MFI = 7). Control L6 mAb (80 μg/ml) gave MFI < 30. These results are representative of four independent experiments.

Binding of L104EA29YIg, L104EIg, and CTLA4Ig to human CD80-transfected CHO cells is approximately equivalent (FIG. 27A). L104EA29YIg and L104EIg bind more strongly to CHO cells stably transfected with human CD86 than does CTLA4Ig (FIG. 27B).

Functional Assays:

Human CD4-positive T cells were isolated by immunomagnetic negative selection (Linsley et al., (1992) J. Exp. Med. 176:1595-1604). Isolated CD4-positive T cells were stimulated with phorbal myristate acetate (PMA) plus CD80-positive or CD86-positive CHO cells in the presence of titrating concentrations of inhibitor. CD4-positive T cells (8-10 x 10^4/well) were cultured in the presence of 1 nM PMA with or without irradiated CHO cell stimulators. Proliferative responses were measured by the addition of 1 μCi/well of [3H]thymidine during the final 7 hours of a 72 hour culture. Inhibition of PMA plus CD80-positive CHO, or CD86-positive CHO, stimulated T cells by L104EA29YIg and CTLA4Ig was performed. The results are shown in FIG. 28. L104EA29YIg inhibits proliferation of CD80-positive PMA treated CHO cells more than CTLA4Ig (FIG. 28A). L104EA29YIg is also more effective than CTLA4Ig at inhibiting proliferation of CD86-positive PMA treated CHO cells (FIG. 28B). Therefore, L104EA29YIg is a more potent inhibitor of both CD80- and CD86-mediated costimulation of T cells.

Figure 29 shows inhibition by L104EA29YIg and CTLA4Ig of allostimulated human T cells prepared above, and further allostimulated with a human B lymphoblastoid cell line (LCL) called PM that expressed CD80 and CD86 (T cells at 3.0x10^4/well and PM at 8.0x10^3/well). Primary allostimulation occurred for 6 days, then the cells were pulsed with ^{3}H-thymidine for 7 hours, before incorporation of radiolabel was determined.

Secondary allostimulation was performed as follows. Seven day primary allostimulated T cells were harvested over lymphocyte separation medium (LSM) (ICN, Aurora, OH) and rested for 24 hours. T cells were then restimulated (secondary), in the presence of titrating amounts of CTLA4Ig or L104EA29YIg, by adding PM in the same ratio as above. Stimulation occurred for 3 days, then the cells were pulsed with radiolabel and harvested as above. The effect of L104EA29YIg on primary allostimulated T cells is shown in FIG. 29A. The effect of L104EA29YIg on secondary allostimulated T cells is

shown in FIG. 29B. L104EA29YIg inhibits both primary and secondary T cell proliferative responses better than CTLA4Ig.

To measure cytokine production (Figure 30), duplicate secondary allostimulation plates were set up. After 3 days, culture media was assayed using ELISA kits (Biosource, Camarillo, CA) using conditions recommended by the manufacturer. L104EA29YIg was found to be more potent than CTLA4Ig at blocking T cell IL-2, IL-4, and γ-IFN cytokine production following a secondary allogeneic stimulus (FIGS. 30A-C).

The effects of L104EA29YIg and CTLA4Ig on monkey mixed lymphocyte response (MLR) are shown in Figure 31. Peripheral blood mononuclear cells (PBMC'S; $3.5x10^4$ cells/well from each monkey) from 2 monkeys were purified over lymphocyte separation medium (LSM) and mixed with 2μg/ml phytohemaglutinin (PHA). The cells were stimulated 3 days then pulsed with radiolabel 16 hours before harvesting. L104EA29YIg inhibited monkey T cell proliferation better than CTLA4Ig.

Table I:

Equilibrium and apparent kinetic constants are given in the following table (values are means ± standard deviation from three different experiments):

Immobilized Protein	**Analyte**	k_{on} (x 10^5) M^{-1} S^{-1}	k_{off} (x 10^{-3}) S^{-1}	K_d nM
CD80Ig	CTLA4Ig	3.44 ± 0.29	2.21 ± 0.18	6.51 ± 1.08
CD80Ig	L104EIg	3.02 ± 0.05	1.35 ± 0.08	4.47 ± 0.36
CD80Ig	L104EA29YIg	2.96 ± 0.20	1.08 ± 0.05	3.66 ± 0.41
CD80Ig	$CTLA4X_{C120S}$	12.0 ± 1.0	230 ±10	195 ± 25
CD80Ig	$L104EA29YX_{C120S}$	8.3 ± 0.26	71 ± 5	85.0 ± 2.5
CD86Ig	CTLA4Ig	5.95 ± 0.57	8.16 ± 0.52	13.9 ± 2.27
CD86Ig	L104EIg	7.03 ± 0.22	4.26 ± 0.11	6.06 ± 0.05
CD86Ig	L104EA29YIg	6.42 ± 0.40	2.06 ± 0.03	3.21 ± 0.23
CD86Ig	$CTLA4X_{C120S}$	16.5 ± 0.5	840 ± 55	511 ± 17
CD86Ig	$L104EA29YX_{C120S}$	11.4 ± 1.6	300 ± 10	267 ± 29

Table II

The effect on CD86Ig binding by mutagenesis of CTLA4Ig at the sites listed was determined by SPR, described supra. The predominant effect is indicated with a "+" sign.

Mutagenesis Site	Effects of Mutagenesis			
	No Effect	Apparent	Slow "on" rate/ slow "off rate	Reduced ligand binding
S25			+	
P26	+			
G27	+			
K28	+			
A29			+	
T30			+	
E31				+
R33				+
K93			+	
L96			+	
M97				+
Y98				+
P99				+
P100				+
P101				+
Y102				+
Y103			+	
L104			+	
G105			+	
I106	+			
G107	+			
Q111	+			

Mutagenesis Site	Effects of Mutagenesis				
	No Effect	Apparent	Slow "on" rate/ slow "off rate	Reduced binding	ligand
Y113	+				
I115	+				

EXAMPLE 3

The following provides a description of phase II clinical studies of human patients administered soluble CTLA4 mutant molecule L104EA29YIg (also known as LEA29Y or LEA) or CTLA4Ig, to relieve at least one symptom associated with rheumatoid arthritis, including reducing: joint swelling, joint tenderness, inflammation, morning stiffness, and pain. The CTLA4Ig molecule used herein begins with methionine at position +1 (or alternatively with alanine at position –1) and ends with lysine at position +357 as shown in Figure 24. DNA encoding an embodiment of the CTLA4Ig molecule has been deposited as ATCC 68629. The L104EA29YIg molecule used herein begins with methionine at position +1 (or alternatively with alanine at position –1) and ends with lysine at position +357 as shown in Figure 19. DNA encoding an embodiment of the L104EA29YIg molecule has been deposited as ATCC PTA 2104.

Additionally, the following provides a description of human patients administered L104EA29YIg or CTLA4Ig to relieve at least one biological surrogate marker associated with rheumatoid arthritis, including reducing erythrocyte sedimentation rates, and serum levels of C-reactive protein and/or IL2 receptor.

Patient Cohorts

A total of 214 patients, including 54 males and 160 females, participated in the study (Figures 1A, 1B). The patients at baseline had a mean disease duration of 3.4 (±2.0) years and had failed at least one Disease Modifying Antirheumatic Drug (DMARD). Stable Nonsteroidal Anti-inflammatory Drugs (NSAIDS) or steroids (≤ 10 mg/day) were permitted and concomitant DMARDS were prohibited. The patients were randomized

into groups of 25 to 32 patients per treatment group. Thirty-two patients received a placebo, 92 received L104EA29YIg, and 90 received CTLA4Ig. The patients who followed protocol guidelines and did not discontinue before day 57 received a total of 4 intravenous infusions, one infusion each on days 1, 15, 29, and 57. All patients were evaluated on days 1, 15, 29, 43, 57, 71, and 85. The doses administered included 0.5, 2.0, or 10.0 mg/kg of L104EA29YIg (denoted as LEA.5, LEA2 and LEA10, respectively in Figures 1A-1E) or of CTLA4Ig (denoted as CTLA.5, CTLA2 and CTLA10, respectively in Figures 1A-1E).

All subjects were monitored for peri-infusional adverse events and global safety by answering a questionnaire listing potential adverse events. The patients were questioned about potential adverse events that may have occurred within twenty-four hours post-infusion. In addition, the patients were encouraged to spontaneously report any adverse events that they experienced. The physicians routinely monitored laboratory samples from the patients for abnormalities in blood chemistry and hematology e.g. assessed the levels of inflammatory response mediators such as cytokines (TNF, IL-6), tryptase and complement. The primary endpoint was the proportion of subjects meeting the ACR 20 criteria on day 85.

Storage of Test Material

The CTLA4Ig and L104EA29YIg were supplied in single-use glass vials containing 200 mg/vial of CTLA4Ig or 100 mg/vial of L104EA29YIg, respectively. Prior to infusion, the CTLA4Ig and L104EA29YIg were diluted to a final concentration of 25 mg/ml with sterile water for injection (SWFI).

Administration Protocol

All infusions were administered intravenously over 1 hour (Figures 1 through 17). All subjects received at least one infusion of study medication.

Group 1: 32 patients, CTLA4Ig or L104EA29YIg matching placebo.

Group 2: 26 patients; dosage 0.5 mg/kg of CTLA4Ig.

Group 3: 32 patients; dosage 2.0 mg/kg of CTLA4Ig.

Group 4: 32 patients; dosage 10.0 mg/kg of CTLA4Ig.

Group 5: 32 patients; dosage 0.5 mg/kg of L104EA29YIg.

Group 6: 29 patients; dosage 2.0 mg/kg of L104EA29YIg.

Group 7: 31 patients; dosage 10.0 mg/kg of L104EA29YIg.

Clinical Monitoring

Patients were evaluated for baseline symptoms of disease activity prior to receiving any infusions. These baseline evaluations included: joint swelling, joint tenderness, inflammation, morning stiffness, disease activity evaluated by patient and physician as well as disability evaluated by Health Questionnaire Assessment (HAQ) (reported as a physical function score in Figure 1C), and pain (Figures 1A to 1D). Additionally, the baseline evaluations included erythrocyte sedimentation rates (ESR), and serum levels of C-reactive protein (CRP) and soluble IL-2 receptor (IL-2r) (Figures 1C and 1D).

The clinical response studies were based on the criteria established by the American College of Rheumatology (ACR). A subject satisfied the ACR20 criterion if there was a 20 percent improvement in tender and swollen joint counts and 20 percent improvement in three of the five remaining symptoms measured, such as patient and physician global disease changes, pain, disability, and an acute phase reactant (Felson, D. T., et al., 1993 *Arthritis and Rheumatism* 36:729-740; Felson, D. T., et al., 1995 *Arthritis and Rheumatism* 38:1-9).

Biomarkers

Potential biomarkers of disease activity (rheumatoid factor, CRP, ESR, soluble IL-2R, soluble ICAM-1, soluble E-selectin, and MMP-3) were also assessed. Validated enzyme immunoassay (EIA) methods were used to determine the serum concentration of IL-2sRα, sICAM-1, sE-selectin and MMP-3. TNFα and IL-6 were assessed at infusion pre and 2 hours post, if necessary.

IL-2sRα, sICAM-1, and sE-selectin were measured using commercially available colorimetric EIA kits from R&D Systems, Inc. (Minneapolis, MN). The lower and upper limits of quantitation were 312-20,000 pg/mL, 40-907 ng/mL and 10-206 ng/mL, respectively. The inter-assay coefficient of variation ranged from 4.48-8.4%, 3.8-5.0% and 5.5-9.0% respectively. According to the kit manufacturer, normal serum values range from 676-2,132 pg/mL, respectively.

MMP-3 was measured using a commercially available colorimetric EIA kit from Amersham Pharmacia Biotech (Piscataway, NJ). The lower and upper limits of quantitation were 30-7,680 ng/mL. The inter-assay coefficient of variation ranged from 6.3-10.6%. According to the kit manufacturer, normal serum values range from 28-99 ng/mL.

IL-6 and TNFα were measured using commercially available chemiluminescent EIA kits from R&D Systems, Inc. (Minneapolis, MN). The lower and upper limits of quantitation were 0.3-3,000 pg/mL and 0.7-7,000 pg/mL, respectively. The inter-assay coefficient of variation ranged from 3.1-5.7% and 6.4-20.7%, respectively. According to the kit manufacturer, normal serum values range from <0.3-12 pg/mL and <0.7-7.5 pg/mL.

Antibody testing

Serum samples were obtained for assessment of drug-specific antibodies prior to dosing on day 1, and approximately on days 15, 29, 57, 85 and 169. Due to high, preexisting titers directed to the immunoglobulin (Ig) portion of the molecule, specific antibody formation against CTLA4Ig and LEA29Y without Ig constant regions was also assessed.

Ninety-six well Immulon II ELISA plates (Dynex, Chantilly, Virginia) were coated with CTLA4Ig, CTLA4Ig without the Ig constant regions, LEA29Y, or LEA29Y without the Ig constant regions at 2, 4, 2, or 1 μg/ml in phosphate buffered saline (PBS), respectively, and incubated overnight at 2-8°C. The plates were washed with PBS containing 0.05% Tween 20 and blocked for 1 hour at 37°C with PBS containing 1% bovine serum albumin (BSA). The plates were then washed and serial dilutions of the test sera or quality control (QC) sera were added to the appropriate wells and incubated for 2 hours at 37°C. Sera was diluted threefold in PBS with 0.25% BSA and 0.05% Tween 20 starting at a 1:10 dilution. Plates were washed and an alkaline-phosphatase-conjugated goat anti-human kappa and lambda (Southern Biotechnology Associates, Inc., Birmingham, Alabama) antibody cocktail was added. Following a 1-hour incubation at 37°C, the plates were washed and 1 mg/ml para-nitrophenyl phosphate in diethanolamine buffer was added to each well. After 30 minutes at 25°C, the reactions were stopped with 3N NaOH and the absorbance (dual wavelength: 405 nm and 550 nm) was recorded. Results were expressed as endpoint titer (EPT), defined as the reciprocal of the highest dilution that resulted in an absorbance reading fivefold greater than or equal to the mean plate-background absorbance. Plate background was determined as the absorbance measurement recorded in the absence of serum. Values were considered positive for seroconversion if they were at least two serial dilutions (ninefold) or greater relative to predose EPT values. Serum QC samples positive for either CTLA4Ig- or LEA29Y-specific antibodies were generated from immunized monkeys. An aliquot of the appropriate QC sample was assayed during each analytical run. Analytical runs were accepted only when the QC samples were within the assay acceptance criteria.

Results

CTLA4Ig and L104EA29YIg were generally well-tolerated at all dose-levels. Peri-infusional adverse events were similar across all dose groups, with the exception of headaches. Headache response of patients on day 85 increased dose-dependently 23%, 44%, and 53% in CTLA4Ig-treated patients, and 34%, 45%, and 61% in L104EA29YIg-treated patients, at 0.5, 2.0, and 10.0 mg/kg respectively. In contrast, 31% of the patients administered placebos experienced headaches.

The percent of patients that discontinued from the clinical study due to arthritis flares and other adverse events is summarized in Figure 2. A much higher percentage of patients on placebo discontinued treatment due to arthritis flare. The CTLA4Ig treated patients discontinued treatment less with increasing doses. Very few patients treated with L104EA29YIg discontinued treatment. These results indicate a good inverse dose-dependent response for CTLA4Ig, and a stronger therapeutic response with L104EA29YIg therapy.

The ACR-20, -50, and -70 responses of patients treated with CTLA4Ig, L104EA29YIg, or placebo at day 85 are summarized in Figure 3A. Similarly, Figures 3B and C describe the ACR-20 responses with 95% confidence limits. The responses appear to be dose-dependent with a clear significant response at 10 mg/kg per body weight of the patient.

The percent of patients having reduced swollen and tender joint counts compared to the patients having no response to treatment with CTLA4Ig, L104EA29YIg, or placebo, is shown in Figures 4A and B. The therapeutic responses appear to be dose-dependent. A larger percentage of patients show improvement of 20, 50, 70, and even 100% in the 2 and 10 mg/kg groups for both products.

The percent of patients having reduced pain, disease activity evaluated by patient and physician mean score units with CTLA4Ig, L104EA29YIg, or placebo, is shown in Figures 5A, B, C, and D. The therapeutic responses, as monitored by the Likert scale,

appear to be dose-dependent in favor of the active treatment groups as compared to placebo on day 85. The Likert scale is a validated verbal rating scale using adjectives to rank the symptoms (The American College of Rheumatology Preliminary Core Set of Disease Activity Measures for Rheumatoid Arthritis Clinical Trials: Arthritis and Rheumatism, June 1993, 36(6):729-740).

The patient and physician assessments of disease activity change from the baseline by at least 2 units, resulting from treatment with CTLA4Ig, L104EA29YIg, or placebo, are shown in Figures 6A and B. The responses appear to be dose-dependent with more marked improvement for the higher doses of active drugs.

The percent reduction in C-reactive protein (CRP) levels in patients treated with CTLA4Ig, L104EA29YIg, or placebo, is shown in Figures 7A and B. The responses appear to be dose-dependent with a clear decrease for the 2 and 10 mg/kg active treatment groups. In addition, Figure 7B showed that the difference is quite significant compared to placebo with 95% confidence intervals. Figure 7C shows the changes in serum level changes from baseline at day 85.

The amount of serum soluble IL-2 receptor in patients treated with CTLA4Ig, L104EA29YIg, or placebo, is shown in Figure 8. The reduction in soluble IL-2 receptor levels appears to be dose-dependent.

The amount of serum soluble ICAM-1 and soluble E-selectin in patients treated with CTLA4Ig, L104EA29YIg, or placebo, is shown in Figure 33. The reduction in soluble ICAM-1 and soluble E-selectin levels appears to be dose-dependent.

The median and mean tender joint counts in patients treated with CTLA4Ig or placebo over time are shown in Figures 9A and B. The change from baseline (e.g., reduction in tender joints) appears to be more important in the 2 and 10 mg/kg treated groups, than in the placebo or 0.5 mg/kg groups.

The median and mean swollen joint counts in patients treated with CTLA4Ig or placebo over time are shown in Figures 10A and B. The change from baseline (e.g., reduction in swollen joints) appears to be more important in the 2 and 10 mg/kg treated groups than placebo or 0.5 mg/kg groups.

The mean pain assessment scores over time in patients treated with CTLA4Ig or placebo are shown in Figure 11. The change from baseline (e.g., reduction in pain) appears to be more important in the 2 and 10 mg/kg treated groups than placebo or 0.5 mg/kg groups.

The mean disease activity assessment scores assessed by patient or physician in patients treated with CTLA4Ig or placebo over time are shown in Figures 12A and B. The change from baseline (e.g., reduction in disease activity) appears to be more important in the 2 and 10 mg/kg treated groups than placebo or 0.5 mg/kg groups.

The median and mean tender joint counts in patients treated with L104EA29YIg (denoted as LEA in the figures) or placebo over time are shown in Figures 13A and B. The change from baseline (e.g., reduction in tender joints) appears to be dose-dependent.

The median and mean swollen joint counts in patients treated with L104EA29YIg (denoted as LEA in the figures) or placebo over time are shown in Figures 14A and B. The change from baseline (e.g., reduction in swollen joints) appears to be more important in the 2 and 10 mg/kg treated groups than placebo or 0.5 mg/kg groups.

The mean pain assessment scores in patients treated with L104EA29YIg (denoted as LEA in the figures) or placebo over time are shown in Figure 15. The change from baseline (e.g., reduction in pain) appears to be dose-dependent.

The mean disease activity assessment scores evaluated by patient or physician in patients treated with L104EA29YIg (denoted as LEA in the figures) or placebo over time are shown in Figures 16A and B. The change from baseline (e.g., reduction in disease activity) appears to be dose-dependent.

The percent improvement of physical disability assessed by HAQ at day 85 for patients treated with CTLA4Ig, L104EA29YIg, or placebo are shown in Figure 17 (Health Assessment Questionnaire (HAQ); Fries, J. F., et al., 1982 *J. of Rheumatology* 9:789-793). There is a clear dose dependent improvement with this parameter.

The changes from baseline for soluble IL-2r and C-reactive protein levels were dose-dependent in both treatment groups. After treatment, soluble IL-2r levels were -2%, -10%, and -22% for CTLA4Ig and –4%, -18%, and -32% for L104EA29YIg at 0.5, 2.0, and 10.0 mg/kg respectively, compared to +3% for the placebo. C-reactive protein levels were +12%, -15%, and –32% for CTLA4Ig and +47%, -33%, and –47% for L104EA29YIg at 0.5, 2.0, and 10.0 mg/kg respectively, compared to +20% for the placebo (Figure 7A).

No clinically remarkable findings with respect to routine hematology testing, chemistry laboratory testing with the exception of slight suppressions in IgA and IgG levels at the higher doses of both drugs, physical findings, or vital signs assessments were observed. Notably, neither medication induced drug-specific antibodies.

Example 4

The following examples describe phase II clinical studies of human patients that will be administered L104EA29YIg, to reduce or prevent structural damage, including bone or joint erosion using validated radiographic scales. This improvement in reducing or preventing structural damage is parallel to the clinical improvement measured by the clinical parameters.

The status of the bone structure is monitored in some of the human patients prior to treatment with CTLA4Ig or L104EA29YIg. These patients are administered between 0.5 and 20 mg/kg of CTLA4Ig or L104EA29YIg chronically every two to twelve weeks (alone or in combination with other agents) to maintain their therapeutic improvement

over time. Radiographs of patients' hands and feet are taken at predefined intervals: 6 months, and then yearly, as recommended by the FDA guidelines. These patients are monitored in long-term extension after 6 and 12 months to determine if treatment with CTLA4Ig or L104EA29YIg reduces the progression of bone deterioration, and then yearly. The patients are monitored by radiographic methods, including X-ray and/or magnetic resonance imaging (MRI), according to standard practice in the art (Larsen, A. K. and M. Eek 1977 *Acta. Radiol. Diag.* 18:481-491; Sharp, J. T., et al., 1985 *Arthritis and Rheumatism* 28:1326-1335). The results of the radiographic data are evaluated for prevention of structural damage, including slowing the progression of bone erosion and cartilage damage, with joint space narrowing and/or prevention of new erosions.

What is claimed:

Claim 1. The use of a first agent and a second agent, wherein

a) the first agent is a soluble CTLA4 fusion molecule comprising an extracellular domain of a CTLA4 molecule, wherein the extracellular domain of the CTLA4 molecule comprises the amino acids shown in Figure 23 beginning with methionine at position +1 or with alanine at position -1 and ending with aspartic acid at position 124 (SEQ ID NO:17 beginning with Met at position 27 or with Ala at position 26 and ending with Asp at 150), and

b) the second agent is selected from a group consisting of corticosteroids, nonsteroidal anti-inflammatory drugs, prednisone, azathioprine, TNFα blockers or antagonists, methotrexate, infliximab, hydroxychloroquine, sulphasalazopryine, gold salts, etanercept, anakinra, cyclophosphamide and leflunomide, in the manufacture of a pharmaceutical composition for treating a rheumatic disease.

Claim 2. The use of a soluble CTLA4 fusion molecule comprising an extracellular domain of a CTLA4 molecule, wherein the extracellular domain of the CTLA4 molecule comprises the amino acids shown in Figure 23 beginning with methionine at position +1 or with alanine at position -1 and ending with aspartic acid at position 124 (SEQ ID NO:17 beginning with Met at position 27 or with Ala at position 26 and ending with Asp at 150) in the manufacture of a pharmaceutical composition for administering to a subject the soluble CTLA4 fusion molecule in an amount of 0.5 mg/kg weight of the subject, 2 mg/kg weight of the subject, 10 mg/kg weight of the subject, 0.5 to 10 mg/kg weight of the subject or 0.1 to 20 mg/kg weight of the subject for treating a rheumatic disease.

Claim 3. The use of claim 1 or 2, wherein treating rheumatic disease improves ACR 20, 50 and/or 70 response rates.

Claims 4. The use of claim 1 or 2, wherein the soluble CTLA4 fusion molecule comprises an extracellular domain of a CTLA4 molecule which binds a B7 antigen expressed on activated B cells joined to a non-CTLA4 molecule.

Claims 5. The use of 4, wherein the non-CTLA4 molecule comprises an amino acid sequence which alters the solubility or affinity of the soluble CTLA4 fusion molecule.

Claim 6. The use of claim 5, wherein the amino acid sequence which alters the solubility or affinity comprises an immunoglobulin moiety.

Claim 7. The use of claim 6, wherein the immunoglobulin moiety is an immunoglobulin constant region or portion thereof.

Claim 8. The use of claim 7, wherein the immunoglobulin constant region or portion thereof is mutated to reduce effector function.

Claim 9. The use of claim 6, wherein the immunoglobulin moiety comprises one or more mutations to reduce effector function.

Claim 10. The use of claim 7, wherein the immunoglobulin constant region comprises a hinge, CH2 and CH3 regions of an immunoglobulin molecule.

Claim 11. The use of claim 7, wherein the immunoglobulin constant region or portion thereof is a human or monkey immunoglobulin constant region.

Claim 12. The use of claim 1 or 2, wherein the CTLA4 fusion molecule is CTLA4Ig shown in Figure 24 beginning with methionine at position +1 or with alanine at position -1 and ending with lysine at position +357 (SEQ ID NO:19 beginning with Met at position 27 or with Ala at position 26 and ending with Lys at 383).

Claim 13. The use of a soluble CTLA4 fusion molecule and a second agent, wherein

a) the soluble CTLA4 fusion molecule is CTLA4Ig beginning with methionine at position +1 or with alanine at position -1 and ending with lysine as position +357 as shown in Figure 24 (SEQ ID NO:19 beginning with Met at position 27 or with Ala at position 26 and ending with Lys at 383) , and

b) the second agent is selected from a group consisting of corticosteroids, nonsteroidal anti-inflammatory drugs, prednisone, azathioprine, TNFα blockers or antagonists, methotrexate, infliximab, hydroxychloroquine, sulphasalazopryine, gold salts, etanercept, anakinra, cyclophosphamide and leflunomide, in the manufacture of a pharmaceutical composition for treating rheumatoid arthritis.

Claim 14. The use of claim 1 or 2 wherein the rheumatic disease is selected from rheumatoid arthritis, polymyositis, scleroderma, mixed connective tissue disease, inflammatory rheumatism, degenerative rheumatism, extra-articular rheumatism, collagen diseases, chronic polyarthritis, psoriasis arthropathica, ankylosing spondylitis, juvenile rheumatoid arthritis, panarteriitis nodosa, systemic lupus erythematosus, progressive systemic scleroderma, periarthritis humeroscapularis, arthritis uratica, chondrocalcinosis, dermatomyositis, muscular rheumatism, myositis and myogelosis.

Claim 15. The use of claim 14, wherein the rheumatic disease is rheumatoid arthritis.

Claim 16. The use of claim 1, 2, or 13, wherein a symptom associated with the rheumatic disease is reduced and wherein the symptom is selected from the group consisting of joint swelling, pain, tenderness, morning stiffness, structural damage, an elevated level of serum C-reactive protein, an elevated level of soluble IL-2r, an elevated level of soluble ICAM-1, an elevated level of soluble E-selectin and an elevated erythrocyte sedimentation rate.

Claim 17. The use of claim 1, 2, 13 or 15, for administering to a subject the soluble CTLA4 fusion molecule in an amount of 0.5 mg/kg weight of the subject.

Claim 18. The use of claim 1, 2, 13 or 15, for administering to a subject the soluble CTLA4 fusion molecule in an amount of 2 mg/kg weight of the subject.

Claim 19. The use of claim 1, 2, 13 or 15, for administering to a subject the soluble CTLA4 fusion molecule in an amount of 10 mg/kg weight of the subject.

Claim 20. The use of claim 1, 2, 13 or 15, for administering to a subject the soluble CTLA4 fusion molecule in an amount of 0.5 to 10 mg/kg weight of the subject.

Claim 21. The use of claim 1, 2, 13 or 15 for administering to a subject the soluble CTLA4 fusion molecule in an amount of 0.1 to 20 mg/kg weight of the subject.

Claim 22. The use of a soluble CTLA4 mutant molecule comprising the sequence shown in Figure 19 beginning with methionine at position +1 or with alanine at position -1 and ending with aspartic acid at position +124 (SEQ ID NO: 9 beginning with Met at position 27 or with Ala at position 26 and

ending with Asp at 150) in the manufacture of a pharmaceutical composition for treating a disease selected from autoimmune diseases, graft transplantation rejection, psoriasis, T cell lymphoma, T cell acute lymphoblastic leukemia, testicular angiocentric T cell lymphoma, benign lymphocytic angiitis, lupus erythematosus, Hashimoto's thyroiditis, primary myxedema, Graves' disease, pernicious anemia, autoimmune atrophic gastritis, Addison's disease, insulin dependent diabetes mellitis, good pasture's syndrome, myasthenia gravis, pemphigus, Crohn's disease, sympathetic ophthalmia, autoimmune uveitis, multiple sclerosis, autoimmune hemolytic anemia, idiopathic thrombocytopenia, primary biliary cirrhosis, chronic action hepatitis, ulceratis colitis, Sjogren's syndrome, rheumatic disease, rheumatoid arthritis, polymyositis, scleroderma, mixed connective tissue disease, inflammatory rheumatism, degenerative rheumatism, extra-articular rheumatism, collagen diseases, chronic polyarthritis, psoriasis arthropathica, ankylosing spondylitis, juvenile rheumatoid arthritis, panarteriitis nodosa, systemic lupus erythematosus, progressive systemic scleroderma, periarthritis humeroscapularis, arthritis uratica, chondrocalcinosis, dermatomyositis, muscular rheumatism, myositis and myogelosis

Claim 23. The use of a soluble CTLA4 mutant molecule L104EA29YIg comprising the sequence shown in Figure 19 beginning with methionine at position +1 or with alanine at position -1 and ending with lysine as position +357 (SEQ ID NO: 9 beginning with Met at position 27 or with Ala at position 26 and ending with Lys at 383) in the manufacture of a pharmaceutical composition for treating a disease selected from autoimmune diseases, graft transplantation rejection, psoriasis, T cell lymphoma, T cell acute lymphoblastic leukemia, testicular angiocentric T cell lymphoma, benign lymphocytic angiitis, lupus erythematosus, Hashimoto's thyroiditis, primary myxedema, Graves' disease, pernicious anemia, autoimmune atrophic gastritis, Addison's disease, insulin dependent diabetes mellitis, good pasture's syndrome, myasthenia gravis, pemphigus, Crohn's disease, sympathetic ophthalmia, autoimmune uveitis, multiple sclerosis, autoimmune hemolytic anemia, idiopathic thrombocytopenia, primary biliary cirrhosis, chronic action hepatitis, ulceratis colitis, Sjogren's syndrome, rheumatic disease, rheumatoid arthritis, polymyositis, scleroderma, mixed connective tissue disease, inflammatory rheumatism, degenerative rheumatism, extra-articular rheumatism, collagen diseases, chronic polyarthritis, psoriasis arthropathica, ankylosing spondylitis, juvenile rheumatoid arthritis, panarteriitis nodosa, systemic lupus erythematosus, progressive systemic scleroderma, periarthritis humeroscapularis, arthritis uratica, chondrocalcinosis, dermatomyositis, muscular rheumatism, myositis and myogelosis.

Claim 24. The use of a soluble CTLA4 mutant molecule comprising the sequence shown in Figure 19 beginning with methionine at position +1 or with alanine at position -1 and ending with aspartic acid at position +124 (SEQ ID NO: 9 beginning with Met at position 27 or with Ala at position 26 and

ending with Asp at 150) in the manufacture of a pharmaceutical composition for treating graft transplantation rejection.

Claim 25. The use of a soluble CTLA4 fusion molecule L104EA29YIg having the sequence shown in Figure 19 beginning with methionine at position +1 or with alanine at position -1 and ending with lysine as position +357 (SEQ ID NO: 9 beginning with Met at position 27 or with Ala at position 26 and ending with Lys at 383) in the manufacture of a pharmaceutical composition for treating graft transplantation rejection.

Claim 26. The use of claim 22, 23, 24 or 25, for administering to a subject the soluble CTLA4 fusion molecule in an amount of 0.5 mg/kg weight of the subject, 2 mg/kg weight of the subject, 10 mg/kg weight of the subject, 0.5 to 10 mg/kg weight of a subject or 0.1 to 20 mg/kg weight of a subject.

Claim 27. The use of claim 16 wherein structural damage is reduced.

Claim 28. The use of a first agent and a second agent, wherein

a) the first agent is CTLA4Ig encoded by a nucleic acid molecule designated ATCC No. 68629, and

b) the second agent is selected from a group consisting of corticosteroids, nonsteroidal anti-inflammatory drugs, prednisone, azathioprine, TNFα blockers or antagonists, methotrexate, infliximab, hydroxychloroquine, sulphasalazopryine, gold salts, etanercept, anakinra, cyclophosphamide and leflunomide,in the manufacture of a pharmaceutical composition for treating rheumatoid arthritis.

Claim 29. The use of a soluble CTLA4 fusion molecule L104EA29YIg encoded by a DNA sequence designated ATCC PTA-2104. in the manufacture of a pharmaceutical composition for treating graft transplantation rejection.

Claim 30. The use of a soluble CTLA4 fusion molecule comprising an extracellular domain of a CTLA4 molecule, which binds a B7 antigen expressed on activated B cells, wherein the extracellular domain of the CTLA4 molecule comprises the amino acids shown in Figure 23 beginning with methionine at position +1 or with alanine at position -1 and ending with aspartic acid at position 124 (SEQ ID NO:17 beginning with Met at position 27 or with Ala at position 26 and ending with Asp at 150) in the manufacture of a pharmaceutical composition for administering to a subject the soluble CTLA4

fusion molecule in an amount of 10 mg/kg weight of the subject, 0.5 to 10 mg/kg weight of the subject, or 0.1 to 20 mg/kg weight of the subject, for treating rheumatoid arthritis.

Claim 31. The use of CTLA4Ig encoded by a nucleic acid molecule designated ATCC No. 68629 in the manufacture of a pharmaceutical composition for administering to a subject the soluble CTLA4 fusion molecule in an amount of 10 mg/kg weight of the subject, 0.5 to 10 mg/kg weight of the subject, or 0.1 to 20 mg/kg weight of the subject, for treating rheumatoid arthritis.

Claim 32. The use of claim 1, 13, 19, 20, 21 or 28 wherein the second agent is methotrexate.

Claim 33. The use of a soluble CTLA4 fusion molecule and a second agent, wherein

a) the soluble CTLA4 fusion molecule is CTLA4Ig beginning with methionine at position +1 or with alanine at position -1 and ending with lysine as position +357 as shown in Figure 24 (SEQ ID NO:19 beginning with Met at position 27 or with Ala at position 26 and ending with Lys at 383), and

b) the second agent is methotrexate, in the manufacture of a pharmaceutical composition for administering to a subject the soluble CTLA4 fusion molecule in an amount of 10 mg/kg weight of the subject for treating rheumatoid arthritis.

Claim 34. The use of claim 2, 17, 18, 19, 20, 21, 26, 30, 31 or 33 wherein the soluble CTLA4 fusion molecule is infused intravenously on days 1, 15, 29 and 57.

SEQUENCE LISTING

<110> Cohen, Robert
Carr, Suzette
Hagerty, David
Peach, Robert J
Becker, Jean-Claude
Bristol-Myers Squibb Company

<120> METHODS FOR TREATING RHEUMATIC DISEASES USING A SOLUBLE CTLA4 MOLECULE

<130> D0030PCT;30436.55WOU1

<140> PCT/US01/21204
<141> 2001-07-02

<150> 60/215,913
<151> 2000-07-03

<160> 19

<170> PatentIn Ver. 2.1

<210> 1
<211> 65
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CTLA4 fused to Oncostatin M, forward primer

<400> 1
ctcagtctgg tccttgcact cctgtttcca agcatggcga gcatggcaat gcacgtggcc 60
cagcc 65

<210> 2
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CTLA4 fused to Oncostatin M, reverse primer

<400> 2
tttgggctcc tgatcagaat ctgggcacgg ttg 33

<210> 3
<211> 72
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:CTLA4 with
Oncostatin M and Hind III

<400> 3
ctagccactg aagcttcacc aatgggtgta ctgctcacac agaggacgct gctcagtctg 60
gtccttgcac tc 72

<210> 4
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oncostatin M
CTLA4 (OMCTLA4) Forward Primer

<400> 4
gaggtgataa agcttcacca atgggtgtac tgctcacaca g 41

<210> 5
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oncostatin M
CTLA4 (OMCTLA4) Reverse Primer

<400> 5
gtggtgtatt ggtctagatc aatcagaatc tgggcacggt tc 42

<210> 6
<211> 1152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:L104EIg

<400> 6
atgggtgtac tgctcacaca gaggacgctg ctcagtctgg tccttgcact cctgtttcca 60
agcatggcga gcatggcaat gcacgtggcc cagcctgctg tggtactggc cagcagccga 120
ggcatcgcta gctttgtgtg tgagtatgca tctccaggca aagccactga ggtccgggtg 180
acagtgcttc ggcaggctga cagccaggtg actgaagtct gtgcggcaac ctacatgatg 240
gggaatgagt tgaccttcct agatgattcc atctgcacgg gcacctccag tggaaatcaa 300
gtgaacctca ctatccaagg actgagggcc atggacacgg gactctacat ctgcaaggtg 360
gagctcatgt acccaccgcc atactacgag ggcataggca acggaaccca gatttatgta 420
attgatccag aaccgtgccc agattctgat caggagccca aatcttctga caaaactcac 480
acatccccac cgtccccagc acctgaactc ctgggggggat cgtcagtctt cctcttcccc 540
ccaaaaccca aggacaccct catgatctcc cggacccctg aggtcacatg cgtggtggtg 600
gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt acgtggacgg cgtggaggtg 660
cataatgcca agacaaagcc gcgggaggag cagtacaaca gcacgtaccg tgtggtcagc 720
gtcctcaccg tcctgcacca ggactggctg aatggcaagg agtacaagtg caaggtctcc 780
aacaaagccc tcccagcccc catcgagaaa accatctcca aagccaaagg gcagccccga 840

```
gaaccacagg tgtacaccct gcccccatcc cgggatgagc tgaccaagaa ccaggtcagc 900
ctgacctgcc tggtcaaagg cttctatccc agcgacatcg ccgtggagtg ggagagcaat 960
gggcagccgg agaacaacta caagaccacg cctcccgtgc tggactccga cggctccttc 1020
ttcctctaca gcaagctcac cgtggacaag agcaggtggc agcaggggaa cgtcttctca 1080
tgctccgtga tgcatgaggc tctgcacaac cactacacgc agaagagcct ctccctgtct 1140
ccgggtaaat ga                                                     1152
```

<210> 7
<211> 383
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:L104EIg

<400> 7

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

Leu Leu Phe Pro Ser Met Ala Ser Met Ala Met His Val Ala Gln Pro
            20                  25                  30

Ala Val Val Leu Ala Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu
         35                  40                  45

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

Gln Ala Asp Ser Gln Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met
 65                  70                  75                  80

Gly Asn Glu Leu Thr Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser
                85                  90                  95

Ser Gly Asn Gln Val Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp
            100                 105                 110

Thr Gly Leu Tyr Ile Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr
        115                 120                 125

Tyr Glu Gly Ile Gly Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu
    130                 135                 140

Pro Cys Pro Asp Ser Asp Gln Glu Pro Lys Ser Ser Asp Lys Thr His
145                 150                 155                 160

Thr Ser Pro Pro Ser Pro Ala Pro Glu Leu Leu Gly Gly Ser Ser Val
                165                 170                 175

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
            180                 185                 190

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
        195                 200                 205

Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
```

```
    210                 215                 220

Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
225                 230                 235                 240

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                245                 250                 255

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
            260                 265                 270

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
        275                 280                 285

Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
    290                 295                 300

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
305                 310                 315                 320

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                325                 330                 335

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
            340                 345                 350

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
        355                 360                 365

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    370                 375                 380
```

<210> 8
<211> 1152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:L104EA29YIg

<400> 8
atgggtgtac tgctcacaca gaggacgctg ctcagtctgg tccttgcact cctgtttcca 60
agcatggcga gcatggcaat gcacgtggcc cagcctgctg tggtactggc cagcagccga 120
ggcatcgcta gctttgtgtg tgagtatgca tctccaggca aatatactga ggtccgggtg 180
acagtgcttc ggcaggctga cagccaggtg actgaagtct gtgcggcaac ctacatgatg 240
gggaatgagt tgaccttcct agatgattcc atctgcacgg gcacctccag tggaaatcaa 300
gtgaacctca ctatccaagg actgagggcc atggacacgg gactctacat ctgcaaggtg 360
gagctcatgt acccaccgcc atactacgag ggcataggca acggaaccca gatttatgta 420
attgatccag aaccgtgccc agattctgat caggagccca aatcttctga caaaactcac 480
acatccccac cgtccccagc acctgaactc ctgggggggat cgtcagtctt cctcttcccc 540
ccaaaaccca aggacaccct catgatctcc cggacccctg aggtcacatg cgtggtggtg 600
gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt acgtggacgg cgtggaggtg 660
cataatgcca agacaaagcc gcgggaggag cagtacaaca gcacgtaccg tgtggtcagc 720
gtcctcaccg tcctgcacca ggactggctg aatggcaagg agtacaagtg caaggtctcc 780
aacaaagccc tcccagcccc catcgagaaa accatctcca aagccaaagg gcagccccga 840
gaaccacagg tgtacaccct gcccccatcc cgggatgagc tgaccaagaa ccaggtcagc 900
```
```

ctgacctgcc tggtcaaagg cttctatccc agcgacatcg ccgtggagtg ggagagcaat 960
gggcagccgg agaacaacta caagaccacg cctcccgtgc tggactccga cggctccttc 1020
ttcctctaca gcaagctcac cgtggacaag agcaggtggc agcaggggaa cgtcttctca 1080
tgctccgtga tgcatgaggc tctgcacaac cactacacgc agaagagcct ctccctgtct 1140
ccgggtaaat ga 1152

<210> 9
<211> 383
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:L104EA29YIg

<400> 9
Met Gly Val Leu Leu Thr Gln Arg Thr Leu Leu Ser Leu Val Leu Ala
1 5 10 15

Leu Leu Phe Pro Ser Met Ala Ser Met Ala Met His Val Ala Gln Pro
20 25 30

Ala Val Val Leu Ala Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu
35 40 45

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

Gln Ala Asp Ser Gln Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met
65 70 75 80

Gly Asn Glu Leu Thr Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser
85 90 95

Ser Gly Asn Gln Val Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp
100 105 110

Thr Gly Leu Tyr Ile Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr
115 120 125

Tyr Glu Gly Ile Gly Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu
130 135 140

Pro Cys Pro Asp Ser Asp Gln Glu Pro Lys Ser Ser Asp Lys Thr His
145 150 155 160

Thr Ser Pro Pro Ser Pro Ala Pro Glu Leu Leu Gly Gly Ser Ser Val
165 170 175

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
180 185 190

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
195 200 205

Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
210 215 220

```
Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
225                 230                 235                 240

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                245                 250                 255

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
            260                 265                 270

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
        275                 280                 285

Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
    290                 295                 300

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
305                 310                 315                 320

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                325                 330                 335

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
            340                 345                 350

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
        355                 360                 365

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    370                 375                 380
```

<210> 10
<211> 1152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: L104EA29LIg

<400> 10
atgggtgtac tgctcacaca gaggacgctg ctcagtctgg tccttgcact cctgtttcca 60
agcatggcga gcatggcaat gcacgtggcc cagcctgctg tggtactggc cagcagccga 120
ggcatcgcta gctttgtgtg tgagtatgca tctccaggca aattgactga ggtccgggtg 180
acagtgcttc ggcaggctga cagccaggtg actgaagtct gtgcggcaac ctacatgatg 240
gggaatgagt tgaccttcct agatgattcc atctgcacgg gcacctccag tggaaatcaa 300
gtgaacctca ctatccaagg actgagggcc atggacacgg gactctacat ctgcaaggtg 360
gagctcatgt acccaccgcc atactacgag ggcataggca acggaaccca gatttatgta 420
attgatccag aaccgtgccc agattctgat caggagccca aatcttctga caaaactcac 480
acatccccac cgtccccagc acctgaactc ctgggggggat cgtcagtctt cctcttcccc 540
ccaaaaccca aggacaccct catgatctcc cggacccctg aggtcacatg cgtggtggtg 600
gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt acgtggacgg cgtggaggtg 660
cataatgcca agacaaagcc gcgggaggag cagtacaaca gcacgtaccg tgtggtcagc 720
gtcctcaccg tcctgcacca ggactggctg aatggcaagg agtacaagtg caaggtctcc 780
aacaaagccc tcccagcccc catcgagaaa accatctcca aagccaaagg gcagccccga 840
gaaccacagg tgtacaccct gcccccatcc cgggatgagc tgaccaagaa ccaggtcagc 900
ctgacctgcc tggtcaaagg cttctatccc agcgacatcg ccgtggagtg ggagagcaat 960

gggcagccgg agaacaacta caagaccacg cctcccgtgc tggactccga cggctccttc 1020
ttcctctaca gcaagctcac cgtggacaag agcaggtggc agcaggggaa cgtcttctca 1080
tgctccgtga tgcatgaggc tctgcacaac cactacacgc agaagagcct ctccctgtct 1140
ccgggtaaat ga 1152

<210> 11
<211> 383
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: L104EA29LIg

<400> 11
Met Gly Val Leu Leu Thr Gln Arg Thr Leu Leu Ser Leu Val Leu Ala
1 5 10 15

Leu Leu Phe Pro Ser Met Ala Ser Met Ala Met His Val Ala Gln Pro
20 25 30

Ala Val Val Leu Ala Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu
35 40 45

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

Gln Ala Asp Ser Gln Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met
65 70 75 80

Gly Asn Glu Leu Thr Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser
85 90 95

Ser Gly Asn Gln Val Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp
100 105 110

Thr Gly Leu Tyr Ile Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr
115 120 125

Tyr Glu Gly Ile Gly Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu
130 135 140

Pro Cys Pro Asp Ser Asp Gln Glu Pro Lys Ser Ser Asp Lys Thr His
145 150 155 160

Thr Ser Pro Pro Ser Pro Ala Pro Glu Leu Leu Gly Gly Ser Ser Val
165 170 175

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
180 185 190

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
195 200 205

Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
210 215 220

```
Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
225                 230                 235                 240

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                245                 250                 255

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
            260                 265                 270

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
        275                 280                 285

Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
    290                 295                 300

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
305                 310                 315                 320

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                325                 330                 335

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
            340                 345                 350

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
        355                 360                 365

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    370                 375                 380
```

<210> 12
<211> 1152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: L104EA29TIg

<400> 12
atgggtgtac tgctcacaca gaggacgctg ctcagtctgg tccttgcact cctgtttcca 60
agcatggcga gcatggcaat gcacgtggcc cagcctgctg tggtactggc cagcagccga 120
ggcatcgcta gctttgtgtg tgagtatgca tctccaggca aaactactga ggtccgggtg 180
acagtgcttc ggcaggctga cagccaggtg actgaagtct gtgcggcaac ctacatgatg 240
gggaatgagt tgaccttcct agatgattcc atctgcacgg gcacctccag tggaaatcaa 300
gtgaacctca ctatccaagg actgagggcc atggacacgg gactctacat ctgcaaggtg 360
gagctcatgt acccaccgcc atactacgag ggcataggca acggaaccca gatttatgta 420
attgatccag aaccgtgccc agattctgat caggagccca aatcttctga caaaactcac 480
acatccccac cgtccccagc acctgaactc ctgggggggat cgtcagtctt cctcttcccc 540
ccaaaaccca aggacaccct catgatctcc cggacccctg aggtcacatg cgtggtggtg 600
gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt acgtggacgg cgtggaggtg 660
cataatgcca agacaaagcc gcgggaggag cagtacaaca gcacgtaccg tgtggtcagc 720
gtcctcaccg tcctgcacca ggactggctg aatggcaagg agtacaagtg caaggtctcc 780
aacaaagccc tcccagcccc catcgagaaa accatctcca aagccaaagg gcagccccga 840
gaaccacagg tgtacaccct gccccatcc cgggatgagc tgaccaagaa ccaggtcagc 900
ctgacctgcc tggtcaaagg cttctatccc agcgacatcg ccgtggagtg ggagagcaat 960
gggcagccgg agaacaacta caagaccacg cctcccgtgc tggactccga cggctccttc 1020
```
```

ttcctctaca gcaagctcac cgtggacaag agcaggtggc agcaggggaa cgtcttctca 1080
tgctccgtga tgcatgaggc tctgcacaac cactacacgc agaagagcct ctccctgtct 1140
ccgggtaaat ga 1152

<210> 13
<211> 383
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: L104EA29TIg

<400> 13
Met Gly Val Leu Leu Thr Gln Arg Thr Leu Leu Ser Leu Val Leu Ala
1 5 10 15

Leu Leu Phe Pro Ser Met Ala Ser Met Ala Met His Val Ala Gln Pro
20 25 30

Ala Val Val Leu Ala Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu
35 40 45

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

Gln Ala Asp Ser Gln Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met
65 70 75 80

Gly Asn Glu Leu Thr Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser
85 90 95

Ser Gly Asn Gln Val Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp
100 105 110

Thr Gly Leu Tyr Ile Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr
115 120 125

Tyr Glu Gly Ile Gly Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu
130 135 140

Pro Cys Pro Asp Ser Asp Gln Glu Pro Lys Ser Ser Asp Lys Thr His
145 150 155 160

Thr Ser Pro Pro Ser Pro Ala Pro Glu Leu Leu Gly Gly Ser Ser Val
165 170 175

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
180 185 190

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
195 200 205

Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
210 215 220

Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser

```
225                 230                 235                 240

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                245                 250                 255

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
            260                 265                 270

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
        275                 280                 285

Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
    290                 295                 300

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
305                 310                 315                 320

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                325                 330                 335

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
            340                 345                 350

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
        355                 360                 365

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    370                 375                 380
```

<210> 14
<211> 1152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: L104EA29WIg

<400> 14

```
atgggtgtac tgctcacaca gaggacgctg ctcagtctgg tccttgcact cctgtttcca 60
agcatggcga gcatggcaat gcacgtggcc cagcctgctg tggtactggc cagcagccga 120
ggcatcgcta gctttgtgtg tgagtatgca tctccaggca aatggactga ggtccgggtg 180
acagtgcttc ggcaggctga cagccaggtg actgaagtct gtgcggcaac ctacatgatg 240
gggaatgagt tgaccttcct agatgattcc atctgcacgg gcacctccag tggaaatcaa 300
gtgaacctca ctatccaagg actgagggcc atggacacgg gactctacat ctgcaaggtg 360
gagctcatgt acccaccgcc atactacgag ggcataggca acggaaccca gatttatgta 420
attgatccag aaccgtgccc agattctgat caggagccca aatcttctga caaaactcac 480
acatccccac cgtccccagc acctgaactc ctgggggggat cgtcagtctt cctcttcccc 540
ccaaaaccca aggacaccct catgatctcc cggacccctg aggtcacatg cgtggtggtg 600
gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt acgtggacgg cgtggaggtg 660
cataatgcca agacaaagcc gcgggaggag cagtacaaca gcacgtaccg tgtggtcagc 720
gtcctcaccg tcctgcacca ggactggctg aatggcaagg agtacaagtg caaggtctcc 780
aacaaagccc tcccagcccc catcgagaaa accatctcca aagccaaagg gcagccccga 840
gaaccacagg tgtacaccct gcccccatcc cgggatgagc tgaccaagaa ccaggtcagc 900
ctgacctgcc tggtcaaagg cttctatccc agcgacatcg ccgtggagtg ggagagcaat 960
gggcagccgg agaacaacta caagaccacg cctcccgtgc tggactccga cggctccttc 1020
ttcctctaca gcaagctcac cgtggacaag agcaggtggc agcaggggaa cgtcttctca 1080
```

tgctccgtga tgcatgaggc tctgcacaac cactacacgc agaagagcct ctccctgtct 1140
ccgggtaaat ga 1152

<210> 15
<211> 383
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: L104EA29WIg

<400> 15

Met	Gly	Val	Leu	Leu	Thr	Gln	Arg	Thr	Leu	Leu	Ser	Leu	Val	Leu	Ala
1				5				10						15	
Leu	Leu	Phe	Pro	Ser	Met	Ala	Ser	Met	Ala	Met	His	Val	Ala	Gln	Pro
			20					25					30		
Ala	Val	Val	Leu	Ala	Ser	Ser	Arg	Gly	Ile	Ala	Ser	Phe	Val	Cys	Glu
		35					40					45			
Tyr	Ala	Ser	Pro	Gly	Lys	Trp	Thr	Glu	Val	Arg	Val	Thr	Val	Leu	Arg
	50					55					60				
Gln	Ala	Asp	Ser	Gln	Val	Thr	Glu	Val	Cys	Ala	Ala	Thr	Tyr	Met	Met
65					70					75					80
Gly	Asn	Glu	Leu	Thr	Phe	Leu	Asp	Asp	Ser	Ile	Cys	Thr	Gly	Thr	Ser
				85					90					95	
Ser	Gly	Asn	Gln	Val	Asn	Leu	Thr	Ile	Gln	Gly	Leu	Arg	Ala	Met	Asp
			100					105					110		
Thr	Gly	Leu	Tyr	Ile	Cys	Lys	Val	Glu	Leu	Met	Tyr	Pro	Pro	Pro	Tyr
		115					120					125			
Tyr	Glu	Gly	Ile	Gly	Asn	Gly	Thr	Gln	Ile	Tyr	Val	Ile	Asp	Pro	Glu
	130					135					140				
Pro	Cys	Pro	Asp	Ser	Asp	Gln	Glu	Pro	Lys	Ser	Ser	Asp	Lys	Thr	His
145					150					155					160
Thr	Ser	Pro	Pro	Ser	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Ser	Ser	Val
				165					170					175	
Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr
			180					185					190		
Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu
		195					200					205			
Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys
	210					215					220				
Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser
225					230					235					240

```
Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                245                 250                 255

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
            260                 265                 270

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
        275                 280                 285

Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
    290                 295                 300

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
305                 310                 315                 320

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                325                 330                 335

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
            340                 345                 350

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
        355                 360                 365

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    370                 375                 380
```

<210> 16
<211> 636
<212> DNA
<213> Homo sapiens

<400> 16
atgggtgtac tgctcacaca gaggacgctg ctcagtctgg tccttgcact cctgtttcca 60
agcatggcga gcatggcaat gcacgtggcc cagcctgctg tggtactggc cagcagccga 120
ggcatcgcca gctttgtgtg tgagtatgca tctccaggca aagccactga ggtccgggtg 180
acagtgcttc ggcaggctga cagccaggtg actgaagtct gtgcggcaac ctacatgatg 240
gggaatgagt tgaccttcct agatgattcc atctgcacgg gcacctccag tggaaatcaa 300
gtgaacctca ctatccaagg actgagggcc atggacacgg gactctacat ctgcaaggtg 360
gagctcatgt acccaccgcc atactacctg ggcataggca acggaaccca gatttatgta 420
attgatccag aaccgtgccc agattctgac ttcctcctct ggatccttgc agcagttagt 480
tcggggttgt ttttttatag ctttctcctc acagctgttt ctttgagcaa aatgctaaag 540
aaaagaagcc ctcttacaac aggggtctat gtgaaaatgc ccccaacaga gccagaatgt 600
gaaaagcaat ttcagcctta ttttattccc atcaat 636

<210> 17
<211> 212
<212> PRT
<213> Homo sapiens

<400> 17
Met Gly Val Leu Leu Thr Gln Arg Thr Leu Leu Ser Leu Val Leu Ala
  1   5   10   15

```
Leu Leu Phe Pro Ser Met Ala Ser Met Ala Met His Val Ala Gln Pro
            20                  25                  30

Ala Val Val Leu Ala Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu
        35                  40                  45

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

Gln Ala Asp Ser Gln Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met
65                  70                  75                  80

Gly Asn Glu Leu Thr Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser
                85                  90                  95

Ser Gly Asn Gln Val Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp
            100                 105                 110

Thr Gly Leu Tyr Ile Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr
        115                 120                 125

Tyr Leu Gly Ile Gly Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu
    130                 135                 140

Pro Cys Pro Asp Ser Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser
145                 150                 155                 160

Ser Gly Leu Phe Phe Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser
                165                 170                 175

Lys Met Leu Lys Lys Arg Ser Pro Leu Thr Thr Gly Val Tyr Val Lys
            180                 185                 190

Met Pro Pro Thr Glu Pro Glu Cys Glu Lys Gln Phe Gln Pro Tyr Phe
        195                 200                 205

Ile Pro Ile Asn
    210
```

<210> 18
<211> 1152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CTLA4Ig

<400> 18
atgggtgtac tgctcacaca gaggacgctg ctcagtctgg tccttgcact cctgtttcca 60
agcatggcga gcatggcaat gcacgtggcc cagcctgctg tggtactggc cagcagccga 120
ggcatcgcta gctttgtgtg tgagtatgca tctccaggca aagccactga ggtccgggtg 180
acagtgcttc ggcaggctga cagccaggtg actgaagtct gtgcggcaac ctacatgatg 240
gggaatgagt tgaccttcct agatgattcc atctgcacgg gcacctccag tggaaatcaa 300
gtgaacctca ctatccaagg actgagggcc atggacacgg gactctacat ctgcaaggtg 360
gagctcatgt acccaccgcc atactacctg ggcataggca acggaaccca gatttatgta 420
attgatccag aaccgtgccc agattctgat caggagccca aatcttctga caaaactcac 480

acatccccac cgtccccagc acctgaactc ctgggtggat cgtcagtctt cctcttcccc 540
ccaaaaccca aggacaccct catgatctcc cggacccctg aggtcacatg cgtggtggtg 600
gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt acgtggacgg cgtggaggtg 660
cataatgcca agacaaagcc gcgggaggag cagtacaaca gcacgtaccg ggtggtcagc 720
gtcctcaccg tcctgcacca ggactggctg aatggcaagg agtacaagtg caaggtctcc 780
aacaaagccc tcccagcccc catcgagaaa accatctcca aagccaaagg gcagccccga 840
gaaccacagg tgtacaccct gcccccatcc cgggatgagc tgaccaagaa ccaggtcagc 900
ctgacctgcc tggtcaaagg cttctatccc agcgacatcg ccgtggagtg ggagagcaat 960
gggcagccgg agaacaacta caagaccacg cctcccgtgc tggactccga cggctccttc 1020
ttcctctaca gcaagctcac cgtggacaag agcaggtggc agcaggggaa cgtcttctca 1080
tgctccgtga tgcatgaggc tctgcacaac cactacacgc agaagagcct ctccctgtct 1140
ccgggtaaat ga 1152

<210> 19
<211> 383
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CTLA4Ig

<400> 19
Met Gly Val Leu Leu Thr Gln Arg Thr Leu Leu Ser Leu Val Leu Ala
1 5 10 15

Leu Leu Phe Pro Ser Met Ala Ser Met Ala Met His Val Ala Gln Pro
20 25 30

Ala Val Val Leu Ala Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu
35 40 45

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

Gln Ala Asp Ser Gln Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met
65 70 75 80

Gly Asn Glu Leu Thr Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser
85 90 95

Ser Gly Asn Gln Val Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp
100 105 110

Thr Gly Leu Tyr Ile Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr
115 120 125

Tyr Leu Gly Ile Gly Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu
130 135 140

Pro Cys Pro Asp Ser Asp Gln Glu Pro Lys Ser Ser Asp Lys Thr His
145 150 155 160

Thr Ser Pro Pro Ser Pro Ala Pro Glu Leu Leu Gly Gly Ser Ser Val
165 170 175

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr

```
            180                 185                 190

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
        195                 200                 205

Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
    210                 215                 220

Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
225                 230                 235                 240

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                245                 250                 255

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
            260                 265                 270

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
        275                 280                 285

Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
    290                 295                 300

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
305                 310                 315                 320

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                325                 330                 335

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
            340                 345                 350

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
        355                 360                 365

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    370                 375                 380
```