METHODS FOR TREATING CARDIOVASCULAR DISEASE 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 cardiovascular diseases. In particular, the invention relates to methods and compositions for treating or preventing cardiovascular diseases by administering to a subject an effective amount of soluble CTLA4 molecules alone, or in conjunction with other therapeutic agents.

BACKGROUND OF THE INVENTION

Approximately 62 million Americans have one or more types of cardiovascular disease with coronary heart disease (CHD) and stroke afflicting more than 17 million patients in the United States alone (Ref: American Heart Association. *2002 Heart and Stroke Statistical Update*. Dallas, Tex.: American Heart Association; 2001). Despite numerous therapeutic options and technological advances, morbidity and mortality from these diseases are exceedingly high; in fact, cardiovascular diseases are the leading cause of death in the United States claiming 2 of every 5 deaths. Consequently, new approaches to the treatment and prevention of cardiovascular diseases are needed.

The concept that atherosclerosis, the leading cause of CHD, is a process of passive accumulation of lipid in arterial walls eventually leading to the development of symptomatic cardiovascular disease is no longer tenable (Ref: Scientific American 2002 (May): 46-55). Instead, this hypothesis is being replaced by evidence that cardiovascular disease represents a chronic inflammatory process (Ref: Circulation 2002; 105:1135-43). It has been proposed that underlying and preceding acute coronary or cerebrovascular events are "vulnerable" (or high-risk) atherosclerotic plaque(s). Inflammation is thought to be a major contributing factor to plaque

instability and rupture leading to unstable angina (UA) and acute myocardial infarction (AMI). Vulnerable plaques have been shown to be frequently present in numerous anatomically distinct locations rather than isolated to a single (culprit) lesion (Ref: Circulation 2003;107:2072-2075). The multifocal nature of vulnerable plaques has been documented in autopsy series and in studies using angiographic, intravascular ultrasound (IVUS), angioscopic, or thermography techniques. Clinical endpoint data (e.g., death, myocardial infarction, stroke) that further support these hypotheses are derived from epidemiological data, retrospective hypothesis-generating analyses of completed clinical trials, and prospective evaluation in clinical trials (summarized below).

Epidemiological data from the Physician's Health Study provides compelling evidence that markers of inflammation (e.g., C-reactive protein [CRP], fibrinogen, interleukin-6, soluble intracellular adhesion molecule-1 [sICAM-1]) are predictive of the future risk of developing AMI (Ref: Circulation 1999;100:1148-1150). Subsequently, more than a dozen population-based epidemiological studies have reported similar observations (Ref: Circulation 2002; 105:1135-43). CRP (and high sensitivity CRP [hs-CRP]) has been the most widely studied inflammatory marker across these epidemiological studies. CRP appears to be an independent predictor of subsequent cardiovascular events (AMI, death) in both primary prevention and secondary prevention patient populations.

Experimental and clinical evidence supports the notion that reduction of inflammation leads to a reduction in clinical events. Aspirin has been shown in the Physician's Health Study to be associated with a reduction of inflammation, as measured by CRP, and is associated with a concomitant reduction of coronary events (Ref: Circulation 1999;100:1148-1150). To what extent anti-inflammatory effects/CRP reduction vs. antiplatelet effects of aspirin had on this finding in a population of apparently healthy men is not known. Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors ("statins"; e.g., pravastatin, simvastatin, atorvastatin, fluvastatin, and lovastatin) serve as a second example of an existing therapy that has anti-inflammatory properties. In addition to their effects on serum lipids, statins also reduce CRP (Ref: Circulation 2002; 105:1135-43). The Pravastatin in the Cholesterol and Recurrent Events (CARE) study provided the first

clinical evidence that statin therapy lowers CRP in a fashion unrelated to low-density lipoprotein (LDL) or high-density lipoprotein (HDL) cholesterol. The magnitude of relative risk reduction for subsequent cardiovascular events in this hypercholesterolemic population was greater among subjects who also had evidence of inflammation (i.e., CRP elevation) as compared with those without evidence of inflammation (Ref: Circulation 1999;100:230-235). This observation was prospectively validated in the Pravastatin Inflammation CRP Evaluation (PRINCE) study and also reported in analysis of several other trials with a variety of statins (Ref: Circulation 2002; 105:1135-43).

Evidence dissociating the benefits of statin therapy in patients with elevations in CRP from those with elevation of cholesterol are derived from the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS; Ref: New Engl J Med 2001;344:1959-1965). This study was a primary prevention study using lovastatin in a population with low to moderate cardiovascular risk. In this study, the magnitude of risk reduction afforded by lovastatin as compared with placebo was nearly as great in the patients with low levels of LDL / high levels of CRP as compared with those with high levels of LDL / low levels of CRP.

The above findings suggest that inflammation represents a novel risk category that is not currently addressed as standard of care in clinical practice guidelines. Since approximately half of all heart attacks occur in populations with normal cholesterol levels, identification and modulation of newly identified risk factors would be an important strategy for reducing cardiovascular morbidity and mortality. The population that fits within this cohort of low-LDL/high-CRP has been estimated to be approximately 25 million Americans (Ref: Circulation 2002; 105:1135-43). The elevation of CRP appears to occur in a graded fashion consistent with subsequent risk of cardiovascular events. Specifically, elevated CRP (>3 mg/dL) is found in 10% of healthy individuals, but is elevated in <20%, >65%, and >90% in patients with chronic stable angina, unstable angina (Braunwald class IIIb), and AMI preceded by USA, respectively (Ref: Circulation 2002; 105:1135-43). Therefore, inflammatory markers represent an opportunity for identification and pharmacological intervention in populations at risk for the development of cardiovascular events.

Other markers of inflammation are emerging that may either replace or augment the utility of CRP measurement. Recently, novel markers of inflammation, including increases in soluble CD40 ligand (sCD40L) and decreases in serum interleukin-10 (IL-10) concentration, have been associated with increased cardiovascular morbidity and mortality. Evidence suggests that CD40L is important in atherosclerotic plaque destabilization. CD40L shed from stimulated lymphocytes is pro-inflammatory causing upregulation of inflammatory cytokines and adhesion molecules. Moreover, CD40L also promotes coagulation by inducing expression of tissue factor in macrophages and endothelial cells and also activates glycoprotein IIb/IIIa (Refs: Proc. Natl. Acad. Sci. 1997;94:1931-1936; Nature 1998;394:200-203; Circulation 2002;106:896-899).

Epidemiological evidence from the Women's Health Study demonstrated elevated serum sCD40L concentrations are associated with a graded and continuous rise in cardiovascular risk (Ref: Circulation 2001;104:2266-2268). The increase in cardiovascular risk was nearly 12-fold higher among women with the highest sCD40L concentrations. Similar findings were also observed in the c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) study (Ref: NEJM 2003;348:1104-1111). In this study, a graded and continuous rise in cardiovascular risk (death or nonfatal myocardial infarction) was noted among placebo-treated subjects based upon quintiles of baseline sCD40L. This difference in cardiovascular risk was evident at both early (24 hour) and late (6 month) endpoint determinations. A similar analysis from the CAPTURE trial investigating the anti-inflammatory cytokine IL-10 revealed that placebo-treated patients with high levels of IL-10 (i.e., high anti-inflammatory cytokine levels) had a reduced risk of death (Ref: Circulation 2003; 107:2109-2114). Patients in the highest quartile of serum concentrations of the anti-inflammatory cytokine IL-10 had a >50% reduction in mortality rate as compared with those patients in the lowest quartile of serum IL-10 levels. Moreover, if at the time of hospital discharge patient populations are dichotomized as either high or low IL-10 levels there was an observed adjusted hazard ratio for mortality of 0.38 (i.e., a 62% relative risk reduction) at 6 months favoring subjects with high (anti-inflammatory) IL-10 levels.

In summary, the concept of cardiovascular diseases occurring as a passive process mediated by lipid deposition alone is antiquated. There is a substantial increase in experimental and clinical evidence that suggests cardiovascular disease is a manifestation of a chronic inflammatory process and that intervention in this inflammation may reduce patient morbidity and mortality. Currently, however, the cellular and molecular mediators of these processes are only now being elucidated. Elucidation for these processes is hampered by the lack of an appropriate preclinical model for acute coronary syndrome (ACS). Despite the lack of knowledge of these precise mechanisms, the anti-inflammatory effects of statins appear to validate the concept that pharmacological intervention in patients with elevated markers inflammation leads to reductions of cardiovascular morbidity and mortality. Notably, these benefits are afforded through the serendipitous pleiotropic anti-inflammatory activities of statins. Further understanding of the cellular and molecular processes may lead to the development of specific and more efficacious anti-inflammatory agents to further reduce cardiovascular morbidity and mortality among these patients.

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.

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-positive 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 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 (SEQ ID NO.: 20) 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; U.S. Patent No. 5,434,131; U.S. Patent No. 6,090,914; U.S. Patent No. 5,773,253.

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 cardiovascular diseases.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for treating cardiovascular diseases, by administering to a subject a molecule that blocks B7 interactions with CTLA4 and/or CD28, thereby inhibiting endogenous B7 molecules on B7-positive cells 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 provides compositions and methods for treating cardiovascular 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 treating (e.g. reducing symptoms of) cardiovascular diseases by administering to a subject suffering from symptoms of cardiovascular disease, soluble CTLA4 molecules such as CTLA4Ig and/or soluble CTLA4 mutant molecule L104EA29YIg and/or a mix of any soluble CTLA molecule. CTLA4Ig and 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, are preferred for use in the methods of the invention.

The present invention also provides a pharmaceutical composition for treating cardiovascular comprising a pharmaceutically acceptable carrier and a biologically effective agent, such as soluble CTLA4 molecules, alone or in conjunction with other therapeutic drugs.

Kits comprising pharmaceutical compositions therapeutic for cardiovascular 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 a cardiovascular disease. For example, the pharmaceutical composition comprises an effective amount of soluble CTLA4 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 other therapeutic agents used in conjunction with the pharmaceutical compositions of the invention.

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 (SEQ ID NOs: 6-7) as described in Example 1, *infra*.

Figure 19: Nucleotide and amino acid sequence of L104EA29YIg (SEQ ID NOs: 8-9) as described in Example 1, *infra*.

Figure 20: Nucleotide and amino acid sequence of L104EA29LIg (SEQ ID NOs: 10-11) as described in Example 1, *infra*.

Figure 21: Nucleotide and amino acid sequence of L104EA29TIg (SEQ ID NOs: 12-13) as described in Example 1, *infra*.

Figure 22: Nucleotide and amino acid sequence of L104EA29WIg (SEQ ID NOs: 14-15) as described in Example 1, *infra*.

Figure 23: Nucleotide and amino acid sequence of CTLA4 receptor (SEQ ID NOs: 16-17).

Figure 24: Nucleotide and amino acid sequence of CTLA4Ig (SEQ ID NOs: 18-19).

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

Figures 29A and 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 and 33B: Reduction in soluble ICAM-1 and soluble E-selectin levels mean change from baseline at Day 85 as described in Example 3, *infra*.

Figure 34: A graph showing the summary of ACR20 response by visit day in response to methotrexate and CTLA4Ig (2 and 10 mg/kg) therapy, as described in Example 5, *infra*.

Figure 35: A graph showing the summary of ACR50 response by visit day in response to methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) therapy, as described in Example 5, *infra*.

Figure 36: A graph showing the summary of ACR70 response by visit day in response to methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) therapy, as described in Example 5, *infra*.

Figure 37: A graph showing the mean ACR-N over time in response to methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) therapy, as described in Example 5, *infra*.

Figure 38: A bar graph showing the ACR response in response to methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) therapy on day 180 with a 95% confidence interval, as described in Example 5, *infra*.

Figure 39: A bar graph showing the proportion of New Active Joints in response to methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) therapy on day 180, as described in Example 5, *infra*.

Figure 40: A bar graph showing ACR response after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) on day 180, as described in Example 5, *infra*.

Figure 41: A graph showing percent improvement in tender joints after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - mean percent improvement from baseline, as described in Example 5, *infra*.

Figure 42: A graph showing percent improvement in swollen joints after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - mean percent improvement from baseline, as described in Example 5, *infra*.

Figure 43: A graph showing percent improvement in pain after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - mean percent improvement from baseline, as described in Example 5, *infra*.

Figure 44: A graph showing percent improvement in regard to disease activity as reported by the subject after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - mean percent improvement from baseline, as described in Example 5, *infra*.

Figure 45: A graph showing percent improvement in regard to disease activity as reported by the physician after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - mean percent improvement from baseline, as described in Example 5, *infra*.

Figure 46: A graph showing percent improvement regarding physical function after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - mean percent improvement from baseline as measured by HAQ, as described in Example 5, *infra*.

Figure 47: A graph showing percent improvement in CRP levels function after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - mean percent improvement from baseline, as described in Example 5, *infra*.

Figure 48: A graph showing percent improvement in CRP levels function after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg) - median percent improvement from baseline, as described in Example 5, *infra*.

Figure 49: A graph showing the difference in ACR response rate on day 180 in two groups after therapy with CTLA4Ig (2 and 10 mg/kg) in comparison to a group treated with methotrexate (MTX) only (95% Confidence Limits), as described in Example 5, *infra*.

Figure 50: A graph showing the change from baseline for SF-36 Physical Health Component on day 180, in two groups after therapy with CTLA4Ig (2 and 10

mg/kg) compared to a group treated with methotrexate only (95% Confidence Limits), as described in Example 5, *infra*.

Figure 51: A graph showing the change from baseline for SF-36 Mental Health Component on Day 180, in two groups after therapy with CTLA4Ig (2 and 10 mg/kg) compared to a group treated with methotrexate only (95% Confidence Limits), as described in Example 5, *infra*.

Figure 52: A bar graph showing CRP levels at day 180 after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg), as described in Example 5, *infra*.

Figure 53: A bar graph showing Rheumatoid Factor levels on day 180 after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg), as described in Example 5, *infra*.

Figure 54: A bar graph showing IL-2r levels on day 180 after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg), as described in Example 5, *infra*.

Figure 55: A bar graph showing IL-6 levels on day 180 after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg), as described in Example 5, *infra*.

Figure 56: A bar graph showing TNFα levels on day 180 after therapy with methotrexate alone or methotrexate and CTLA4Ig (2 and 10 mg/kg), as described in Example 5, *infra*.

Figure 57: A table of the univariate methotrexate dose at screening/enrollment for treatment group BMS 10 - treated with CTLA4Ig at 10 mg/kg body weight as described in Example 5, *infra*.

Figure 58: A table of the univariate methotrexate dose at screening/enrollment for treatment group BMS 2 - treated with CTLA4Ig at 2 mg/kg body weight as described in Example 5, *infra*.

Figure 59: A table of the univariate methotrexate dose at screening/enrollment for the placebo group, as described in Example 5, *infra*.

Figure 60: A table of the univariate methotrexate dose up to and including day 180 of the study for treatment group BMS 10 - treated with CTLA4Ig at 10 mg/kg body weight as described in Example 5, *infra*.

Figure 61: A table of the univariate methotrexate dose up to and including day 180 of the study for treatment group BMS 2 - treated with CTLA4Ig at 2 mg/kg body weight as described in Example 5, *infra*.

Figure 62: A table of the univariate methotrexate dose up to and including day 180 of the study for the placebo group, as described in Example 5, *infra*.

Figure 63: A bar graph showing the difference in modified ACR response rates on day 180 in two groups after therapy with etanercept alone (25 mg twice weekly) or in combination with CTLA4Ig (2mg/kg), as described in Example 6, *infra*.

Figure 64A-C: Graphs showing percentage improvement of individual components of the modified ACR criteria as assessed on each visit day after therapy with etanercept alone (25 mg twice weekly) or in combination with CTLA4Ig (2 mg/kg) as described in Example 6, *infra*. A. Tender Joint Count. B. Swollen Joint Count. C. Pain Assessment.

Figure 65: A. A graph showing the change from baseline for SF-36 Physical Health Component on day 180, in two groups after therapy with etanercept (25 mg biweekly) alone or in combination with CTLA4Ig (2 mg/kg) (95% Confidence Limits), as described in Example 6, *infra*. B. A graph showing the change from baseline for SF-36 Mental Health Component on day 180, in two groups after therapy with etanercept (25 mg biweekly) alone or in combination with CTLA4Ig (2 mg/kg) (95% Confidence Limits), as described in Example 6, *infra*.

Figure 66: Nucleotide sequence of a CTLA4Ig encoding a signal peptide; a wild type amino acid sequence of the extracellular domain of CTLA4 starting at methionine at position +1 to aspartic acid at position +124, or starting at alanine at position -1 to aspartic acid at position +124; and an Ig region (SEQ ID NO.: 21).

Figure 67: Amino acid sequence of a CTLA4Ig having a signal peptide; a wild type amino acid sequence of the extracellular domain of CTLA4 starting at methionine at position +1 to aspartic acid at position +124, or starting at alanine at position -1 to aspartic acid at position +124; and an Ig region (SEQ ID NO.: 22).

Figure 68: A schematic diagram showing the disposition of subjects into three cohorts as described in Example 7, *infra*.

Figure 69: A Kaplan-Meier plot of the cumulative proportion of subjects who discontinued for any reason during the first 12 months of the study, as described in Example 7, *infra*.

Figure 70: A Kaplan-Meier plot of the cumulative proportion of subjects who discontinued due to lack of efficacy during the first 12 months of study, as described in Example 7, *infra*.

Figure 71A: A graph showing the ACR Responses on Day 180 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 71B: A graph showing the 95 Percent Confidence Intervals for Differences in ACR Responses on Day 180 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 72A: A graph showing the ACR Responses on Day 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 72B: A graph showing the 95 Percent Confidence Intervals for Differences in ACR Responses on Day 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 73A: A graph summarizing the ACR 20 Response by Visit during a one year interval for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 73B: A graph summarizing the ACR 50 Response by Visit during a one year interval for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 73C: A graph summarizing the ACR 70 Response by Visit during a one year interval for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 74: A graph showing the Mean ACR-N over a one year time interval for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 75: A graph showing the Proportion of New Active Joints at Day 180 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 76A: A graph showing the Frequency of New Tender Joints per Subject at Day 180 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 76B: A graph showing the Frequency of New Tender Joints per Subject at Day 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 77A: A graph showing the Frequency of New Swollen Joints per Subject at Day 180 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 77B: A graph showing the Frequency of New Swollen Joints per Subject at Day 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 78: A graph showing the Proportion of New Active Joints at Day 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 79: Graphs showing the: A) Change from Baseline in the Physical Health Domains on Day 180, and B) Change from Baseline in the Mental Health Domains on 180, for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 80: Graphs showing the: A) Change from Baseline in the Physical Health Domains on Day 360, and B) Change from Baseline in the Mental Health Domains on Day 360, for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 81: A graph showing the Soluble IL-2r Levels at Baseline, Days 180 and 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 82: A graph showing the Rheumatoid Factor Levels at Baseline, Days 180 and 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 83: A graph showing the ICAM-1 Levels at Baseline, Days 180 and 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 84: A graph showing the e-Selectin Levels at Baseline, Days 180 and 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 85: A graph showing the Serum IL-6 at Baseline, Days 180 and 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 86A: A graph showing the CRP Levels at Baseline, Days 180 and 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 86B: A graph showing the TNFα Levels at Baseline, Days 180 and 360 for patients administered methotrexate alone or methotrexate and CTLA4Ig (2 or 10 mg/kg body weight) as described in Example 7, *infra*.

Figure 87: Percentage of activated T-cells in atherectomy specimens.

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. In a further example, a ligand for the B7 molecule is a CTLA4 and/or CD28 molecule. The interaction of a molecule and its ligand can be regulated by compositions of the invention. For example, CTLA4 interaction with its ligand B7 can be blocked by administration of CTLA4Ig molecules. Alternatively, Tumor Necrosis Factor (TNF), a ligand, interacts with its receptor , the TNF receptor (TNFR), and can be blocked by administration of etanercept or other TNF/TNFR blocking molecules.

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 in U.S. Patent Nos. 5,434,131, 5,844,095, and 5,851,795 herein incorporated by reference in their entirety), 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 as shown in Figure 23. 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 that binds B7, or a portion thereof, joined to an immunoglobulin constant region (Ig), or a portion thereof. 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, compositions and/or kits 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. encoded by DNA deposited with ATCC accession number 68629), wherein the extracellular domain of CTLA4 is fused to an immunoglobulin (Ig) moiety such as IgCγ1 (IgCgamma1), IgCγ2 (IgCgamma2), IgCγ3 (IgCgamma3), IgCγ4 (IgCgamma4), IgCμ(IgCmu), IgCα1 (IgCalpha1), IgCα2 (IgCalpha2), IgCδ (IgCdelta) or IgCεIgCepsilon), 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) (as described in U.S. Patent No. 5,844,095, herein incorporated by reference in its entirety), or fragments and derivatives thereof; hybrid (chimeric) fusion proteins such as CD28/CTLA4Ig (as described in U.S. Patent No. 5,434,131, herein incorporated by reference in its entirety), 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, herein incorporated by reference in its entirety), 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, compositions and/or kits 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

Amino Acid	Symbol	One Letter Symbol	Codons
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. The non-CTLA4 molecule, attached to the extracellular domain of a CTLA4 molecule can alter the solubility or affinity of the CTLA4 molecule. 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 molecule e.g. CTLA4 or CD28, preferably the extracellular domain of CTLA4 or CD28 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) (Freeman et al, 1989, J Immunol. 143:2714-2722, herein incorporated by reference in its entirety), B7-2 (CD86) (Freeman et al, 1993, Science 262:909-911 herein incorporated by reference in its entirety; Azuma et al, 1993, Nature 366:76-79 herein incorporated by reference in its entirety) that may recognize

and bind CTLA4 and/or CD28. A B7 molecule can be expressed on an activated B cell.

As used herein, "CD28" refers to the molecule that recognizes and binds B7 as described in U.S. Serial No. 5,580,756 and 5,521,288 (herein incorporated by reference in their entirety).

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 similarity 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. A derivative means any change to the amino acid sequence and/or chemical quality of the amino acid e.g., amino acid analogs.

As used herein, to "regulate" an immune response is to activate, stimulate, up-regulate, inhibit, block, down-regulate or modify the immune response. The cardiovascular diseases described herein, may be treated by regulating an immune response e.g., by regulating functional CTLA4- and/or CD28- positive cell interactions with B7-positive cells. For example, a method for regulating an immune response comprises contacting the B7-positive cells with a soluble CTLA4 molecule of the invention so as to form soluble CTLA4/B7 complexes, the soluble CTLA4 molecule interfering with reaction of an endogenous CTLA4 and/or CD28 molecule with said B7 molecule.

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 immune 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 molecules that block B7 interactions with CTLA4 and/or CD28 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)). Also included are small molecules that block B7 interactions with CTLA4 and/or CD28. Blocking B7 interactions can be detected by art-recognized tests such as determining reduction of immune disease (e.g., cardiovascularic disease) or inflammatory disease associated symptoms, by determining reduction in T-cell/B7-cell interactions, or by determining reduction in B7 interaction with CTLA4 and/or CD28. Blockage may be partial or total.

As used herein, an "effective amount" of a molecule is defined as an amount that blocks the interaction of the molecule with its ligand. For example, an effective amount of a molecule that blocks B7 interaction with CTLA4 and/or CD28 may be defined as the amount of the molecule that, when bound to B7 molecules on B7-positive cells, inhibit B7 molecules from binding endogenous ligands such as CTLA4 and CD28. Alternatively, an effective amount of a molecule that blocks B7 interaction with CTLA4 and/or CD28 may be defined as the amount of the molecule that, when bound to CTLA4 and/or CD28 molecules on T cells, inhibit B7 molecules from binding endogenous ligands such as CTLA4 and CD28. The inhibition or blockage may be partial or complete.

As used herein, "treating" a disease means to manage a disease by medicinal or other therapies. Treatment of a disease may ameliorate or alleviate the symptoms of a disease, reduce the severity of a disease, alter or prevent the course of disease progression, prevent disease occurrence, and/or ameliorate or alleviate or cure the basic disease problem. Symptoms of cardiovascular disease include, but are not limited to: dysrhythmias; chest pain; myocardial ischemia; angina; reduced exercise tolerance; fatigue; dyspnea on exertion; paroxysmal nocturnal dyspnea, claudication; transient ischemic attacks and quality of life.For example, to treat a cardiovascular disease may be accomplished by regulating an immune response e.g., by regulating functional CTLA4- and/or CD28- positive cell interactions with B7-positive cells. Alternatively, treating a cardiovascualar disease may be accomplished by preventing the disease from occurring or progressing through the use of the compositions described herein.

As used herein, "cardiovascular disease" has the meaning commonly used in the field, and includes, but is not limited to, the following diseases or conditions: thromboembolic disorders, including arterial cardiovascular thromboembolic disorders, venous cardiovascular thromboembolic disorders, and thromboembolic disorders in the chambers of the heart; ahtherosclerosis; restensosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; cardiovascular inflammation; vascular inflammation; coronary heart disease (CHD); unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); first or recurrent myocardial infarction; acute myocardial infarction (AMI); myocardial infarction; non-Q wave myocardial infarction; non-STE myocardial infarction; coronary artery disease; cardiac ischemia; ischemia; ischemic sudden death; transient ischemic attack; stroke; atherosclerosis; peripheral occlusive arterial disease; venous thrombosis; deep vein thrombosis; thrombophlebitis; arterial embolism; coronary arterial thrombosis; cerebral arterial thrombosis; cerebral embolism; kidney embolism; pulmonary embolism; thrombosis resulting from (a) prosthetic valves or other implants, (b) indwelling catheters, (c) stents, (d) cardiopulmonary bypass, (e) hemodialysis, or (f) other procedures in which blood is exposed to an artificial surface that promotes thrombosis; thrombosis resulting from atherosclerosis, surgery or surgical

complications, prolonged immobilization, arterial fibrillation, congenital thrombophilia, cancer, diabetes, effects of medications or hormones, and complications of pregnancy; cardiac arrhytmias including supraventricular arrhythmias, atrial arrhythmias, atrial flutter, atrial fibrillation; other diseases listed in Heart Disease: A Textbook of Cardiovascular Medicine, 2 Volume Set, 6th Edition, 2001, Eugene Braunwald, Douglas P. Zipes, Peter Libby, Douglas D. Zipes.

Preferred cardiovascular diseases are: atherosclerosis; coronary heart disease (CHD); restensosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; cardiovascular inflammation; vascular inflammation; unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); myocardial infarction; acute myocardial infarction (AMI), including first or recurrent myocardial infarction, non-Q wave myocardial infarction, non-ST elevation myocardial infarction, and ST-segment elevation myocardial infarction.

More preferred cardiovascular diseases are: atherosclerosis; coronary heart disease (CHD); unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); myocardial infarction; acute myocardial infarction (AMI), including first or recurrent myocardial infarction, non-Q wave myocardial infarction, and non-ST-segmentelevation myocardial infarction, and ST-segment elevation myocardial infarction.

As used herein, "gene therapy" is a process to treat a disease by genetic manipulation. Gene therapy involves introducing a nucleic acid molecule into a cell and the cell expressing a gene product encoded by the nucleic acid molecule. For example, as is well known by those skilled in the art, introducing the nucleic acid molecule into a cell may be performed by introducing an expression vector containing the nucleic acid molecule 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, nucleotide sequences of interest may be introduced into a cell *in vivo* using 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 molecule into a viral vector, production of the recombinant virus or viral particle, and infection of the subject with the recombinant 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 introduction of the liposomes, or liposomes combined with a hemagglutinating Sendai virus, into 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.

As used herein, "alleviate" refers to lessening or making less severe, one or more of the symptoms of a disease, such as one or more symptoms of a cardiovascular disease, including, but not limited to: dysrhythmias; chest pain; myocardial ischemia; angina; reduced exercise tolerance; fatigue; dyspnea on exertion; paroxysmal nocturnal dyspnea, claudication; transient ischemic attacks and quality of life.

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 cardiovascular diseases by administering to a subject an effective amount of a molecule that blocks B7 interactions with CTLA4 and/or CD28. For example, such ligands include: soluble CTLA4 molecules (such as CTLA4Ig, CTLA4-E7, CTLA4-p97, CTLA4-env gp120, and mutant CTLA4 molecules such as, CTLA4/CD28Ig, L104EA29YIg, L104EA29LIg, L104EA29TIg and/or L104EA29WIg), soluble CD28 molecules, soluble B7-1 molecules, soluble B7-2 molecules, and monoclonal antibodies that recognize and bind B7, CD28 and/or CTLA4 (e.g., an anti-CTLA4

monoclonal antibody, an anti-CD28 monoclonal antibody, an anti-B7-1 monoclonal antibody or an anti-B7-2 monoclonal antibody.

An effective amount of a molecule that blocks B7 interaction with CTLA4 and/or CD28 may be defined as the amount of anti-B7 monoclonal antibodies, soluble CTLA4 and/or soluble CD28 molecules that, when bound to B7 molecules on B7-positive cells, inhibit B7 molecules from binding endogenous ligands such as CTLA4 and CD28. The inhibition may be partial or complete.

Alternatively, an effective amount of a molecule that blocks B7 interaction with CTLA4 and/or CD28 may be defined as the amount of anti-CTLA4 monoclonal antibody, anti-CD28 monoclonal antibody or soluble B7 (B7-1 or B7-2) molecules that, when bound to CTLA4 and/or CD28 molecules on T cells, inhibit B7 molecules from binding endogenous ligands such as CTLA4 and CD28. The inhibition may be partial or complete.

An effective amount of a molecule that blocks B7 interaction with CTLA4 and/or CD28 is an amount about 0.1 to 100 mg/kg weight of a subject. In another embodiment, the effective amount is an amount about 0.5 to 100 mg/kg weight of a subject, 0.5 to 5 mg/kg weight of a subject, 0.1 to 5 mg/kg weight of a subject, about 5 to 10 mg/kg weight of a subject, about 10 to 15 mg/kg weight of a subject, about 15 to 20 mg/kg weight of a subject, about 20 to 25 mg/kg weight of a subject, about 25 to 30 mg/kg weight of a subject, about 30 to 35 mg/kg weight of a subject, about 35 to 40 mg/kg weight of a subject, about 40 to 45 mg/kg of a subject, about 45 to 50 mg/kg weight of a subject, about 50 to 55 mg/kg weight of a subject, about 55 to 60 mg/kg weight of a subject, about 60 to 65 mg/kg weight of a subject, about 65 to 70 mg/kg weight of a subject, about 70 to 75 mg/kg weight of a subject, about 75 to 80 mg/kg weight of a subject, about 80 to 85 mg/kg weight of a subject, about 85 to 90 mg/kg weight of a subject, about 90 to 95 mg/kg weight of a subject, or about 95 to 100 mg/kg weight of a subject.

In an embodiment, the effective amount of a molecule that blocks B7 interaction with CTLA4 and/or CD28 is an amount about 2 mg/kg to about 10 mg/kg weight of a subject. The preferred amount is 10 mg/kg weight of a subject. In another embodiment, the effective amount is an amount about 0.1 to 4 mg/kg weight of a subject. In another embodiment the effective amount is an amount about 0.1 to 0.5

mg/kg weight of a subject, about 0.5 to 1.0 mg/kg weight of a subject, about 1.0 to 1.5 mg/kg weight of a subject, about 1.5 to 2.0 mg/kg weight of a subject, about 2.0 to 2.5 mg/kg weight of a subject, about 2.5 to 3.0 mg/kg weight of a subject, about 3.0 to 3.5 mg/kg weight of a subject, about 3.5 to 4.0 mg/kg weight of a subject, about 4.0 to 4.5 mg/kg weight of a subject, about 4.5 to 5.0 mg/kg weight of a subject, about 5.0 to 5.5 mg/kg weight of a subject, about 5.5 to 6.0 mg/kg weight of a subject, about 6.0 to 6.5 mg/kg weight of a subject, about 6.5 to 7.0 mg/kg weight of a subject, about 7.0 to 7.5 mg/kg weight of a subject, about 7.5 to 8.0 mg/kg weight of a subject, about 8.0 to 8.5 mg/kg weight of a subject, about 8.5 to 9.0 mg/kg weight of a subject, about 9.0 to 9.5 mg/kg weight of a subject, about 9.5 to 10.0 mg/kg weight of a subject.

In another embodiment, the effective amount is an amount about 0.1 to 20 mg/kg weight of a subject. In another embodiment, the effective amount is an amount about 0.1 to 2 mg/kg weight of a subject, about 2 to 4 mg/kg weight of a subject, about 4 to 6 mg/kg weight of a subject, about 6 to 8 mg/kg weight of a subject, about 8 to 10 mg/kg weight of a subject, about 10 to 12 mg/kg weight of a subject, about 12 to 14 mg/kg weight of a subject, about 14 to 16 mg/kg weight of a subject, about 16 to 18 mg/kg weight of a subject or about 18 to 20 mg/kg weight of a subject.

In another embodiment, the effective amount is about 2 mg/kg weight of a subject. In yet another embodiment, the effective amount is about 10 mg/kg weight of a subject.

In a specific embodiment, the molecule that blocks B7 interaction with CTLA4 and/or CD28 is a soluble CTLA4 molecule and the effective amount of a soluble CTLA4 molecule is about 2 mg/kg weight of a subject. In another specific embodiment, the effective amount of a soluble CTLA4 molecule is about 10 mg/kg weight of a subject. In another specific embodiment, an effective amount of a soluble CTLA4 is 500 mg for a subject weighing less than 60 kg, 750 mg for a subject weighing between 60-100 kg, and 1000 mg for a subject weighing more than 100 kg.

An effective amount of the molecule that blocks B7 interaction with CTLA4 and/or CD28 is soluble CTLA4 may be administered to a subject daily, weekly, monthly and/or yearly, in single or multiple times per hour/day/week/month/year, depending on need. For example, in one embodiment, the molecule may initially be

administered once every two weeks for a month, and then once every month thereafter.

In a preferred embodiment, the cardiovascular disease is: atherosclerosis; coronary heart disease (CHD); unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); first or recurrent myocardial infarction; acute myocardial infarction (AMI); myocardial infarction; non-Q wave myocardial infarction; or non-STE myocardial infarction.

Herein (Examples 3-7) is presented clinical data supporting the use of molecules that block CD28-B7 interactions in the prevention or treatment of cardiovascular diseases. In clinical studies for RA, CTLA4Ig has lowered CRP, IL-6, and TNF-α, all markers of inflammation that also correlate with cardiovascular diseases. These findings suggest that a novel mechanism of action – blocking the CD28-B7 pathway – may be useful to treat cardiovascular diseases. Further studies are discussed in Examples 8 and 9. We present as our invention methods for treating cardiovascular diseases by administering to a subject an effective amount of a molecule that blocks B7 interactions with CTLA4 and/or CD28.

While some supporting data discussed herein for this new method of treating cardiovascular diseases is derived from the published medical literature, our invention is not diminished. The concept that immunology/inflammation may play a critical role in cardiovascular diseases is a relatively recent and incomplete paradigm shift. The breadth of research across cardiology, immunology, and infectious disease is substantial, so discoveries in one field do not become integrated across the divide of these disciplines. Conversely, once a new discovery is made its implications into treatments and complete mechanisms of actions may not be immediately transparent to investigators/scientists. Additionally, it has been reported in one study that the dominant T-cell population in patients with USA were $CD4^{+}CD28^{null}$ cells (Ref: Circulation 2000;102:2883-2888). This finding suggests that interference with CD28-B7 mediated signaling would not be efficacious in UA patients.

Inhibition of CD28-B7 interaction, such as by CTLA4Ig or L104EA29YIg, to reduce cardiovascular morbidity and mortality or improve cardiovascular function and quality of life is new. The use of these agents across the spectrum of cardiovascular diseases is supported by: (1) cellular and biochemical similarities between patients

with rheumatoid arthritis (RA) and atherosclerosis/unstable angina; (2) the mechanism of action of CTLA4Ig and L104EA29YIg seeming appropriate for intervention in cardiovascular diseases; (3) anti-inflammatory effects of CTLA4Ig and/or L104EA29YIg in animal models and clinical studies presented herein; (4) recently reported finding on the importance of B7-CD28 interaction in atherosclerosis. Much of these data exists in disparate preclinical studies (cellular and animal models), as well as in clinical studies presented herein, and is summarized below.

(1) Cellular and biochemical similarities between patients with rheumatoid arthritis and atherosclerosis/unstable angina

There are biochemical and cellular similarities between these two pathological processes (Table 1). Additionally, the premature deaths of patients with RA have been recently linked to acute coronary syndromes (Refs: J Rheumatology 1999;26:2562-2571; Rheumatology 1999;38:668-674).

TABLE 1

Biochemical and Cellular Similarities Between Rheumatoid Arthritis and Atherosclerosis/Unstable Angina

	Atherosclerosis/ Unstable Angina	Rheumatoid Arthritis
Biochemical Similarities		
Tumor Necrosis Factor-α (TNF-α)	↑	↑
Metalloproteinase expression	↑	↑
Interleukin-6 (IL-6)	↑	↑
C-reactive Protein (CRP)	↑	↑↑
Adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin)	↑	↑
Endothelin	↑	↑
Cellular Similarities		
Mast-cell activation	↑	↑
T-cell activation	↑	↑
B-cell activation	0 or ↑	0 or ↑
Possible Antigens		
Heat shock proteins (HSPs)	Reported	Reported
Infections agents	Reported	Reported
Oxidized-LDL	Reported	
Collagen II		Reported

	Atherosclerosis/ Unstable Angina	Rheumatoid Arthritis
Cartilage antigens		Reported

Notes: Adapted from Paceri and Teh, (Ref: Circulation 1999;100:2124-2126). The symbols 0 denote no appreciable increase or decrease in parameter from control populations; ↑ denotes increase in parameter from control populations; Reported denotes presence of supporting evidence available in the medical literature.

These similarities suggest that there may be common pathophysiological processes underlying each of these diseases.

(2) The mechanisms of action of CTLA4Ig and L104EA29YIg seem appropriate for intervention in cardiovascular diseases

T-cells are present in early atheroma as well as vulnerable/ruptured plaques

There are lines of evidence that support the notion that T-cell activation is important across the spectrum of cardiovascular diseases from atherosclerosis to acute plaque rupture. T-cells are among the most common cells present in human atherosclerotic lesions (Ref: New Engl J Med 1999; 340:115-126) and are present in both early lesions and in vulnerable or ruptured plaques. Pathological studies of vulnerable or ruptured plaques have shown that T-cells (mostly $CD4^+$) are present and located in the shoulder region of the atheromatous lesion - the most likely site of tissue erosion (Refs: Am. J. Cardiol. 1991;68:36B-50B. Circulation 1994;89:36-44).

Cytokines elaborated by T-cells are poised to promote inflammation and plaque rupture

T- cells are known to secrete inflammatory cytokines including interferon-γ (IFN-γ); TNFα, and interleukin-2 (IL-2) (Refs: Atherosclerosis 1986;6:131-138. J Clin Invest 1985;76:125-131). The actions of T-cells in atheromatous plaques are thought to be two-fold. First, T-cells can regulate the actions of macrophages that in turn are thought to release digestive enzymes, disrupt the covering matrix and smooth muscle layer, and promote plaque rupture. Secondly, T-cells can directly produce a reduction of collagen synthesis in the fibrous caps of atheroma by secreting IFN-γ to

vascular smooth muscle cells. Collectively, these T-cell activities are thought to represent a coordinated process that promotes atheromatous plaque instability and rupture.

Within atheroma, T-cells are activated in situ to a degree that parallels the severity of clinical symptoms

T-cells are not only present in atheroma, but there is also evidence for their *in situ* activation within these lesions. In a study of T-cell activation in coronary atheroma, van der Wal *et. al.* has shown from atherectomy specimens that T-cells are activated as evidenced by IL-2 receptor (IL-2r) expression and IL-2 secretion (Ref: Heart 1998;80:14-18). The increase in T-cell expression of IL-2r in this study was in proportion to the severity of clinical symptoms of patients undergoing atherectomy. Figure 87 shows the percentage of activated T-cells in atherectomy specimens.

HSP60 or other novel antigens may directly promote cardiovascular inflammation

Studies have suggested a possible link between infectious agents and the establishment of cardiovascular diseases. A number of infectious agents have been suggested including, *Chlamydia pneumoniae (Cp), Helicobacter pylori,* cytomegalovirus (CMV), adenoviruses, coxsackieviruses, several Herpesviridae, and a variety of dental infections have been liked to CHD. The evidence substantiating causality among these agents with respect to coronary atherosclerosis, however, varies widely. *C. pneumoniae,* a human respiratory pathogen, has most often been found in association with CHD.

The role of persistent elevation of CRP, and presence of antibodies to *C. pneumoniae* (Cp) or antibodies to human HSP60 (hu-HSP60) has been investigated in a nested case-control study of the Helsinki Heart Study (Ref: Circulation 2003;107:2566-2570). This study, a prospective double-blinded placebo-controlled primary prevention trial, randomized 4081 subjects and observed 241 coronary events (coronary death or non-fatal MI) over a 8.5-year follow-up. The importance of persistent seropositivity (as defined by an elevation of CRP, serum antibodies to Cp or serum antibodies to hu-HSP60 at both baseline and 3 to 6 months prior to the index

coronary event) for the prediction of subsequent coronary events was investigated (Table 2).

TABLE 2

Odds Ratios (OR) for the Development of Coronary Death or Non-fatal MI with Persistence of Risk Factors in the Helsinki Heart Study

	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
CRP-		
Cp-	1	1
Cp+	1.55 (0.85 – 2.80)	1.62 (0.87 – 3.00)
CRP+		
Cp-	2.36 (1.16 – 4.79)	1.96 (0.92 – 4.18)
Cp+	5.38 (2.32 – 12.46)	4.47 (1.84 – 10.83)
CRP-		
hu-HSP60-	1	1
hu-HSP60+	0.92 (0.46 – 1.85)	0.87 (0.42 – 1.80)
CRP+		
hu-HSP60-	2.07 (1.04 – 4.14)	1.78 (0.85 – 3.74)
hu-HSP60+	6.06 (2.23 – 16.47)	4.36 (1.53 – 12.39)

These data indicate that in a population of middle-aged males with no known coronary heart disease, persistently elevated seropositivity for Cp or hu-HSP60 when present with an elevated CRP, predicted subsequent coronary events (Table 2). Of note, patients who were persistently CRP+, Cp+, and hu-HSP60+ had an adjusted OR of 16.87 (2.06 – 137.9) for the development of subsequent coronary events. Only 1/138 control patients met this criteria for a persistent elevation in all 3 markers, whereas 17 were identified in index cases. These findings suggest the presence of novel antigenic risk factors for the prospective identification of individuals at risk of developing cardiovascular events.

A separate recent study, reported that chlamydial heat shock protein 60 (Cp-HSP60) is strongly associated with ACS (Ref: Biasucci, *et. al.*Circulation 2003;107:3015-3017). Unlike the asymptomatic patients in the Helsinki Heart Study, this study investigated patients at the other end of the CHD spectrum. In this study, 219 patients admitted to a Coronary Care Unit (CCU) with either UA (Braunwald Class IIIb) or AMI were compared to healthy patients or patients with stable angina (SA). The presence of an antibody response in patients to Cp-HSP60 (anti-Cp-

HSP60) was measured in 100 control subjects, 40 subjects with SA, 179 subjects with UA, and 40 subjects with AMI (Table 3).

TABLE 3

Serological Characteristics of Study Population as Reported by Biasucci, *et.al.*

	Control Subjects (N = 100)	Stable Angina Patients (N = 40)	Unstable Angina Patients (N = 179)	AMI Patients (N = 40)
Cp seropositivity	30%	60%	68%	65%
HSP60 seropositivity	0%	20%	98%	99%
CRP > 3 mg/dL	8%	25%	59%	62%
Median CRP, mg/dL	1.4	2.1	5.05	6.02

Note: The antibody used for HSP60 seropositivity determination in this study does not discriminate between Cp-HSP60 and hu-HSP60. Because of this lack of specificity these data do not support a direct association between anti-HSP60 response and infection.

The above findings are supportive of the notion that a specific antigenic response exists in patients with UA and AMI. These two studies represent extremes of patient populations in the spectrum of cardiovascular disease. The Helsinki Heart Study shows that patients without history of coronary heart (or other major) diseases could be identified as having an increased risk of coronary death or non-fatal MI based upon CRP elevation and a persistent antibody response to Cp or hu-HSP60. The prevalence of these risk factors in a healthy primary prevention population, however, was low. In stark contrast, patients in the study by Biasucci, *et. al.* indicates that HSP60 seropositivity is nearly universal in patients with UA or AMI. In addition, the presence of 20% seropositivity to HSP60 in patients with stable angina suggests that a graded relationship may exist between the presence of antibodies to HSP-60 and cardiovascular events.

Antigenic response to HSP60 is mediated through CD28-B7 interactions

In animal models, macrophages (a common cell type in human atheroma) have been identified as the main inducer of IFN-γ in T-cells upon administration of either mouse or hu-HSP60 (Ref: Int. Immunol. 2002;14:1247-1253). This process appears to occur through a classic "two-signal" model for T-cell activation involving CD28-

B7 pathways. In this model, the activation of T-cells by either murine or hu-HSP60 is blocked by administration of CTLA4Ig (Ref: Int. Immunol. 2002;14:1247-1253). Given the high sequence homologies between human, murine, chlamydial and bacterial heat shock proteins, this finding may be conserved across species. In fact, it may be this high homology between the HSPs of infectious agents and hu-HSPs that may serve to promote autoimmunity through molecular mimicry.

HSP60 signaling through CD28-B7 may afford an opportunity for intervention in human disease

There appears to be a number of similarities between preclinical models surrounding HSP60 and that aforementioned clinical trials (Helsinki Heart Study; Biasucci, *et. al.*). First, murine models have shown that the strongest autoimmune response (i.e., T-cell proliferation and antibody production to self HSP60) are achieved only by concurrent administration of both murine (self) HSP60 and Cp-HSP60 (Ref: Infection and Immunity 1997;65:1669-1674). This preclinical finding in a unrelated animal model is very similar to the observed finding in the highest risk group in the Helsinki Heart Study. Of note, Cp-HSP60 and hu-HSP60 co-localization within in human atheromatous plaques has been reported (Refs: Circulation 1998;98:300-307. J Infect Dis 1997:176:292-295). In these plaques, HSP60s may subsequently regulate TNF-α, matrix metalloproteinase expression, and activate endothelium and smooth muscle cells (Ref: Circulation 2003;107:3015-3017). In addition, co-administration of both murine HSP60 and Cp-HSP60 in this animal model results in a 6-fold decline in lymphocyte IL-10 production and a 12-fold increase in the ratio of IFN-γ/IL-10 production. The coincident decline in IL-10 levels may translate into a clinically meaningful observations as low serum IL-10 concentrations have been associated with a concentration-dependent risk of death following AMI (Ref: Heeschen, *et. al.* Circulation 2003; 107:2109-2114). Given the parallels between these animal studies and data from clinical trials, inhibition of CD28-B7 signaling through the use of CTLA4Ig or L104EA29YIg may represent a specific mechanistically rational intervention for prevention and/or treatment of cardiovascular disease.

Use of CTLA4Ig or L104EA29YIg for prevention and/or treatment of cardiovascular disease may not be limited to HSP60

HSP60 serves as a hypothetical model for the mechanistic rationale of CTLA4Ig or L104EA29YIg in the prevention and/or treatment of cardiovascular disease. HSP60 however, does not represent the sole antigenic stimulation that may be interrupted by CTLA4Ig or L104EA29YIg treatment. For example, recent epidemiological evidence from the Women's Health Study (Ref: Circulation 2001;104:2266-2268) and the observational data from the CAPTURE study (Ref: NEJM 2003;348:1104-1111) have independently demonstrated the existence of a graded and continuous rise in cardiovascular risk (death or nonfatal myocardial infarction) based upon serum sCD40L levels. Consequently, CD40-CD40L interactions are becoming recognized as important mediators of inflammation in cardiovascular disease. There is also evidence that suggests interactions between the CD28-B7 pathways and CD40-CD40L pathways in patients with unstable angina. Specifically, it has been shown that CD40L expressing (CD40L+) T-cells are present in atheromatous lesions (Ref: Proc. Natl. Acad. Sci. 1997;94:1931-1936). *In vitro* ligation of CD40 on atheroma-associated cells has been shown to increase the production of pro-inflammatory cytokines, matrix metalloproteinases, adhesion molecules, and tissue factor (Ref: Nature 1998;394:200-203). In other model systems, CD40 interaction with CD40L on antigen presenting cells (APC) increases expression of B7-1 (CD80) and B7-2 (CD86) - the stimulatory molecules for CD28-mediated T-cell activation (J. Immunol. 2000;165:3506-3518). In addition, *ex-vivo* treatment of T-cells from patients with unstable angina using stimulatory antibodies against CD3 and CD28 stimulates the release of sCD40L (Ref: Circulation 1999;100:614-620). Together, these observations may suggest that CD28-B7 and CD40-CD40L signaling pathways may collectively work to promote conditions that predispose to atherosclerotic plaque progression and rupture. Furthermore, interference with this process through use of CTLA4Ig or L104EA29YIg may be therapeutically beneficial. Finally, other, yet undiscovered, CD28-B7-mediated processes (perhaps via novel ischemia-related antigens, see below) may have important roles in cardiovascular disease; use of CTLA4Ig or L104EA29YIg in these process may be clinically useful.

(3) Anti-inflammatory effects of CTLA4Ig and/or L104EA29YIg in animal models and clinical studies

Inhibition of CD28-B7 signaling in animal models of renal ischemia suggests that novel self antigens are important in ischemia

The effects of CTLA4Ig on inhibition of ischemia/reperfusion injury has been evaluated in a rat model (Ref: J Clin Invest 1997;100:1199-1203). In this model, rats underwent a unilateral nephrectomy and the contralateral kidney was rendered ischemic for 45 minutes. Coadministration of CTLA4Ig, but not control immunoglobulin, largely blocked the infiltration of CD4+ T-cells, macrophages, and major histocompatability (MHC) II cells into the ischemic kidney. Coincident with this finding, was a reduction of inflammatory "Th1" cytokines (IL-2, IFN-γ, IL2r), macrophage-associated cytokines (TNF-α. IL-6, and inducible nitric oxide synthetase), and chemoattractants/growth factors (MCP-1, RANTES, TGFβ). In addition to these findings, CTLA4Ig almost completely abrogates early (i.e., elevations of plasma creatinine) or late (i.e., urine protein measurements) renal dysfunction. What is particularly striking about this observation is the ability of CTLA4Ig to inhibit this process in a model that is devoid of alloantigen. Alternatively stated, inhibition of CD28-B7 interactions in a non-transplant model of renal ischemia is afforded by CTLA4Ig administration and suggests that novel self-antigens that act through CD28-B7 pathways are important in ischemic injury. Efficacy in this animal model is supportive of a role for the use of CTLA4Ig in models of coronary ischemia that likely has some parallel physiology.

Anti-inflammatory aspects of CTLA4Ig and L104EA29YIg are consistent with their use in the prevention and/or treatment of CV disease

Data presented herein (Examples 3-7) for the clinical development of CTLA4Ig and L104EA29Ig in patients with RA are encouraging with respect to favorable alterations in inflammatory markers. It is noteworthy that patients with RA and patients with atherosclerosis or UA have a number of biochemical, cellular, and antigenic similarities (see above, Table 1). In the phase II clinical development

program in RA several markers of inflammation (CRP, IL-6, TNF-α) that are shared in patients with UA were measured. In patients with RA, administration of CTLA4Ig at either 2 mg/kg or 10 mg/kg resulted in a dose-dependent decline in CRP, IL-6, TNF-α at 180 days as compared with placebo. See Figures 52, 55, and 56. This effect appears to be durable and was shown to persist throughout 360 days treatment with CTLA4Ig . See Figures 85, 86A, and 86B.

Further studies are discussed in Examples 8 and 9.

(4) Newly reported findings on the importance of B7-CD28 interactions in atherosclerosis

Since the filing of the patent application to which this patent application claims priority, two reports have appeared in the scientific literature that strongly support the role of CD28-B7 interactions in the development, progression, and instability of atheromatous lesions. First, Buono, *et. al. (Circulation 2004; 109:2009-2015)* characterized the development and progression of atheromatous lesions and plaque-antigen-specific T-cell responses in a low-density lipoprotein receptor (LDLR)-deficient (*Ldlr*$^{-/-}$) murine model. *Ldlr*$^{-/-}$ mice develop accelerated atherosclerosis when fed a cholesterol-enriched diet. In this study, *Ldlr*$^{-/-}$ mice were crossbred with B7-1$^{-/-}$B7-2$^{-/-}$ mice and the progeny were then intercrossed to generate a compound mutant B7-1$^{-/-}$B7-2$^{-/-}$ *Ldlr*$^{-/-}$ line that is completely devoid of CD80 and CD86. This unique model allowed for investigation of the role of B7-CD28 interactions in an animal model prone to develop atherosclerosis. As expected, when these animals were fed a cholesterol enriched diet, both the *Ldlr*$^{-/-}$ and the B7-1$^{-/-}$B7-2$^{-/-}$ *Ldlr*$^{-/-}$ mice had significantly elevated cholesterol as compared to *Ldlr*$^{-/-}$ mice fed a control diet. Importantly, however, there were no differences observed between the cholesterol levels in the *Ldlr*$^{-/-}$ mice fed a cholerterol enriched diet as compared with the B7-1$^{-/-}$B7-2$^{-/-}$ *Ldlr*$^{-/-}$ mice fed a cholesterol enriched diet. Atherosclerotic lesions in these mice were quantitated from the aortic arch and descending aorta using computerized image analysis from each of the 3 groups. At 8 weeks, there was minimal atherosclerosis in *Ldlr*$^{-/-}$ mice receiving the control diet. *Ldlr*$^{-/-}$ mice receiving a cholesterol enriched diet, however, had a marked increase in atherosclerotic lesisons in both the aortic arch and descending aorta. In contrast, B7-

$1^{-/-}$B7-$2^{-/-}$ *Ldlr*$^{-/-}$ mice receiving a cholesterol enriched diet had a significant reduction of atherosclerotic lesions as compared to *Ldlr*$^{-/-}$ mice receiving a cholesterol enriched diet. After 20 weeks, there was progression of atherosclerosis in all 3 treatment groups and the magnitude of difference observed bewteen the *Ldlr*$^{-/-}$ mice and the B7-$1^{-/-}$B7-$2^{-/-}$ *Ldlr*$^{-/-}$ mice was somewhat diminished. Nonetheless, a statistically significant reduction in atherosclerosis between the *Ldlr*$^{-/-}$ mice and the B7-$1^{-/-}$B7-$2^{-/-}$ *Ldlr*$^{-/-}$ mice in the aortic arch persisted at 20 weeks.

In addition to these morphological observations, there also appears to be functional differences between T-cells from *Ldlr*$^{-/-}$ mice and B7-$1^{-/-}$B7-$2^{-/-}$ *Ldlr*$^{-/-}$ mice. Specifically, *ex-vivo* assays of CD4+ T-cells from *Ldlr*$^{-/-}$ mice and B7-$1^{-/-}$B7-$2^{-/-}$ *Ldlr*$^{-/-}$ mice demonstrate that only *Ldlr*$^{-/-}$ mice fed a cholesterol enriched diet produced substantial (~5-fold higher) amounts of IFN-γ when stimulated with mHSP60. This finding suggests that under conditions of hypercholesterolemia and atherosclerosis, T-cells from *Ldlr*$^{-/-}$ mice were primed to respond to self-HSP60.

In a second publication, Afek *et. al. (Experimental and Molecular Pathology 2004; 76:219-223)* utilize immunohistochemical methods to characterize the expression of CD80 and CD86 within atheroma of apolioprotein E knockout (ApoE$^{-/-}$) mice. ApoE$^{-/-}$ mice develop atherosclerosis according to their maturation age. These investigators evaluated the presence of CD80 and CD86 within atheromatous lesions at various stages of maturation. In this model, CD80 and CD86 positive immunostaining was present in both early and more advanced atheromatous lesions. Moreover, CD80 and CD86 staining was found to be colocalized with oxidized LDL – a putative autoantigen for T-cell activation.

Collectively, these two literature reports support and extend the material previously presented in the priority patent application. First, the notion that T-cells are present in atheroma and are activiated *in* situ is strengthed by the finding of CD80 and CD86 colocalization within both early and advanced atheroma. Second, the data obtained from the B7-$1^{-/-}$B7-$2^{-/-}$ *Ldlr*$^{-/-}$ mice clearly and elegantly demonstrate that interruption of B7-CD28 signaling influences the development and progression of atherosclerotic disease in a well accepted model of atherosclerosis. Finally, the observation that *ex-vivo* stimulation of CD4+ T-cells from mHSP60 results in substantial IFN-γ production supports the prior hypothesis that HSP60 serve as a

novel antigen to promote cardiovascular inflammation and plaque instability (i.e., through the effects of IFN-γ on matrix metalloproteinases and collagen synthesis) through B7-CD28-dependent processes.

Compositions

The present invention provides compositions for treating cardiovascular diseases comprising molecules that block B7 interactions with CTLA4 and/or CD28, such as soluble CTLA4 molecules. Examples of soluble CTLA4 include CTLA4Ig (Figure 24) and soluble CTLA4 mutant molecules such as L104EA29YIg (Figure 19), L104EA29LIg (Figure 20), L104EA29Tig (Figure 21), and L104EA29WIg (Figure 22).

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 a CTLA4Ig molecule (Figure 24) (Linsley, P. S., et al., 1994 *Immunity* 1:793-80). Examples of immunoglobulin domains that may be fused to CTLA4 include, but are not limited to IgCγ1 (IgCgamma1), IgCγ2 (IgCgamma2), IgCγ3 (IgCgamma3), IgCγ4 (IgCgamma4), IgCμ (IgCmu), IgCα1 (IgCalpha1), IgCα2 (IgCalpha2), IgCδ (IgCdelta) or IgCε (IgCepsilon).

For clinical protocols, it is preferred that the immunoglobulin moiety 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 moiety 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 moiety 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*, can be fusion molecules comprising human Ig, e.g., IgC(gamma)1 (i.e. IgCγ1) moieties fused to the mutated CTLA4 fragments.

In one embodiment, the soluble CTLA4 mutant molecules comprise IgCγ1 (IgCgamma1) fused to an extracellular domain of CTLA4 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 domain 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 (IgCgamma1) 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

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 IgCγ1 (IgCgamma1) 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 soluble CTLA4 proteins of the invention, and fragments thereof, can be generated by chemical synthesis methods. The principles of solid phase chemical synthesis of polypeptides are well known in the art and may be found in general texts relating to this area (Dugas, H. and Penney, C. 1981 *Bioorganic Chemistry*, pp 54-92, Springer-Verlag, New York). The soluble CTLA4 proteins may be synthesized by solid-phase methodology utilizing an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.) and synthesis cycles supplied by Applied

Biosystems. Protected amino acids, such as t-butoxycarbonyl-protected amino acids, and other reagents are commercially available from many chemical supply houses.

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 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 comprising 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 substituted with tyrosine, and serine at position +25 is substituted with 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. For example, a CTLA4Ig molecule can comprise a GCT or GCC codon, encoding alanine, at nucleotide position +49 to +51 as shown in Figure 24. In another example, a CTLA4Ig molecule can comprise a GGT or GGG codon, encoding glycine, at nucleotide position +436 to +438 as shown in Figure 24. In yet another example, a CTLA4Ig molecule can comprise a CGG or CGT codon, encoding arginine, at nucleotide position +631 to +633 as shown in Figure 24. 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.

The nucleic acid molecules of the invention also include derivative nucleic acid molecules which differ from DNA or RNA molecules, and anti-sense molecules. Derivative molecules include peptide nucleic acids (PNAs), and non-nucleic acid molecules including phosphorothioate, phosphotriester, phosphoramidate, and methylphosphonate molecules, that bind to single-stranded DNA or RNA in a base pair-dependent manner (Zamecnik, P. C., et al., 1978 *Proc. Natl. Acad. Sci.* 75:280284; Goodchild, P. C., et al., 1986 *Proc. Natl. Acad. Sci.* 83:4143-4146). Peptide nucleic acid molecules comprise a nucleic acid oligomer to which an amino acid residue, such as lysine, and an amino group have been added. These small molecules, also designated anti-gene agents, stop transcript elongation by binding to their complementary (template) strand of nucleic acid (Nielsen, P. E., et al., 1993 *Anticancer Drug Des* 8:53-63). Reviews of methods for synthesis of DNA, RNA, and their analogues can be found in: *Oligonucleotides and Analogues,* eds. F. Eckstein, 1991, IRL Press, New York; *Oligonucleotide Synthesis*, ed. M. J. Gait, 1984, IRL Press, Oxford, England. Additionally, methods for antisense RNA technology are described in U. S. patents 5,194,428 and 5,110,802. A skilled artisan can readily obtain these classes of nucleic acid molecules using the herein described soluble CTLA4

polynucleotide sequences, see for example *Innovative and Perspectives in Solid Phase Synthesis* (1992) Egholm, et al. pp 325-328 or U. S. Patent No. 5,539,082.

Additionally, the invention provides a vector, which comprises the nucleotide sequences of the invention. The term vector includes, but is not limited to, plasmids, cosmids, and phagemids. In one embodiment, the vector can be an autonomously replicating vector comprising a replicon that directs the replication of the rDNA within the appropriate host cell. Alternatively, the vector can direct integration of the recombinant vector into the host cell. Various viral vectors may also be used, such as, for example, a number of well known retroviral and adenoviral vectors (Berkner 1988 *Biotechniques* 6:616-629).

The vectors can permit expression of the soluble CTLA4 transcript or polypeptide sequences in prokaryotic or eukaryotic host cells. The vectors include expression vectors, comprising an expression control element, such as a promoter sequence, which enables transcription of the inserted soluble CTLA4 nucleic acid sequences and can be used for regulating the expression (e.g., transcription and/or translation) of an operably linked soluble CTLA4 sequence in an appropriate host cell. Expression control elements are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, enhancers, transcription terminators, and other transcriptional regulatory elements. Other expression control elements that are involved in translation are known in the art, and include the Shine-Dalgarno sequence (e.g., prokaryotic host cells), and initiation and termination codons.

Specific initiation signals may also be required for efficient translation of a soluble CTLA4 sequence. These signals include the ATG-initiation codon and adjacent sequences. In cases where the soluble CTLA4 initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only the coding sequence, or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG-initiation codon may be provided. Furthermore, the initiation codon should be in the correct reading-frame to ensure translation of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf, D., et al, 1994

Results Probl. Cell. Differ. 20:125-62; Bittner, et al., 1987 *Methods in Enzymol.* 153:516-544).

The preferred vectors for expression of the soluble CTLA4 sequences in eukaryote host cells include expression control elements, such as the baculovirus polyhedrin promoter for expression in insect cells. Other expression control elements include promoters or enhancers derived from the genomes of plant cells (e. g., heat shock, RUBISCO, storage protein genes), viral promoters or leader sequences or from plant viruses, and promoters or enhancers from the mammalian genes or from mammalian viruses.

The preferred vector includes at least one selectable marker gene that encodes a gene product that confers drug resistance such as resistance to ampicillin or tetracyline. The vector also comprises multiple endonuclease restriction sites that enable convenient insertion of exogenous DNA sequences. Methods for generating a recombinant expression vector encoding the soluble CTLA4 proteins of the invention are well known in the art, and can be found in Sambrook et al., (*Molecular Cloning; A Laboratory Manual*, 2nd edition, Sambrook, Fritch, and Maniatis 1989, Cold Spring Harbor Press) and Ausubel et al. (1989 *Current Protocols in Molecular Biology*, John Wiley & Sons, New York N.Y.).

The preferred vectors for generating soluble CTLA4 transcripts and/or the encoded soluble CTLA4 polypeptides are expression vectors which are compatible with prokaryotic host cells. Prokaryotic cell expression vectors are well known in the art and are available from several commercial sources. For example, pET vectors (e.g., pET-21, Novagen Corp.), BLUESCRIPT phagemid (Stratagene, LaJolla, CA), pSPORT (Gibco BRL, Rockville, MD), or ptrp-lac hybrids may be used to express soluble CTLA4 polypeptides in bacterial host cells.

Alternatively, the preferred expression vectors for generating soluble CTLA4 transcripts and/or the encoded soluble CTLA4 polypeptides are expression vectors which are compatible with eukaryotic host cells. The more preferred vectors are those compatible with vertebrate cells. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-

1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), and similar eukaryotic expression vectors.

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. Exemplary animal cells include cells from bovine, ovine, porcine, murine, equine, monkey and ape. 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 whole plants, cell culture, or callus, from tobacco, 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 comprising pharmaceutically effective amounts of a molecule that blocks B7 interaction with CTLA4 and/or CD28 such as soluble CTLA4 molecules, CD28 molecules, B7 (B7-1 or B7-2) molecules, anti-CTLA4 monoclonal antibodies, anti-CD28 monoclonal antibodies or anti-B7 (B7-1 or B7-2) monoclonal antibodies. The pharmaceutical compositions of the invention are useful for treatment of cardiovascular diseases. In certain embodiments, cardiovascular 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 of the invention may additionally include one or more additional, other, or second therapeutic agents. By "administered in combination" or "combination therapy" it is meant that a compound of the present invention and one or more additional therapeutic agents are both administered to the mammal being treated. When administered in combination each component may be administered at the same time or sequentially in any order at different points in time. Thus, each component may be administered separately but sufficiently closely in time so as to provide the desired therapeutic effect.

Additional therapeutic agents, when employed in combination with the molecules of the present invention, may be used, for example, in those amounts indicated in the Physicians' Desk Reference (PDR) or as otherwise determined by one of ordinary skill in the art.

Additional, other, or second therapeutic agents include anti-coagulant or coagulation inhibitory agents, anti-platelet or platelet inhibitory agents, thrombin inhibitors, Vitamin K antagonists, glycoprotein IIb/IIIa receptor antagonists, thrombolytic or fibrinolytic agents, anti-arrythmic agents, anti-hypertensive agents, angiotensin converting enzyme inhibitors (ACE-Is), angiotensin receptor blockers (ARBs), beta-blockers, calcium channel blockers (L-type and T-type), cardiac glycosides, diruetics, mineralocorticoid receptor antagonists, phospodiesterase inhibitors, cholesterol/lipid lowering agents and lipid profile therapies, anti-diabetic agents, anti-depressants, anti-inflammatory agents (steroidal and non-steroidal), anti-osteoporosis agents, hormone replacement therapies, oral contraceptives, anti-obesity agents, anti-anxiety agents, anti-proliferative agents, anti-tumor agents, anti-ulcer and gastroesophageal reflux disease agents, growth hormone and/or growth hormone secretagogues, thyroid mimetics (including thyroid receptor antagonist), anti-infective agents, anti-viral agents, anti-bacterial agents, and anti-fungal agents.

Anticoagulant agents (or coagulation inhibitory agents) that may be used in combination with the compositions of this invention include warfarin and heparin (either unfractionated heparin or any commercially available low molecular weight

heparin), synthetic pentasaccharide, direct acting thrombin inhibitors including hirudin and argatroban as well as factor Xa inhibitors.

The term anti-platelet agents (or platelet inhibitory agents), as used herein, denotes agents that inhibit platelet function, for example by inhibiting the aggregation, adhesion or granular secretion of platelets. Agents include, but are not limited to, the various known non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen, naproxen, sulindac, indomethacin, mefenamate, droxicam, diclofenac, sulfinpyrazone, piroxicam, and pharmaceutically acceptable salts or prodrugs thereof. Of the NSAIDS, aspirin (acetylsalicyclic acid or ASA) and piroxicam are preferred. Other suitable platelet inhibitory agents include IIb/IIIa antagonists (e.g., tirofiban, eptifibatide, and abciximab), thromboxane-A2-receptor antagonists (e.g., ifetroban), thromboxane-A2-synthetase inhibitors, PDE-III inhibitors (e.g., dipyridamole), and pharmaceutically acceptable salts or prodrugs thereof.

The term anti-platelet agents (or platelet inhibitory agents), as used herein, is also intended to include ADP (adenosine diphosphate) receptor antagonists, preferably antagonists of the purinergic receptors P_2Y_1 and P_2Y_{12}, with P_2Y_{12} being even more preferred. Preferred P_2Y_{12} receptor antagonists include ticlopidine and clopidogrel, including pharmaceutically acceptable salts or prodrugs thereof. Clopidogrel is an even more preferred agent. Ticlopidine and clopidogrel are also preferred compounds since they are known to be gentle on the gastro-intestinal tract in use.

The term thrombin inhibitors (or anti-thrombin agents), as used herein, denotes inhibitors of the serine protease thrombin. By inhibiting thrombin, various thrombin-mediated processes, such as thrombin-mediated platelet activation (that is, for example, the aggregation of platelets, and/or the granular secretion of plasminogen activator inhibitor-1 and/or serotonin) and/or fibrin formation are disrupted. A number of thrombin inhibitors are known to one of skill in the art and these inhibitors are contemplated to be used in combination with the present compounds. Such inhibitors include, but are not limited to, boroarginine derivatives, boropeptides, heparins, hirudin, argatroban, and melagatran, including pharmaceutically acceptable salts and prodrugs thereof. Boroarginine derivatives and boropeptides include N-

acetyl and peptide derivatives of boronic acid, such as C-terminal α-aminoboronic acid derivatives of lysine, ornithine, arginine, homoarginine and corresponding isothiouronium analogs thereof. The term hirudin, as used herein, includes suitable derivatives or analogs of hirudin, referred to herein as hirulogs, such as disulfatohirudin. The term thrombolytics or fibrinolytic agents (or thrombolytics or fibrinolytics), as used herein, denote agents that lyse blood clots (thrombi). Such agents include tissue plasminogen activator (natural or recombinant) and modified forms thereof, anistreplase, urokinase, streptokinase, tenecteplase (TNK), lanoteplase (nPA), factor VIIa inhibitors, PAI-1 inhibitors (i.e., inactivators of tissue plasminogen activator inhibitors), alpha2-antiplasmin inhibitors, and anisoylated plasminogen streptokinase activator complex, including pharmaceutically acceptable salts or prodrugs thereof. The term anistreplase, as used herein, refers to anisoylated plasminogen streptokinase activator complex, as described, for example, in EP 028,489, the disclosure of which is hereby incorporated herein by reference herein. The term urokinase, as used herein, is intended to denote both dual and single chain urokinase, the latter also being referred to herein as prourokinase.

Examples of suitable anti-arrythmic agents for use in combination with the present compounds include: Class I agents (such as propafenone); Class II agents (such as carvadiol and propranolol); Class III agents (such as sotalol, dofetilide, amiodarone, azimilide and ibutilide); Class IV agents (such as ditiazem and verapamil); K^+ channel openers such as I_{Ach} inhibitors, and I_{Kur} inhibitors (e.g., compounds such as those disclosed in WO01/40231).

Examples of suitable anti-hypertensive agents for use in combination with the compounds of the present invention include: alpha adrenergic blockers; beta adrenergic blockers; calcium channel blockers (e.g., diltiazem, verapamil, nifedipine, amlodipine and mybefradil); diruetics (e.g., chlorothiazide, hydrochlorothiazide, flumethiazide, hydroflumethiazide, bendroflumethiazide, methylchlorothiazide, trichloromethiazide, polythiazide, benzthiazide, ethacrynic acid tricrynafen, chlorthalidone, furosemide, musolimine, bumetanide, triamtrenene, amiloride, spironolactone); renin inhibitors; ACE inhibitors (e.g., captopril, zofenopril, fosinopril, enalapril, ceranopril, cilazopril, delapril, pentopril, quinapril, ramipril, lisinopril); AT-1 receptor antagonists (e.g., losartan, irbesartan, valsartan); ET

receptor antagonists (e.g., sitaxsentan, atrsentan and compounds disclosed in U.S. Patent Nos. 5,612,359 and 6,043,265); Dual ET/AII antagonist (e.g., compounds disclosed in WO 00/01389); neutral endopeptidase (NEP) inhibitors; vasopepsidase inhibitors (dual NEP-ACE inhibitors) (e.g., omapatrilat, gemopatrilat and nitrates).

Examples of suitable calcium channel blockers (L-type or T-type) for use in combination with the compounds of the present invention include diltiazem, verapamil, nifedipine, amlodipine and mybefradil.

Examples of suitable cardiac glycosides for use in combination with the compounds of the present invention include digitalis and ouabain.

Examples of suitable diruetics for use in combination with the compounds of the present invention include: chlorothiazide, hydrochlorothiazide, flumethiazide, hydroflumethiazide, bendroflumethiazide, methylchlorothiazide, trichloromethiazide, polythiazide, benzthiazide, ethacrynic acid tricrynafen, chlorthalidone, furosemide, musolimine, bumetanide, triamtrenene, amiloride, and spironolactone.

Examples of suitable mineralocorticoid receptor antagonists for use in combination with the compounds of the present invention include sprionolactone and eplerenone.

Examples of suitable phospodiesterase inhibitors for use in combination with the compounds of the present invention include: PDE III inhibitors (such as cilostazol); and PDE V inhibitors (such as sildenafil).

Examples of suitable cholesterol/lipid lowering agents and lipid profile therapies for use in combination with the compounds of the present invention include: HMG-CoA reductase inhibitors (e.g., pravastatin, lovastatin, atorvastatin, simvastatin, fluvastatin, NK-104 (a.k.a. itavastatin, or nisvastatin or nisbastatin) and ZD-4522 (a.k.a. rosuvastatin, or atavastatin or visastatin)); squalene synthetase inhibitors; fibrates; bile acid sequestrants (such as questran); ACAT inhibitors; MTP inhibitors; lipooxygenase inhibitors; choesterol absorption inhibitors; and cholesterol ester transfer protein inhibitors (e.g., CP-529414).

Examples of suitable anti-diabetic agents for use in combination with the compounds of the present invention include: biguanides (e.g., metformin); glucosidase inhibitors (e.g., acarbose); insulins (including insulin secretagogues or insulin sensitizers); meglitinides (e.g., repaglinide); sulfonylureas (e.g., glimepiride,

glyburide and glipizide); biguanide/glyburide combinations (e.g., glucovance), thiozolidinediones (e.g., troglitazone, rosiglitazone and pioglitazone), PPAR-alpha agonists, PPAR-gamma agonists, PPAR alpha/gamma dual agonists, SGLT2 inhibitors, inhibitors of fatty acid binding protein (aP2) such as those disclosed in WO00/59506, glucagon-like peptide-1 (GLP-1), and dipeptidyl peptidase IV (DP4) inhibitors.

Examples of suitable anti-depressant agents for use in combination with the compounds of the present invention include nefazodone and sertraline.

Examples of suitable anti-inflammatory agents for use in combination with the compounds of the present invention include: steroid compounds such as corticosteroids and glucocorticoids, including prednisone and dexamethasone; etanercept (Enbrel®); infliximab (Remicade®); protein tyrosine kinase (PTK) inhibitors; cyclooxygenase inhibitors (including NSAIDs, and COX-1 and/or COX-2 inhibitors); aspirin; indomethacin; ibuprofen; prioxicam; naproxen; celecoxib; and/or rofecoxib.

Examples of suitable anti-osteoporosis agents for use in combination with the compounds of the present invention include alendronate and raloxifene.

Examples of suitable hormone replacement therapies for use in combination with the compounds of the present invention include estrogen (e.g., congugated estrogens) and estradiol.

Examples of suitable anti-coagulants for use in combination with the compounds of the present invention include heparins (e.g., unfractioned and low molecular weight heparins such as enoxaparin and dalteparin).

Examples of suitable anti-obesity agents for use in combination with the compounds of the present invention include orlistat and aP2 inhibitors (such as those disclosed in WO00/59506).

Examples of suitable anti-anxiety agents for use in combination with the compounds of the present invention include diazepam, lorazepam, buspirone, and hydroxyzine pamoate.

Examples of suitable anti-proliferative agents for use in combination with the compounds of the present invention include cyclosporin A, paclitaxel, adriamycin; epithilones, cisplatin, and carboplatin.

Examples of suitable anti-ulcer and gastroesophageal reflux disease agents for use in combination with the compounds of the present invention include famotidine, ranitidine, and omeprazole.

Additional therapeutic agents also include: collagen, dnaJ, molecules that block TNF function (e.g., pegsunercept), molecules that block cytokine function (e.g., AMG719), molecules that block LFA-1 function (e.g., efalizumab) and stem cell transplants. These other treatments are currently being studied in clinical trials (www.clinicaltrials.gov) to determine their effect on rheumatoid arthritis.

Collagen, for example in the form of bovine II collagen, may be orally administered to a patient suffering from cardiovascular disease in order to alleviate one or more symptoms of cardiovascular disease.

DnaJ is a small peptide which mimics a protein contained in a gene in many patients with cardiovascular disease. The peptide is derived from *E. coli* bacteria heat shock protein. DnaJ may be orally administered to a patient suffering from cardiovascular disease in order to alleviate one or more symptoms of cardiovascular disease.

TNF is a molecule involved in the inflammatory response of patients with rheumatoid arthritis and possibly cardiovascular disease. Conceivably, any molecule that blocks TNF function e.g., by blocking TNF binding to the TNF receptor (TNFR), may help modify the progression of cardiovascular disease and alleviate some of its symptoms. Several TNF blockers such as infliximab and etanercept, have been shown to be efficacious in treating cardiovascular disease. Other TNF blockers such as pegsunercept are being developed and tested (Phase II clinical trial) for their efficacy in treating cardiovascular disease.

Cytokines e.g., Interleukin-1 (IL-1), are cell secreted molecules involved in mediating immune responses. Conceivably, any molecule that blocks cytokine function e.g., by blocking IL-1 interaction with its receptor, may help modify the progression of cardiovascular disease and alleviate one or more of its symptoms. Anakinra, a recombinant protein that blocks IL-1 interaction with its receptor (IL-1R) has been shown to be efficacious in treating cardiovascularoid arthritis. An IL-1 inhibitor, AMG719, is being developed and tested (Phase II clinical trial) for its efficacy in treating rheumatoid arthritis.

Lymphocyte function associated molecule 1 (LFA-1) is a molecule composed of two subunits, CD11a and CD18, which functions by mediating lymphocyte adhesion to various cell types such as endothelium. Conceivably, interference of LFA-1 function may help modify the progression of cardiovascular disease and alleviate one or more of its symptoms. An anti-LFA-1 antibody, efalizumab, is being developed and tested (Phase II clinical trial) for its efficacy in treating rheumatoid arthritis.

Blockage of TNF, cytokine or LFA-1 interaction to their ligands by a potentially therapeutic molecule can be determined by any number of assays known to those skilled in the art. For example, competition assays may be used to test blockage by the molecule of interest e.g., a molecule can be exposed to a TNF/TNFR binding pair in order to compete with TNF to bind to TNFR. Alternatively, functional assays can be performed to test blockage e.g., a molecule can be tested for its ability to inhibit an inflammatory cascade, or any part of an inflammatory reaction such as swelling, redness or pain, caused by a cytokine.

Additional therapeutic agents also include: p38 MAP kinase inhibitor, 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. ATCC accession number 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 agent; the soluble CTLA4 molecules with two other agents; the soluble CTLA4 molecules with three other agents; and the like. The determination of the optimal combination and dosages can be determined and optimized using methods well known in the art.

Some specific combinations for co-administration include the following: CTLA4Ig or L104EA29YIg and CD80 monoclonal antibodies (mAbs); CTLA4Ig or L104EA29YIg and CD86 mAbs; CTLA4Ig or L104EA29YIg, CD80 mAbs, and CD86 mAbs; CTLA4Ig or L104EA29YIg and gp39 mAbs; CTLA4Ig or L104EA29YIg and CD40 mAbs; CTLA4Ig or L104EA29YIg and CD28 mAbs; CTLA4Ig or L104EA29YIg, CD80 and CD86 mAbs, and gp39 mAbs; CTLA4Ig or L104EA29YIg, CD80 and CD86 mAbs and CD40 mAbs; and CTLA4Ig or 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.

Additional therapeutic agents also include: 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; a dihydrofolic acid reductase inhibitor such as 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.

Administration of the compounds of the present invention (i.e., a first therapeutic agent) in combination with at least one additional therapeutic agent (i.e., a second therapeutic agent), preferably affords an efficacy advantage over the compounds and agents alone, preferably while permitting the use of lower doses of each (i.e., a synergistic combination). A lower dosage minimizes the potential of side effects, thereby providing an increased margin of safety. It is preferred that at least one of the therapeutic agents is administered in a sub-therapeutic dose. It is even more preferred that all of the therapeutic agents be administered in sub-therapeutic doses. Sub-therapeutic is intended to mean an amount of a therapeutic agent that by itself does not give the desired therapeutic effect for the condition or disease being treated. Synergistic combination is intended to mean that the observed effect of the combination is greater than the sum of the individual agents administered alone.

A pharmaceutical composition comprising soluble CTLA4 can be used for methods for blocking B7 interaction with CTLA4 and/or CD28; or for treating cardiovascular diseases. Effective amounts of soluble CTLA4 in the pharmaceutical composition range about 0.1 to 100 mg/kg weight of the subject. In another embodiment, the effective amount is an amount about 0.5 to 100 mg/kg weight of a subject, 0.5 to 5 mg/kg weight of a subject, about 5 to 10 mg/kg weight of a subject, about 10 to 15 mg/kg weight of a subject, about 15 to 20 mg/kg weight of a subject, about 20 to 25 mg/kg weight of a subject, about 25 to 30 mg/kg weight of a subject, about 30 to 35 mg/kg weight of a subject, about 35 to 40 mg/kg weight of a subject, about 40 to 45 mg/kg of a subject, about 45 to 50 mg/kg weight of a subject, about 50 to 55 mg/kg weight of a subject, about 55 to 60 mg/kg weight of a subject, about 60 to 65 mg/kg weight of a subject, about 65 to 70 mg/kg weight of a subject, about 70 to 75 mg/kg weight of a subject, about 75 to 80 mg/kg weight of a subject, about 80 to 85 mg/kg weight of a subject, about 85 to 90 mg/kg weight of a subject, about 90 to 95 mg/kg weight of a subject, or about 95 to 100 mg/kg weight of a subject.

In an embodiment, the effective amount of soluble CTLA4 is an amount about 2 mg/kg to about 10 mg/kg weight of a subject. In another embodiment, the effective amount is an amount about 0.1 to 4 mg/kg weight of a subject. In another

embodiment the effective amount is an amount about 0.1 to 0.5 mg/kg weight of a subject, about 0.5 to 1.0 mg/kg weight of a subject, about 1.0 to 1.5 mg/kg weight of a subject, about 1.5 to 2.0 mg/kg weight of a subject, about 2.0 to 2.5 mg/kg weight of a subject, about 2.5 to 3.0 mg/kg weight of a subject, about 3.0 to 3.5 mg/kg weight of a subject or about 3.5 to 4.0 mg/kg weight of a subject. In another embodiment, the effective amount is an amount about 0.1 to 20 mg/kg weight of a subject. In another embodiment, the effective amount is an amount about 0.1 to 2 mg/kg weight of a subject, about 2 to 4 mg/kg weight of a subject, about 4 to 6 mg/kg weight of a subject, about 6 to 8 mg/kg weight of a subject, about 8 to 10 mg/kg weight of a subject, about 10 to 12 mg/kg weight of a subject, about 12 to 14 mg/kg weight of a subject, about 14 to 16 mg/kg weight of a subject, about 16 to 18 mg/kg weight of a subject or about 18 to 20 mg/kg weight of a subject. In an embodiment, the effective amount is 2 mg/kg weight of a subject. In another embodiment, the effective amount is about 10 mg/kg weight of a subject.

In a specific embodiment, an effective amount of soluble CTLA4 is 500 mg for a subject weighing less than 60 kg, 750 mg for a subject weighing between 60-100 kg and 1000 mg for a subject weighing more than 100 kg.

The present invention also provides pharmaceutical compositions comprising the molecules of the inventon e.g., CTLA4Ig and an acceptable carrier or adjuvant which is known to those of skill of the art. The pharmaceutical compositions preferably include suitable carriers and adjuvants which include any material which when combined with the molecules of the invention (e.g., a soluble CTLA4 molecule, such as, CTLA4Ig or L104EA29Y) retain 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.

In a further embodiment of the invention, the present invention provides kits (i.e., a packaged combination of reagents with instructions) containing the molecules of the invention useful for blocking B7 interactions with its ligands and/or for treating a cardiovascular disease.

The kit can contain a pharmaceutical composition that includes one or more agents, for example, a soluble CTLA4 molecule alone, or with a second agent, and an acceptable carrier or adjuvant, e.g., pharmaceutically acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use. The agents may be provided as dry powders, usually lyophilized, including excipients that upon dissolving will provide a reagent solution having the appropriate concentration.

Second agents can include those described above as additional agents, such as anti-coagulant or coagulation inhibitory agents, anti-platelet or platelet inhibitory agents, thrombin inhibitors, thrombolytic or fibrinolytic agents, anti-arrythmic agents, anti-hypertensive agents, calcium channel blockers (L-type and T-type), cardiac glycosides, diruetics, mineralocorticoid receptor antagonists, phospodiesterase inhibitors, cholesterol/lipid lowering agents and lipid profile therapies, anti-diabetic agents, anti-depressants, anti-inflammatory agents (steroidal and non-steroidal), anti-osteoporosis agents, hormone replacement therapies, oral contraceptives, anti-obesity agents, anti-anxiety agents, anti-proliferative agents, anti-tumor agents, anti-ulcer and gastroesophageal reflux disease agents, growth hormone and/or growth hormone

secretagogues, thyroid mimetics (including thyroid receptor antagonist), anti-infective agents, anti-viral agents, anti-bacterial agents, and anti-fungal agents.

The kit comprises a container with a label and/or instructions. Suitable containers include, for example, bottles, vials, and test tubes. The containers can be formed from a variety of materials such as glass or plastic. The container can have a sterile access port (for example the container can be an intravenous solution bag or a vial having a stopper pierceable by a needle such as a hypodermic injection needle). The container can hold a pharmaceutical composition such as a pharmaceutical composition having an agent that is effective for blocking B7 interactions with its ligand and/or treating an cardiovascular disease.

The kit can also comprise a second container comprising one or more second agents as described herein and/or a pharmaceutically acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The kit may also suitably include a label and/or instructions on, or associated with the container. The label can provide directions for carrying out the preparation of the agents for example, dissolving of the dry powders, and/or treatment for a specific cardiovascular disease. The label and/or instructions may indicate that administration of the composition of the invention and a second agent may be at the same time or may be sequentially in any order at different points in time.

The label and/or the instructions can indicate directions for either in vivo or in vitro use of the pharmaceutical composition. The label and/or the instructions can indicate that the pharmaceutical composition is used alone, or in combination with a second agent.

The label can indicate appropriate dosages for the molecules of the invention. For example, the label can indicate that dosages for a molecule that is effective for blocking B7 interactions with its ligand and/or treating an cardiovascular disease is about 0.1 to 100 mg/kg weight of the subject, about 0.5 to 100 mg/kg weight of a subject, 0.5 to 5 mg/kg weight of a subject, about 5 to 10 mg/kg weight of a subject, about 10 to 15 mg/kg weight of a subject, about 15 to 20 mg/kg weight of a subject,

about 20 to 25 mg/kg weight of a subject, about 25 to 30 mg/kg weight of a subject, about 30 to 35 mg/kg weight of a subject, about 35 to 40 mg/kg weight of a subject, about 40 to 45 mg/kg of a subject, about 45 to 50 mg/kg weight of a subject, about 50 to 55 mg/kg weight of a subject, about 55 to 60 mg/kg weight of a subject, about 60 to 65 mg/kg weight of a subject, about 65 to 70 mg/kg weight of a subject, about 70 to 75 mg/kg weight of a subject, about 75 to 80 mg/kg weight of a subject, about 80 to 85 mg/kg weight of a subject, about 85 to 90 mg/kg weight of a subject, about 90 to 95 mg/kg weight of a subject, about 95 to 100 mg/kg weight of a subject, about 2 to 10 mg/kg weight of a subject, about 0.1 to 4 mg/kg weight of a subject, about 0.1 to 0.5 mg/kg weight of a subject, about 0.5 to 1.0 mg/kg weight of a subject, about 1.0 to 1.5 mg/kg weight of a subject, about 1.5 to 2.0 mg/kg weight of a subject, about 2.0 to 2.5 mg/kg weight of a subject, about 2.5 to 3.0 mg/kg weight of a subject, about 3.0 to 3.5 mg/kg weight of a subject, about 3.5 to 4.0 mg/kg weight of a subject, about 4.0 to 4.5 mg/kg weight of a subject, about 4.5 to 5.0 mg/kg weight of a subject, about 5.0 to 5.5 mg/kg weight of a subject, about 5.5 to 6.0 mg/kg weight of a subject, about 6.0 to 6.5 mg/kg weight of a subject, about 6.5 to 7.0 mg/kg weight of a subject, about 7.0 to 7.5 mg/kg weight of a subject, about 7.5 to 8.0 mg/kg weight of a subject, about 8.0 to 8.5 mg/kg weight of a subject, about 8.5 to 9.0 mg/kg weight of a subject, about 9.0 to 9.5 mg/kg weight of a subject, about 9.5 to 10.0 mg/kg weight of a subject, about 0.1 to 2 mg/kg weight of a subject, about 2 to 4 mg/kg weight of a subject, about 4 to 6 mg/kg weight of a subject, about 6 to 8 mg/kg weight of a subject, about 8 to 10 mg/kg weight of a subject, about 10 to 12 mg/kg weight of a subject, about 12 to 14 mg/kg weight of a subject, about 14 to 16 mg/kg weight of a subject, about 16 to 18 mg/kg weight of a subject, about 18 to 20 mg/kg weight of a subject, about 0.5 mg/kg weight of the subject, 2 mg/kg weight of the subject, 10 mg/kg weight of the subject, about 0.5 mg/kg to 100 weight of the subject, about 0.5 to 10 mg/kg weight of a subject, about 0.1 to 20 mg/kg weight of a subject, about 500 mg for a subject weighing less than 60 kg, 750 mg for a subject weighing between 60-100 kg or 1000 mg for a subject weighing more than 100 kg

The label and/or the instructions can also indicate that the pharmaceutical composition can be used alone, or in combination, with a second agent to treat a

condition of choice, e.g., cardiovascular diseases, at the same time or sequentially in any order at different points in time.

In a specific embodiment of the invention, the kit comprises a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an effective amount of a first agent, wherein the first agent is a molecule that blocks B7 interaction with CTLA4 and/or CD28 such as soluble CTLA4 molecules, CD28 molecules, B7 (B7-1 or B7-2) molecules, anti-CTLA4 monoclonal antibodies, anti-CD28 monoclonal antibodies or anti-B7 (B7-1 or B7-2) monoclonal antibodies. 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).

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 regulate functional CTLA4- and CD28- positive cell interactions with B7-positive cells, e.g., by interfering with reaction of an endogenous CTLA4 and/or CD28 molecule with a B7 molecule. Suitable amounts of soluble CTLA4 for use in the methods of the invention are described supra.

The present invention further provides methods for treating cardiovascular diseases. The methods comprise administering a therapeutic composition of the invention, such as soluble CTLA4 molecules of the invention, to a subject in an amount effective to relieve at least one of the symptoms associated with cardiovascular diseases. Examples of soluble CTLA4 include CTLA4Ig and soluble CTLA4 mutant molecule e.g. L104EA29YIg. Symptoms of cardiovascular disease include, but are not limited to, dysrhythmias; ischemia; angina; reduced exercise tolerance; fatigue; dyspnea on exertion; and transient ischemic attacks. Additionally, the invention may provide long-term therapy for cardiovascular diseases by blocking the T-cell/B7-positive cell interactions, thereby blocking T-cell activation/stimulation by co-stimulatory signals such as B7 binding to CD28, leading to induction of T-cell anergy or tolerance.

Cardiovascular diseases include, but are not limited to, the following diseases or conditions: thromboembolic disorders, including arterial cardiovascular thromboembolic disorders, venous cardiovascular thromboembolic disorders, and thromboembolic disorders in the chambers of the heart; ahtherosclerosis; restensosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; cardiovascular inflammation; vascular inflammation; coronary heart disease (CHD); unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); first or recurrent myocardial infarction; acute myocardial infarction (AMI); myocardial infarction; non-Q wave myocardial infarction; non-STE myocardial infarction; coronary artery disease; cardiac ischemia; ischemia; ischemic sudden death; transient ischemic attack; stroke; atherosclerosis; peripheral occlusive arterial disease; venous thrombosis; deep vein thrombosis; thrombophlebitis; arterial embolism; coronary arterial thrombosis; cerebral arterial thrombosis; cerebral embolism; kidney embolism; pulmonary embolism; thrombosis resulting from (a) prosthetic valves or other implants, (b) indwelling catheters, (c) stents, (d) cardiopulmonary bypass, (e) hemodialysis, or (f) other procedures in which blood is exposed to an artificial surface that promotes thrombosis; thrombosis resulting from atherosclerosis, surgery or surgical complications, prolonged immobilization, arterial fibrillation, congenital thrombophilia, cancer, diabetes, effects of medications or hormones, and complications of pregnancy; cardiac arrhytmias including supraventricular arrhythmias, atrial arrhythmias, atrial flutter, atrial fibrillation; other diseases listed in Heart Disease: A Textbook of Cardiovascular Medicine, 2 Volume Set, 6th Edition, 2001, Eugene Braunwald, Douglas P. Zipes, Peter Libby, Douglas D. Zipes.

Preferred cardiovascular diseases are: atherosclerosis; coronary heart disease (CHD); restensosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; cardiovascular inflammation; vascular inflammation; unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); myocardial infarction; acute myocardial infarction (AMI), including first or recurrent myocardial infarction, non-Q wave myocardial infarction, non-ST-segment elevation myocardial infarction and ST-segment elevation myocardial infarction.

More preferred cardiovascular diseases are: atherosclerosis; coronary heart disease (CHD); unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); myocardial infarction; acute myocardial infarction (AMI), including first or recurrent myocardial infarction, non-Q wave myocardial infarction, non-ST-segment elevation myocardial infarction and ST-segment elevation myocardial infarction.

Preferred cardiovascular diseases are carcardiovascular diseases mediated by T cell interactions with B7-positive cells.

Also preferred are cardiovascular diseases associated with at least one marker of inflammation. By associated with a marker of inflammation, is meant that the marker (presence, absence or specific concentrations) correlates statisitically with: the risk of the future development of disease, or the risk of disease recurrence, or the current level of disease activity. Markers of inflammation include, but are not limited to, CRP, hsCRP, IL-10, CD40L, sCD40L, IL-6, sICAM-1, TNF-α, white blood cell count, fibrinogen, and serum amyloid A. Preferred markers of inflammation are CRP, hsCRP, IL-6, and TNF-α; most preferred is hsCRP. High CRP, high hsCRP, low IL-10, high sCD40L, high IL-6, high sICAM-1, highTNF-α, high white blood cell count, high fibrinogen, and high serum amyloid A are known to be indicators of inflammation.

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.

Preferred embodiments of the invention comprises use of CTLA4Ig or the soluble CTLA4 mutant molecule L104EA29YIg to regulate functional CTLA4- and CD28- positive cell interactions with B7-positive cells, to treat or prevent cardiovascular diseases, including recurrent cardiovascular events (e.g., myocardial infarction), 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 (Figure 19). The L104EA29YIg molecule may encompass further mutations beyond the two specified herein.

Suitable amounts of the molecule used to block the B7 interaction with CTLA4 and/or CD28 are described supra. The molecule used to block the B7/CTLA4 interaction may be a soluble CTLA4 such as CTLA4Ig, CTLA4Ig/CD28Ig or L104EA29YIg, a soluble CD28 such as CD28Ig, a soluble B7 (B7-1 or B7-2) such as B7Ig, anti-CTLA4 monoclonal antibodies, anti-CD28 monoclonal antibodies or anti-B7 monoclonal antibodies.

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: angina; recurrent angina; unstable angina; ischemic time; hospitalization for cardiovascular reasons; acute myocardial infarction; recurrent myocardial infarction; need for urgent revascularization; need for percutaneous coronary intervention; all cause mortality; cardiovascular mortality; transent ischemic attack; and stroke.

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

The present invention provides various methods, local or systemic, for administering the therapeutic compositions of the invention such as soluble CTLA4 molecule alone or in conjunction with an additional therapeutic agent. The methods include intravenous, intramuscular, intraperitoneal, oral, inhalation and subcutaneous methods, as well as implantable pump, continuous infusion, bolus infusion, gene therapy, liposomes, suppositories, topical contact, vesicles, capsules, biodegradable polymers, drug-eluting stents, hydrogels, controlled release patch. 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.

The therapeutic composition of the invention, such as soluble CTLA4 molecule, may be administered at the same time, or may be administered sequentially in any order at different points in time, as an additional therapeutic agent.

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. Suitable amounts of soluble CTLA4 are described supra. 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. In an embodiment, soluble CTLA4 may be administered to a subject daily, weekly, monthly and/or yearly, in single or multiple times per day/week/month/year, depending on need. For example, in one embodiment, the molecule may initially be administered once every two weeks for a month, and then once every month thereafter.

Dosage of a therapeutic agent is dependant upon many factors including, but not limited to, the type of tissue affected, the type of cardiovascular 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 from about 0.1 to 100 mg/kg weight of the patient/day. Suitable amounts of soluble CTLA4 are described supra.

The invention also encompasses the use of the compositions of the invention together with other therapeutic agents or pharmaceutical agents to treat cardiovascular diseases. For example, cardiovascular diseases may be treated with molecules of the invention in conjunction with, but not limited to, the additional therapeutic agents listed above in the discussion of compositions of the invention.

Where the soluble CTLA4 mutant molecules of the invention are administered in conjunction with other agents, e.g. as hereinabove specified, dosages of the co-administered agent will of course vary depending on the type of co-drug employed, 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 in any order (i.e. at the same time or sequentially in any order at different points in time), 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, comprising a soluble CTLA4 molecule, in free form or in pharmaceutically acceptable salt form, to be used concomitantly or in sequence in any order (i.e. at the same time or sequentially in any order at different points in time), with at least one pharmaceutical composition comprising another therapeutic agent. The kit may comprise instructions for its administration. The kits of the invention can be used in any method of the present invention.

The invention also provides methods for producing the soluble CTLA4 mutant 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 eukaryote genomes, including 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.

EXAMPLES

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, CTCAGTCTGGTCCTTGCACTCCTGTTTCCAAGCATGGCGAGCATGGCAATG CACGTGGCCCAGCC (SEQ ID NO:1) (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 TTTGGGCTCCTGATCAGAATCTGGG CACGGTTG (SEQ ID NO: 2) (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, CTAGCCACTGAAGCTTCACCAATGGGTG TACTGCTCACACA-GAGGACGCTGCTCAGTCTGGTCCTTGCACTC (SEQ ID NO: 3) 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 $k_s=dR/dt=k_{on}\cdot Ck_{off}$, 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 (SEQ ID NO: 4) was chosen to match sequences in the vector; and the reverse primer, GTGGTGTATTGGTCTAGATCAATCAGAATCTGGGCACGGTTC (SEQ ID NO: 5) 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 (gamma-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 Apparent Effect	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	+		
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 L104EA29YIG 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). Similarly, a subject satisfied the ACR50 or ACR70 criterion if there was a 50 or 70 percent improvement, respectively, in tender and swollen joint counts and 50 or 70 percent improvement, respectively, in three of the five remaining symptoms measured, such as patient and physician global disease changes, pain, physical disability, and an acute phase reactant such as CRP or ESR.

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 L104EA29YIG 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, L104EA29YIG, or L104EA29YIG 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 L104EA29YIG-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 from 0.5 to 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.

EXAMPLE 5

A Study to Evaluate the Safety and Clinical Efficacy of Two Different Doses of CTLA4Ig Administered Intravenously to Subjects with Active Rheumatoid Arthritis While Receiving Methotrexate

Rheumatoid Arthritis (RA) treatment is rapidly changing with an increased willingness to use more aggressive therapies to achieve larger increases in efficacy and higher success rates. The ultimate goal is to improve the subject condition in a

more intensive way, by raising the rate of major and complete clinical response, to treatment and maintaining this benefit with acceptable safety.

Methotrexate remains the cornerstone of the RA treatment. It was the first agent that demonstrated early onset of action, superior efficacy and tolerability compared to the classical DMARDs (e.g. gold, hydroxychloroquine, sulfasalazine) used to treat RA. Clinical benefit may be seen as early as 3 weeks after initiating treatment, and the maximal improvement is generally achieved by 6 months. However, methotrexate has a number of limitations. For example, despite its increased tolerability, the window between efficacy and liver toxicity is quite narrow. Subjects treated with methotrexate require careful monitoring and unacceptable toxicity is often the reason for discontinuation of treatment.

Methotrexate also does not appear to efficiently control disease progression or joint deterioration. For some subjects, practitioners feel compelled to add a second DMARD with the hope of increasing efficacy despite the risk of increased toxicity. Alternatively, co-treatment with methotrexate and a costimulator blocker (e.g. CD80 and CD86 blockers such as CTLA4Ig) that target the auto-immune mechanism that lies upstream of the cytokine inflammatory cascade, may also increase efficacy.

As noted in Example 3, above, significant clinical responses and reductions in surrogate markers of disease activity were observed for CTLA4Ig at doses of 2 and 10 mg/kg with a good tolerability profile. It has also been confirmed that the composition CTLA4Ig, used in Example 3 above, did not induce any side effects. As a result, it was decided to continue the clinical development of CTLA4Ig for rheumatoid arthritis in Phase IIB.

The following provides a description of a Phase IIB clinical study of human patients administered soluble CTLA4 molecule with methotrexate, and the results of the study after six months.

This Example describes a twelve month study in which primary efficacy was assessed after all subjects completed six months of treatment or discontinue therapy. Efficacy, safety, and disease progression were also assessed throughout the duration of the study.

The study utilized a randomized, double blind, placebo controlled, parallel dosing design. The study was designed to evaluate the safety, clinical activity,

immunogenicity and pharmacokinetics of two doses of CTLA4Ig: 2 or 10 mg/kg. A total of approximately 330 subjects with active RA and receiving methotrexate were randomized to 1 of 3 dosing arms: CTLA4Ig at 2 mg/kg (N = 110), 10 mg/kg (N = 110) and placebo control group (N = 110) given monthly infusions for 12 months. All groups continued on weekly methotrexate treatment (10-30 mg weekly) (Figures 57-62).

CTLA4Ig or a placebo were also administered on Day 15. Each dose of study medication was infused intravenously over approximately 30 minutes. The primary efficacy endpoint was the ACR 20 response rate after 6 months.

For the first 6 months, subjects were not allowed to alter their doses of corticosteroids, glucocorticoids or NSAIDs. Increases in methotrexate were also not permitted during the first six months. Decreases in methotrexate were permitted only if it was felt to be causing toxicity. Subjects were treated with methotrexate for at least 6 months, and at a stable dose for 28 days prior to first treatment of CTLA4Ig or placebo. DMARDs other than methotrexate were not permitted. Low-dose stable corticosteroids use (at 10 mg daily or less) and/or use of stable non-steroidal anti-inflammatory drugs (NSAIDs), including acetyl salicylic acid (ASA), was allowed. Analgesics that did not contain ASA or NSAIDs were permitted in subjects experiencing pain not adequately controlled by the baseline and study medications, except for 12 hours before a joint evaluation. Decreases in NSAIDs were permitted but only if due to adverse events such as gastrointestinal toxicity.

Test Product, Dose and Mode of Administration, Duration of Treatment

CTLA4Ig at 2 mg/kg or 10 mg/kg was infused every two weeks for the first month, and monthly thereafter for 12 months.

All subjects received weekly doses of methotrexate (10-30 mg) for at least six months prior to randomization and maintained at the entry dose for the first 6 months of the trial. Doses could only be decreased for toxicity during the first six months.

Criteria for Evaluation

The primary endpoint of the first stage of the study was the proportion of subjects meeting the American College of Rheumatology criteria for 20%

improvement (ACR 20) on Day 180 (month six). The ACR 20 definition of improvement is a 20% improvement from baseline in the number of tender and swollen joint counts, and a 20% improvement from baseline in 3 of the following 5 core set measurements: subject global assessment of pain, subject global assessment of disease activity, physician global assessment of disease activity, subject assessment of physical function and acute phase reactant value (C-reactive protein (CRP)). The evaluation for 50% improvement (ACR 50) and 70% improvement (ACR 70) follow similarly. Subjects who discontinued the study due to lack of efficacy (i.e. worsening RA) were considered as ACR non-responders from that time on. For all subjects who dropped out for other reasons, their ACR response at the time of discontinuation was carried forward.

Statistical Methods

Two doses of CTLA4Ig (2 mg/kg and 10 mg/kg) were compared with the placebo control group. All subjects were maintained at the same stable entry doses of methotrexate. The primary analysis was the comparison of CTLA4Ig 10 mg/kg with placebo. Sample sizes were based on a 5% level (2-tailed) of significance. Based on published studies, the placebo plus methotrexate control ACR 20 response rate at 6 months is about 25%. A sample of 107 subjects (adjusted for a possible 15% dropout) per treatment arm was determined to yield a 94% power to detect a difference of 25% at the 5% level (two-tailed). Similarly, the sample was determined to yield a power of 95% and 90% to detect differences of 20% and 14% in ACR 50 and ACR 70, respectively. If the comparison between CTLA4Ig 10 mg/kg and placebo was significant with regards to ACR 20, then the comparison between CTLA4Ig 2 mg/kg and placebo was carried out. This second testing should have a power of 88%. This sequentially rejective procedure based on Chi-square tests was also used to test for differences in ACR 50 and ACR 70 responses.

All efficacy analyses were based on a data set containing all available assessments from all subjects who received at least one dose of study medication.

Percent changes from baseline were also reported for the individual components of the ACR. For subjects who discontinued, their last observation was carried forward.

Results

Demography and Baseline Characteristics

TABLE III

Subject Disposition and Demographics

	Methotrexate + CTLA4Ig 10 mg/kg	Methotrexate + CTLA4Ig 2 mg/kg	Methotrexate + Placebo
Enrolled/Randomized	115	105	119
Completed	99 (86.1%)	82 (78.1%)	78 (65.5 %)
Discontinued	16 (13.9%)	23 (21.9%)	41 (34.5%)
- Adverse Events	2 (1.7%)	7 (6.7%)	7 (5.9%)
- Lack of Efficacy	12 (10.4%)	13 (12.4%)	29 (24.4%)
- Other	2 (1.7%)	3 (2.9%)	5 (4.2%)
Age (yrs) - Mean (Range)	55.8 (17 - 83)	54.4 (23 - 80)	54.7 (23 - 80)
Weight (kg) - Mean (Range)	77.8 (40.1 - 144)	78.7 (48.4 - 186.8)	79.9 (44 - 140)
Sex	75% females	63 % females	66% females
Race	87% white	87% white	87% white
Duration of Disease (yrs) Mean ± SD	9.7 ± 9.8	9.7 ± 8.1	8.9 ± 8.3

Demographic and baseline clinical characteristics were similar among the treatment groups. Sixty three to 75 percent of subjects were female, 87% were Caucasian. The mean duration of the disease at entry was 9.7 ± 9.8, 9.7 ± 8.1, and 8.9 ± 8.3 years respectively in the 10, 2 mg/kg and the control group. The mean weight in kg was very similar between 77.8 and 79.9 kg with a range of 40.1 to 186.8 kg (Table III).

After 6 months, more subjects had discontinued from the control group (35.5%) than from the active treatment groups; 13.9% and 21.9% for the 10 and 2 mg/kg treated groups, respectively. The main reason was lack of efficacy: with 24.3% discontinuing in the control group, as opposed to 12.4% and 10.4% discontinuing in the 2 and 10 mg/kg groups, respectively. The discontinuation rate due to adverse events was lower in the 10 mg/kg group with 1.7%, while it was 6.7% and 5.9% in the 2 mg/kg and the control groups, respectively.

During the first 3-4 months, the discontinuations appeared at a faster rate in the control group compared to the active-treatment groups. After Day 120, the discontinuations for all treatment groups stabilized for the duration of the primary treatment period (six months).

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TABLE IV

Baseline Clinical Characteristics

	Methotrexate + CTLA4Ig 10 mg/kg (n = 115)	Methotrexate + CTLA4Ig 2 mg/kg (n = 105)	Methotrexate + Placebo (n = 119)
Tender Joints (mean ± SD)	30.8 ± 12.2	28.2 ± 12.0	29.2 ± 13.0
Swollen Joints (mean ± SD)	21.3 ± 8.4	20.2 ± 8.9	21.8 ± 8.8
Pain (VAS 100 mm) (mean ± SD)	62.1 ± 21.4	64.5 ± 22.3	65.2 ± 22.1
Physical Function (MHAQ score of 0 to 3) (mean ± SD)	1.0 ± 0.5	1.0 ± 0.5	1.0 ± 0.6
Subject global assessment (VAS 100 mm) (mean ± SD)	60.1 ± 20.7	59.4 ± 23.7	62.8 ± 21.6
Physician global assessment (VAS 100 mm) (mean ± SD)	62.1 ± 14.8	61.0 ± 16.7	63.3 ± 15.5
CRP (mg/dL)	2.9 ± 2.8	3.2 ± 2.6	3.2 ± 3.2
Morning Stiffness (in min.)	97.9 ± 63.1	104.1 ± 63.9	106.0 ± 64.2

The mean number of tender and swollen joints at baseline was comparable among the three treatment groups. The mean number of tender joints and swollen joints in the 10 mg group was 30.8 ± 12.2 and 21.3 ± 8.4, respectively. The mean number of tender joints and swollen joints in the 2 mg group was 28.2 ± 12.0, and 20.2 ± 8.9, respectively. The mean number of tender joints and swollen joints in the control group was 29.2 ± 13.0, and 21.8 ± 8.8, respectively. These assessments and all other clinical assessments were similar among all treatment groups (Table IV).

ACR Responses and Core Components

TABLE V

ACR Response at 6 months

	Methotrexate + CTLA4Ig 10 mg/kg (n = 115)	Methotrexate + CTLA4Ig 2 mg/kg (n = 105)	Methotrexate + Placebo (n = 119)
ACR 20	60.0%	41.9%	35.3%
Difference from control group	24.7	6.6	-
95% CI	11.9, 37.5	-6.2, 19.4	-
p-value	< 0.001	0.31	-
ACR 50	36.5%	22.9%	11.8%
Difference from control group	24.8	11.1	-
95% CI	13.8, 35.7	1.2, 20.9	-
p-value	< 0.001	0.027	-
ACR 70	16.5%	10.5%	1.7%
Difference from control group	14.8	8.8	-
95% CI	7.5, 22.2	2.7, 14.9	-
p-value	< 0.001	0.005	-

The improvements in ACR 20, 50, and 70 response rates in the 10 mg/kg treatment group, at six months relative to the methotrexate control group, were statistically significant (Figures 34-38, 40). The improvements in ACR 50, and ACR 70 for the 2 mg/kg group were also statistically significant. The difference in ACR 20 response between the 2 mg/kg group and the control group was 6.6%. This difference was not statistically significant, p = 0.31 (Table V, Figure 49).

Figures 34-37 presents the ACR response rates from Day 1 to Day 180. Figures 38 and 40 presents the ACR20, -50 and -70 response rates on day 180 for the various treatment groups. The ACR 50 and ACR 70 response rates suggest the possibility that maximal efficacy may not have been achieved at 10 mg/kg.

Figure 39 shows the proportion of new tender and swollen joints at day 180 of the study after therapy with methotrexate alone or in combination with CTLA4Ig (administered at 2 or 10 mg/kg body weight of subject).

Figure 46 shows the mean percent improvement in physical function from baseline as measured by HAQ.

TABLE VI

Individual ACR Components at Day 180

(Mean Percent Improvement)

Core Components	Methotrexate + CTLA4Ig 10 mg/kg (n = 115)	Methotrexate + CTLA4Ig 2 mg/kg (n = 105)	Methotrexate + Placebo (n = 119)
Tender Joints	59.9%	43.3%	32.1%
Swollen Joints	54.9%	45.1%	33.4%
Pain	46.4%	22.7%	8.4%
Physical Function (mHAQ)	41.5%	17.3%	14.1%
Subject global assessment	40.8%	9.6%	17.6%
Physician global assessment	52.0%	38.6%	25.6%
CRP	31.5%	16.2%	-23.6%

The 2 and 10 mg/kg dose groups demonstrated some degree of efficacy among all clinical components of the ACR response criteria (Table VI; Figures 41-45, 47-48); the subject's global assessment in the 2 mg/kg dose group being the only exception. The reduction of tender and swollen joints appears dose-dependent. The number of tender joints was decreased by 59.9%, 43.3% and 32.1% in the 10 mg/kg, 2 mg/kg and control groups, respectively. A similar pattern was observed for the swollen joint counts with a decrease of 54.9%, 45.1% and 33.4% in the 10 mg/kg, 2 mg/kg and control groups, respectively. The greatest differences relative to the control group were observed with the pain assessment which decreased 46.4% and 22.7% relative to baseline for 10 mg and 2 mg/kg CTLA4Ig, respectively, compared to 8.4% in the control group. The mean CRP decreased 31.5% and 16.2% relative to baseline in the 10 and 2 mg/kg groups compared to an increase of 23.6% in the control group.

Health-Related Quality of Life

The impact of CTLA4Ig on health-related quality of life (HRQOL) was measured by the Medical Outcomes Study Short Form-36 (SF-36). The SF-36 was administered to all subjects at baseline, 90 and 180 days. The SF-36 consists of 36 items which covers eight domains (physical function, role-physical, bodily pain, general health, vitality, social function, role emotional, and mental health). These individual domains are used to derive the physical and mental component summary scores which range from 0 to 100, with higher scores indicating better quality of life.

Absolute differences of 5 or more in the SF-36 scores were considered clinically meaningful.

Compared to subjects treated with placebo, subjects in the CTLA4Ig 10 mg/kg group also experienced statistically significantly greater improvement in all 8 domains of the SF-36 (Figure 50-51). For subjects treated with CTLA4Ig 2 mg/kg, the improvements were also greater than those treated with placebo, but the differences were not statistically significant (Figure 50-51).

Baseline SF-36 scores were comparable between the three treatment groups. Improvements in quality of life show a clear dose-response trend after 6 months of treatment. Subjects in the CTLA4Ig 10 mg/kg treatment group demonstrated clinically and statistically significant improvements from baseline in all 8 domains of the SF-36. The greatest effects were shown in the role-physical, bodily pain, and role-emotional domains. This positive finding was consistent with the efficacy results. For subjects treated with CTLA4Ig 2 mg/kg, improvements from baseline were also statistically significant for all domains except mental health.

Pharmacokinetics

Pharmacokinetic Parameter Values

	CMAX (µG/ML)	TMAX (H)	AUC(TAU) (µG.H/ML)	T-HALF (Days)	CLT (ML/H/KG)	VSS (L/KG)
2 mg/kg						
MEAN	57.96	0.50*	10176.14	13.50	0.23	0.07
SD	16.93	(0.00,4.00)	3069.84	5.91	0.13	0.04
N	15	15	15	15	15	15
10 mg/kg						
MEAN	292.09	0.50*	50102.56	13.11	0.22	0.07
SD	67.78	(0.00,4.00)	15345.95	5.32	0.09	0.03
N	14	14	14	14	14	14

* Median (minimum,maximum)

The pharmacokinetics of CTLA4Ig were derived from serum concentration versus time data between dosing days 60 and 90. Samples were collected prior to dosing on day 60, at 0.5, and 4 h after dosing, on days 67, 74, 81, and prior to dosing

on Day 90. The preliminary data indicate that both Cmax and AUC values increase in a proportion comparable to the dose increment. For nominal doses increasing in a 1:5 proportion, both the Cmax and AUC values increased in the proportion of 1:5.04 and 1:4.92, respectively. T-HALF, CLT, and Vss values appeared to be comparable and dose independent.

Mean Vss values were 0.07 L/kg for both dose levels, which was approximately 1.6-fold the plasma volume.

Pharmacodynamics

TABLE VII

Mean Baseline Values for Pharmacodynamic Biomarkers

Biomarker	Methotrexate + CTLA4Ig 10 mg/kg (n = 115)	Methotrexate + CTLA4Ig 2 mg/kg (n = 105)	Methotrexate + Placebo (n = 119)
CRP (mg/dL)	2.9	3.2	3.2
RF (IU/L)	207	274	179
IL-2r (pg/ml)	1388	1407	1398
IL-6 (pg/ml)	26.7	31.7	21.4
TNFα (pg/ml)	11.8	6.0	11.9

Serum levels of pharmacodynamic biomarkers were analyzed at various times during the study. Baseline values are shown in Table VII. The values on Day 180 relative to baseline are shown in the Figures 52-56.

CRP levels decreased from baseline in both CTLA4Ig-treated groups more than in the control group, with greater reduction observed in the 10 mg/kg dosing group (see Figures 47, 48 and 52).

Rheumatoid factor levels decreased from baseline in both CTLA4Ig-treated groups more than in the control group, with greater reduction observed in the 10 mg/kg dosing group (see Figure 53).

Soluble IL-2r levels decreased from baseline in both CTLA4Ig-treated groups more than in the control group, with greater reduction observed in the 10 mg/kg dosing group (see Figure 54).

Serum IL-6 levels decreased from in both CTLA4Ig-treated groups more than in the control group (see Figure 55).

The effects of CTLA4Ig on serum TNFα levels were inconclusive. The 2 mg/kg group increased and the 10 mg/kg group decreased relative to the control group (see Figure 56).

Safety

CTLA4Ig was well tolerated at all doses. There were no deaths, malignancies or opportunistic infections in any subjects receiving CTLA4Ig. Serious adverse events (SAEs) and non-serious adverse events (NSAEs) were similar or less frequent in the active-treatment groups compared to the control group.

Fewer subjects in the 10 mg/kg group discontinued due to adverse events relative to the control group (1.7% vs 5.9%, respectively). The discontinuations due to adverse events in the 2 mg/kg were similar to the control group (6.7% vs 5.9%, respectively). The SAEs followed a pattern similar to the discontinuations due to adverse events.

No serious adverse events in the 10 mg/kg dose group were considered related to the study drug.

Immunogenicity

No anti-drug antibody responses were detected through Day 180 at both dose levels of CTLA4Ig.

CTLA4Ig significantly reduced the signs and symptoms of rheumatoid arthritis in subjects receiving methotrexate as assessed by ACR response criteria. The effects of CTLA4Ig appear to increase in proportion to dose level. The improvement from baseline in all ACR core components is higher in the 10 mg/kg group than the 2 mg/kg group. CTLA4Ig at 10 mg/kg doses demonstrated clinically and statistically significant improvements in all 8 domains of the SF-36. All pharmacodynamic biomarkers assayed appeared to decrease in proportion to CTLA4Ig dose level except for TNFα. CTLA4Ig was safe and well tolerated in subjects with rheumatoid arthritis receiving methotrexate. The adverse event profile for both CTLA4Ig doses was similar to the control group.

EXAMPLE 6

A Study of a co-stimulation blocker, CTLA4Ig, Given Monthly In Combination with Etanercept to Patients with Active Rheumatoid Arthritis

The following Example provides a description of the administration of CTLA4Ig, in combination with etanercept, to treat patients with active Rheumatoid Arthritis.

Etanercept, along with infliximab, comprises a new generation of Rheumatoid Arthritis drugs which targets Tumor Necrosis Factor (TNF. Etanercept is a dimeric fusion protein having an extracellular portion of the TNF receptor linked to the Fc portion of human immunoglobulin (IgG1). This fusion protein binds to TNF, blocks its interactions with cell surface TNF receptors and render TNF molecules biologically inactive.

This example describes a twelve month study in which efficacy was assessed after all subjects completed six months of treatment or discontinued therapy. Efficacy, safety and disease progression were also assessed throughout the duration of the study.

The study utilized a randomized, double-blind, placebo controlled, parallel dosing design. A total of approximately 141 subjects with active RA and receiving etanercept (25 mg twice weekly) were randomized to 1 of 2 dosing groups: 1) a group receiving CTLA4Ig at 2 mg/kg (n = 94) plus etanercept or 2) a placebo group receiving etanercept only (n = 47).

Test Product, Dose and Mode of Administration, Duration of Treatment

All subjects received etanercept (25 mg twice weekly) for at least 3 months prior to treatment.

Infusions of CTLA4Ig were given on Days 1, 15, 30, and monthly thereafter, for 6 months (primary treatment phase). Each dose of study medication was infused intravenously for approximately 30 minutes.

The primary treatment phase of the study took place during the first six months of treatment. During this period, subjects were required to remain on stable doses of etanercept (25 mg twice weekly). DMARDs other than etanercept were not

permitted. Low-dose stable corticosteroid (at 10 mg daily or less) and/or stable non-steroidal anti-inflammatory drug (NSAID), including acetyl salicylic acid (ASA), use was allowed. Analgesics (that do not contain ASA or NSAIDs) were permitted in subjects experiencing pain that was not adequately controlled by the baseline and study medications, except for 12 hours before a joint evaluation.

Criteria for Evaluation

The primary endpoint of this study was to collect data regarding the proportion of subjects meeting modified American College of Rheumatology (ACR) criteria for 20% improvement (ACR 20) after six months. The modified ACR 20 criteria were used to accommodate the low CRP levels in this study's subject population. The modified ACR criteria were defined as 1) a greater than 20% improvement in tender and swollen joint count and 2) a greater than 20% improvement in 2 of the remaining 4 core data set measures (global pain, physician, subject, functional assessment). CRP, which is normally a part of the standard ACR core data sets, was not included in the modified ACR criteria due to the low levels of CRP in subjects using TNF blockers, such as etanercept. The standard ACR criteria, and two alternative criteria (SF-36 Physical Health and SF-36 Mental Health) were also evaluated as secondary endpoints.

Statistical Methods

Treatment of a group of patients with CTLA4Ig 2 mg/kg in combination with etanercept was compared with a control group treated with placebo plus etanercept. Based on previous studies with etanercept in similar patient populations, it was assumed that the modified ACR 20 response rate (modified criteria for evaluation) at 6 months would be 35% in the control group. This is the rate of response expected among subjects who did not respond adequately to etanercept therapy. Using a 2:1 randomization, a sample of 141 (adjusted for a possible 10% dropout) subjects (47 control/94 CTLA4Ig) yields a 90% power to detect a difference of 30% at the 5% level of significance (2-tailed, based on a chi-square test with no adjustment for continuity correction).

Similarly, the sample was determined to yield a power of 91% and 83% to detect differences of 30 and 25% in ACR 50 and 70, respectively. However, due to slow enrollment, only 122 subjects were randomized and 121 treated and analyzed (one subject was randomized but never received treatment).

Demography and Baseline Characteristics

TABLE 1

Subject Disposition at Day 180

	CTLA4Ig + etanercept	Placebo + etanercept	TOTAL
Randomized*	85	36	121
Completed	68 (80%)	22 (61%)	90 (74%)
Discontinued	17 (20%)	14 (39%)	31 (26%)
Adverse Events	6 (7.0%)	1 (2.7%)	7 (6%)
Lack of Efficacy	6 (7.0%)	12 (33%)	18 (15%)
Other	5 (5.8%)	1 (2.7%)	6 (5%)

*Excludes one subject that did not receive treatment

After six months, the proportion of total discontinuations were higher (39%) in the placebo plus etanercept treatment group compared to the CTLA4Ig plus etanercept group (20%). The difference was driven by a higher rate of discontinuation due to lack of efficacy in the placebo plus etanercept group (Table 1).

Demographic characteristics were similar between treatment groups. The majority of subjects were female and Caucasian. The mean duration of the disease was 13 years and the mean age was 52 years (Table 2).

TABLE 2

Mean Baseline Demographic and Clinical Characteristics

	CTLA4Ig + etanercept N = 85	Placebo + etanercept N = 36	TOTAL N = 121
Mean Age: yrs (Range)	50 (24 - 74)	55 (28 - 72)	52 (24 - 74)
Mean Weight: kg (Range)	81 (45 - 154)	79 (46 - 126)	81(45 - 154)
Gender: female: n (%)	66 (78%)	26 (72%)	92 (76%)
Race: Caucasian - n (%)	80 (94%)	36 (100%)	116 (96%)

	CTLA4Ig + etanercept N = 85	Placebo + etanercept N = 36	TOTAL N = 121
Mean Duration of Disease: yrs ± sd	13.0 ± 10.1	12.8 ± 8.6	13.0 ± 9.7
Tender Joints (out of 68) - mean ± sd	28.7±14.0	29.5±13.7	28.9± 13.8
Swollen Joints - (out of 66) - mean ± sd	19.6±9.4	20.3±11.0	19.8 ± 9.9

Baseline clinical characteristics were similar between treatment groups including a mean of 29 tender joints and 20 swollen joints. With the exception of CRP values, which were lower, the baseline characteristics were typical of subjects with active rheumatoid arthritis and enrolled in clinical studies (Table 2).

ACR Responses and Core Components

The improvements in the ACR 20 and ACR 70 responses in the CTLA4Ig + etanercept group were statistically significant compared to the CTLA4Ig + placebo group (Table 3 and Figure 63).

TABLE 3

Modified ACR Response at Day 180 - number of subjects (%)*

	ACR 20	ACR 50	ACR 70
CTLA4Ig + etanercept***	48.2%	25.9%	10.6%
Diff. from Placebo + etanercept	20.5%	6.4%	10.6%
95% CI	(1.2, 39.7)	(-10.2, 23.1)	(0.4, 20.8)
p-Value	0.037**	0.448	0.042**

* See Criteria for Evaluation

**$p < 0.05$ (probability for ACR response in CTLA4Ig + etanercept vs. placebo + etanercept)

*** N = 85 and N = 36 for CTLA4Ig + etanercept: and Placebo + etanercept, respectively

By two months of treatment, numerically higher responses on all components of the ACR criteria were observed for the CTLA4Ig plus etanercept group. Three of the seven ACR components are shown in Figure 64A-C.

The mean improvements in the individual components of the ACR criteria on Day 180 were consistently greater in the CTLA4Ig plus etanercept treatment group compared to the placebo plus etanercept group (Table 4).

TABLE 4

Mean Percent (SE) Improvement in Individual ACR Components at Day 180

ACR Component	CTLA4Ig + etanercept N=85	Placebo + etanercept N=36
Tender Joints	42% (5.5)	24% (8.3)
Swollen Joints	37% (5.0)	21% (8.1)
Pain	34% (4.3)	-1% (10.8)
Physical Function (MHAQ)	31% (5.2)	-5% (13.8)
Subject Global Assessment	27% (5.4)	3% (9.5)
Physician Global Assessment	43% (4.3)	27% (5.8)

Quality of Life

Compared to baseline, subjects in the CTLA4Ig plus etanercept group demonstrated statistically significant improvements at Day 180 in all 8 subscales of the SF-36 - compared to only one (physical function) in subjects in the placebo plus etanercept group. The absolute changes in HRQOL subscales were considered clinically meaningful.

Compared to the placebo plus etanercept group, subjects in the CTLA4Ig plus etanercept group experienced statistically significantly greater improvement in 4 subscales of the SF-36: role-physical, bodily pain, vitality, and social function (Figure 65). Improvements in the other 4 subscales were also greater than the placebo plus etanercept group, although they were not statistically significant.

Safety

No deaths or opportunistic infections occurred during the first six months of this study. Among the most frequently reported adverse events, headache, upper respiratory infection, musculo/skeletal pain, nausea/vomiting, hypertension, and diarrhea occurred at a higher rate in the CTLA4Ig plus etanercept group compared to the placebo plus etanercept group. Sinus abnormalities and rash were slightly higher in the CTLA4Ig plus etanercept group, as well.

More subjects in the CTLA4Ig plus etanercept group experienced serious adverse events (SAE) (7.1%) than the etanercept plus placebo group (2.8%). However, no SAEs were considered related to the study drug.

Two subjects receiving CTLA4Ig and etanercept had a dermatological malignancy. One subject had a basal cell carcinoma that was excised after the Day 150 visit. The other subject had a squamous cell carcinoma which was a pre-existing lesion that the subject decided to have removed after the Day 120 visit. Another subject experienced angioedema that was considered by the investigator to be a drug reaction to azithromycin.

All adverse events (AEs) leading to discontinuation were of either of mild or of moderate intensity. One discontinuation in the CTLA4Ig plus etanercept group, due to a tremor, was considered a serious adverse event.

Immunogenicity

No subjects receiving CTLA4Ig seroconverted for CTLA4Ig or CTLA4-T specific antibodies. No significant change in GMTs for CTLA4Ig or CTLA4-T specific antibodies was observed.

Comparison between CTLA4Ig/etanercept and CTLA4Ig/methotrexate ACR responses

TABLE 5

CTLA4Ig + etanercept vs. CTLA4Ig + methotrexate ACR responses (% improvement):

	CTLA4Ig + Etanercept[a] (IM101-101)		CTLA4Ig + Methotrexate[b] (IM101-100)		
	2 mg/kg N = 85	0 mg/kg[c] N = 36	10 mg/kg N = 115	2 mg/kg N = 105	0 mg/kg[c] N = 119
ACR 20	48.2%[d]	27.8%	60.0%[d]	41.9%	35.3%
ACR 50	29.3%	19.4%	36.5%[d]	22.9%[d]	11.8%
ACR 70	10.6%[d]	0%	16.5%[d]	10.5%[d]	1.7%

[a] Modified ACR. See criteria for evaluation

[b] Standard ACR criteria

[c] Placebo + Background therapy (etanercept or methotrexate)

[d] $p<0.05$ for the difference in ACR response vs placebo + background therapy

The efficacy of CTLA4Ig plus etanercept at 2 mg/kg was similar to that observed in subjects receiving the same dose of CTLA4Ig plus methotrexate therapy (Example 5). However, the criteria for evaluation in the methotrexate (Example 5) trial was the standard ACR, that includes CRP among the core components, while in the etanercept trial (Example 6) the criteria for evaluation was the modified ACR, that excludes CRP.

Conclusion

Preliminary assessment of the study at six months found that CTLA4Ig (2 mg/kg) in combination with etanercept reduced the signs and symptoms of rheumatoid arthritis, as compared with etanercept alone. The increases in the modified ACR20 and ACR 70 assays were statistically significant. Efficacy of CTLA4Ig plus etanercept therapy was observed within one month of the start of treatment. CTLA4Ig was generally safe and well tolerated when administered in combination with etanercept with the safety profile similar to etanercept therapy alone. CTLA4Ig was not immunogenic during the six month trial period. Additionally, the efficacy of CTLA4Ig therapy in combination with etanercept (Example 6) was similar to the same dose of CTLA4Ig with methotrexate (Example 5).

EXAMPLE 7

One-Year Results of a Phase IIB, Multicenter, Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Safety and Clinical Efficacy of Two Different Doses of BMS-188667 Administered Intravenously to Subjects with Active Rheumatoid Arthritis While Receiving Methotrexate

The following Example provides the one-year results from a Phase IIB, multi-center, randomized, double-blind, placebo controlled clinical study to evaluate the safety and clinical efficacy of administering two different doses of CTLA4Ig in combination with methotrexate to treat patients with active Rheumatoid Arthritis (RA). The study presented in this example is a continuance of the six-month study presented in Example 5.

Based on preliminary efficacy results from Example 3, supra, and the standard practice of adding other therapies to MTX in the treatment of RA, this study was designed to test the hypothesis that CTLA4Ig (BMS-188667) combined with MTX may have greater clinical efficacy when compared with MTX plus placebo in RA subjects who still have active disease despite MTX treatment.

The results presented in this clinical study report are based on data from an analysis performed after all subjects completed 6 months of treatment and again after all subjects completed 12 months of treatment.

Throughout this Example, the 10 mg/kg CTLA4Ig plus MTX group may be referred to as the 10 mg/kg group, the 2 mg/kg plus MTX group is referred to as the 2 mg/kg group, and the CTLA4Ig (BMS-188667) placebo plus MTX group is referred to as the placebo group.

Study Methodology

This study compared the clinical efficacy of two different doses (10 and 2 mg/kg) of CTLA4Ig (BMS-188667) combined with methotrexate (MTX) or with MTX plus placebo in subjects with active RA as assessed by ACR at 6 month and 12 month intervals. This study enrolled adult subjects with active RA who had had an inadequate response to MTX.

Results after one-year of monitoring subjects with active rheumatoid arthritis who were intravenously administere: 1) CTLA4Ig at a dosage of 2 mg/kg body weight with methotrexate, 2) CTLA4Ig at a dosage of 10 mg/kg body weight with methotrexate, or 3) a placebo with methotrexate (hereinafter known as placebo), are presented herein.

Subjects with active RA, despite treatment with MTX and who met the inclusion/exclusion criteria for this study were randomized 1:1:1 to receive one of the following treatments on a background of MTX therapy: CTLA4Ig (BMS-188667) 10 mg/kg, CTLA4Ig (BMS-188667) 2 mg/kg, or placebo. Subjects must have been treated with MTX (10 mg to 30 mg weekly) for at least 6 months, at a stable dose for 28 days prior to Day 1.

Treatment Groups: Subjects were randomized 1:1:1 to one of three treatment groups:

1) Group 1: CTLA4Ig (BMS-188667) 10 mg/kg by intravenous infusion

2) Group 2: CTLA4Ig (BMS-188667) 2 mg/kg by intravenous infusion

3) Group 3: CTLA4Ig (BMS-188667) placebo by intravenous infusion

Infusion doses were based upon the subject's body weight from the pre-treatment visit immediately prior to the Day 1 visit (for a subject on MTX monotherapy, the weight was obtained at the screening visit; for a subject on MTX combination therapy [in combination with other DMARDs], the weight was obtained from the washout visit, Day -2). The infusion doses were not modified during Day 1 to Day 360.

Infusions were to occur at approximately the same time of day throughout the study. All doses of study medication were administered in a fixed volume of 75 mL at a constant rate over approximately 30 minutes. The intravenous bag and line were flushed with 25 mL of dextrose 5% in water (D5W) solution at the end of each infusion. All intravenous infusions were administered with the subject in the seated position. Subjects were observed for Adverse Events (Aes) and changes in vital signs (blood pressure, heart rate, body temperature) from the start of each infusion (pre-dose, 15, 30, 45, 60, 75, 90, 120 minutes) and for a minimum of 2 hours after the start of the infusion. The observation period could be extended, if clinically indicated.

During the primary phase (Day 1 to Day 180) of the study, concomitant administration of selected medications was allowed. The permitted medications included:

- MTX: Continued use of current dose (no increases, and decreases only for toxicity)
- Systemic (non-topical) corticosteroids: Provided that the dose was stable and the total dose was less than or equal to the equivalent of prednisone 10 mg/day. Intra-articular injections were to be avoided; however, if necessary, up to two intra-articular injections were permitted. NOTE: A joint that received an intra-articular injection was counted as "active" in ALL subsequent assessments/evaluations.
- NSAIDs, including ASA: Provided the dose was stable

- Acetaminophen, combination products including acetaminophen and narcotic analgesics (i.e., acetaminophen with codeine phosphate, acetaminophen with propoxyphene napsylate, acetaminophen with oxycodone hydrochloride, acetaminophen with oxycodone bitartrate, etc.), or tramadol: For subjects experiencing pain not adequately controlled by baseline or study medication (except for 12 hours before a joint evaluation)

Table 1 is a schedule of study procedures and evaluations.

TABLE 1

Schedule of Study Procedures and Evaluations

Treatment Period	Pretreatment (Day)		Treatment Day[e,f,j,k]													
Visit Day	Screen (-28 to -2)	(-2)	1	15	30	60	90	120	150	180	210	240	270	300	330	360
Screening assessments																
Informed consent	X															
Complete History and Physical	X															X[i]
CXR	X[a]															
ECG	X[a]															X
Stabilize/Withdraw prohibited medications (if necessary)[b]	X															
Enroll Subject	X	X[m]														
Randomize Subject[k]			X													
Dosing[b]			X	X	X	X	X	X	X	X	X	X	X	X	X	
Interim Assessments[f]																
Duration of morning stiffness	X		X	X	X	X	X	X	X	X		X		X		X
Interim History and Physical			X	X	X	X	X	X	X	X		X		X		
Tender joint count	X	X	X	X	X	X	X	X	X	X		X		X		X
Swollen joint count	X	X	X	X	X	X	X	X	X	X		X		X		X
Subject's assessment of pain	X		X	X	X	X	X	X	X	X		X		X		X
Subject's global assess of disease activity	X		X	X	X	X	X	X	X	X		X		X		X
Physician's global assess of disease activity	X		X	X	X	X	X	X	X	X		X		X		X
Subject's assess of physical function	X		X	X	X	X	X	X	X	X		X		X		X
Short form-36 health questionnaire (SF-36)	X		X				X			X						X
Subjects response to therapy							X			X						X
Safety Assessments																
Adverse event monitoring			X	X	X	X	X	X	X	X	X	X	X	X	X	X[o]
Weight[g]	X	X														X
Mammogram (females only)[l]	X															X
Vital signs	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Labs																
CBC	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X

Treatment Period	Pretreatment (Day)		Treatment Day[c,f],[b]													
Visit Day	Screen (-28 to -2)	(-2)	1	15	30	60	90	120	150	180	210	240	270	300	330	360
Chemistry panel	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Urinalysis	X															X
Urine/serum pregnancy test[d]	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Hepatitis B surface antigen	X															
Hepatitis C antibody	X															
Pharmacodynamics (PD)																
Rheumatoid factor	X		X							X						X
CRP	X	X	X	X	X	X	X	X	X	X		X		X		X
IL-2R			X		X	X	X	X	X	X		X		X		X
Exploratory cytokines (ICAM-1 e-Selectin, IL-6 and TNFα)			X				X			X						X
Pharmacokinetics			X			X	X			X						
Immunoglobulin determinations																
Quantitative immunoglobulins (IgG, IgA, IgM)			X							X						X
Immunogenicity																
Anti-BMS-188667Ab testing			X		X		X			X			X			X
Radiographic assessments[a]																
X-rays (hands/wrists and feet)			X							X						X

[a] Chest X-ray and ECG was performed if not performed within 6 months or not on file.

[b] If subject was being treated with DMARDs on top of methotrexate therapy and did not meet initial entry criteria, the DMARDs must have been washed out for at least 28 days prior to Day 1.

[c] This visit was required only if the subject was on MTX therapy.

[d] Urine or serum pregnancy test performed within 48 hours prior to dosing, for all women of child bearing potential. Serum pregnancy test was to be processed locally.

[e] Subjects who discontinued must have had an "early termination" visit. Assessments at this visit were identical to assessments performed on Day 360. The assessments for this visit replaced what might have been scheduled on the day of discontinuation. Changes in *current* DMARD, steroid, or NSAIDs therapy were **not** permitted until **after** these assessments were performed. Subjects were to be contacted 30 days after discontinuation to capture safety data (adverse events).

[f] Every effort must have been made to insure the same evaluator completed the assessment for each subject.

[g] Most recent weight should have been used to calculate study drug dosage. All doses administered during the study were be based on this weight.

[h] For Day 15, a +/- 3 day visit window was permitted. For subsequent visits, a +/- 7 day visit window was permitted.

[i] Complete physical examination only.

[j] All assessments should have been performed or administered prior to study drug administration unless otherwise indicated.

[k] The results of all assessments must have been reviewed for eligibility requirements before contacting the Central Randomization System for randomization.

[l] See Section 2.1.4.3 of the protocol for mammography rationale. If not performed within 6 months (documentation must be on file) prior to signing consent. Subjects who discontinued from the study after Day 1 required a follow-up mammogram on the one year anniversary of the mammogram that was performed during the screening period.

[m] Subject's body weight was provided to central randomization system.

[n] No radiographic assessments were required at the termination visit for subjects who discontinued within the first nine months of treatment.

[o] Subjects who were terminated early had adverse events and concomitant medications recorded 30 and 60 days after the last dose of study medication.

Efficacy Assessments

Clinical Measurements and Responses

Clinical response was assessed using the American College of Rheumatology (ACR) Core Data Set and Response Definitions. For this assessment, data were collected on seven components: 1) tender joint count (standardized 68 joint count); 2) swollen joint count (standardized 66 joint count); 3) subject global assessment of pain; 4) subject global assessment of disease activity; 5) physician global assessment of disease activity; 6) subject assessment of physical function (MHAQ); and 7) an acute phase reactant value CRP.

The ACR 20, ACR 50, and ACR 70 definition of response corresponds to a 20%, 50%, or 70% improvement, respectively, over baseline in tender and swollen joints (components 1 and 2) and a 20%, 50%, and 70% improvement, respectively, in three of the five remaining core data set measures (components 3 to 7). A Major Clinical Response is defined as maintenance of an ACR 70 response over a

continuous 6-month period. See Table 1 for the days that data for each component was collected.

The primary efficacy analysis tested for differences in ACR 20 response between the two CTLA4Ig (BMS-188667) treatment groups and the placebo group at 6 months (Day 180). A sequential testing procedure was employed. First, a Chi-square test was used to compare the data for the 10 mg/kg CTLA4Ig group with the data for the placebo group at the 0.05 level of significance. If this was significant, the data for the 2 mg/kg CTLA4Ig group was compared with the placebo group at the 0.05 level. This testing procedure preserved the overall alpha level at 5%. Similar analyses were carried out for the ACR 50 and ACR 70 responses at 6 months. Differences in ACR 20, ACR 50 and ACR 70 responses between each CTLA4Ig (BMS-188667) treatment group and the placebo group were summarized using point estimates and 95% confidence intervals. Subjects who discontinued the study due to lack of efficacy (i.e., worsening RA) were considered ACR non-responders at all subsequent time points. For all subjects who discontinued for other reasons, their last ACR response was carried forward.

ACR 20, ACR 50, and ACR 70 response rates on Day 360 were compared between each CTLA4Ig (BMS-188667) treatment group and placebo at the Dunnett-adjusted 0.027 (two-tailed) level of significance.

The proportion of responders achieving an ACR 20 response at each time point was also plotted over time, and the Cochran Mantel-Haenszel test (W.G. Cochran, 1954, Some Methods of Strengthening the Common Chi-Square Test, Biometrics 10:417-451; N. Mantel and W. Haenszel, 1959, Biostatistical Aspects of the Analysis of Data from Retrospective Studies of Disease, J Nat Cancer Inst, 22:719-748) was used to compare the frequency of subjects achieving an ACR 20 response in each CTLA4Ig (BMS-188667) group versus the placebo group.

ACR 20, ACR 50, and ACR 70 responses on Days 15, 30, 60, 90, 120, 150, 180, 240, 300, and 360 were also presented for the two CTLA4Ig (BMS-188667) groups and the placebo group. The differences in ACR responses between the CTLA4Ig (BMS-188667) groups and placebo group were summarized using 95% confidence intervals. The ACR data plotted over time were used to assess onset-of-action and to determine time to maximal response.

A Major Clinical Response was defined as the maintenance of an ACR 70 response over a continuous 6-month period. At the 12-month analysis, the proportion of subjects who achieved a Major Clinical Response among the three groups was summarized.

In order to assess the integrity of the planned analyses, all subjects who received study medication and discontinued the study for any reason were considered ACR non-responders at all scheduled study visits subsequent to discontinuation.

The cumulative index, ACR-N, was evaluated at each follow-up assessment, and the AUC was assessed for up to 6 months and up to 12 months. The trapezoidal rule was used to compute the AUC. The ACR-N AUC was compared between the two CTLA4Ig (BMS-188667) treatment groups and the placebo group using an analysis of variance (ANOVA) for 6- and 12-month data. This allowed for the assessment of subject response throughout the study. These analyses were carried out on the LOCF data sets.

The distributional assumptions regarding the normality of the ACR-N AUC data were checked using the Shapiro-Wilks test on standardized residuals from the ANOVA model at the 10% level of significance.

Surrogate biomarkers were also used to assess the efficacy of the CTLA4Ig + MTX or placebo + MTX treatment regimens. Potential biomarkers for immunomodulation or inflammation in RA include CRP, soluble IL-2r, RF, soluble ICAM-1, E-selectin, serum IL-6, and TNFα. These parameters were summarized by treatment group, using frequencies and mean change from baseline to Day 180 and Day 360.

An Adverse Event (AE) was defined as any new or worsening illness, sign symptom or clinically significant laboratory test abnormality noted by the Investigator during the course of the study, regardless of causality. A serious adverse event (SAE) was defined as an AE that met any of the following criteria: was fatal; was life-threatening; resulted in or prolonged hospitalization; resulted in persistent or significant disability or incapacity, was cancer, was a congenital anomaly/birth defect, resulted in an overdose, resulted in the development of drug dependency or drug abuse, or was an important medical event.

Vital sign measurements were obtained at screening and at each study visit during and following study drug administration. Vital sign measurements (seated blood pressure, heart rate, and body temperature) were summarized by treatment group using means.

The two CTLA4Ig (BMS-188667) treatment groups (10 and 2 mg/kg) were compared with the placebo group. The primary analysis was the comparison of 6-month ACR response rate for 10 mg/kg and placebo groups, to be followed by the comparison of 2 mg/kg with placebo only if the former was significant. Sample sizes were based on a 5% level (two-tailed) of significance. The ACR 20 response rate for a placebo group at 6 months was estimated to be about 25% (Weinblatt M, Kremer JM, Bankhurst AD *et. al.* A trial of etanercept, a recombinant TNF:Tc fusion protein in patients with RA receiving methotrexate. NEJM 1999; 340: 253-259). A sample of 107 subjects per treatment group (adjusted for a possible 15% discontinuation rate) was determined to yield a 94% power to detect a difference of 25% at the 5% level (two-tailed). Table 2 summarizes the power needed to detect the specified treatment differences in ACR 20, ACR 50, and ACR 70 responses at 6 months.

TABLE 2

Response Rates and Power with 107[a] Subjects per Group

Response	Control Rate (%)	Treatment Difference	Power (%)
ACR 20	25	25	94
ACR 50	5	20	95
ACR 70	1	14	90

[a] Sample size was adjusted for a possible 15% discontinuation rate; actual sample size was 91.

If the primary comparisons of the 10 mg/kg CTLA4Ig with placebo were significant, then for the comparison of the 2 mg/kg CTLA4Ig with placebo groups, the power of the test would be at least 0.88, 0.90, and 0.81 for the comparison involving ACR 20, ACR 50, and ACR 70 responses at 6 months, respectively (Koch DD, Gansky SA. Statistical considerations for multiplicity in confirmatory protocols. Drug Info Journal 1996; 30: 523-534).

Statistical Analyses

Study Population

Disposition of Subjects

Of 524 subjects who were enrolled in this study, 339 subjects were randomized: 115 to the 10 mg/kg group; 105 to the 2 mg/kg group; and 119 to the placebo group (Figure 68). The most frequent reason for not being randomized was failure to meet inclusion and/or exclusion criteria.

Primary Phase (Days 1-180)

A total of 256 subjects (75.5% of those randomized) completed the primary phase of the study; 83 subjects discontinued during this period (Table 3). Overall, discontinuation was more than 2-fold higher with placebo compared with 10 mg/kg CTLA4Ig group. Discontinuation due to lack of efficacy and discontinuation due to an AE were also more than 2-fold higher with placebo than with 10 mg/kg CTLA4Ig group.

TABLE 3

Reasons for Discontinuation: Primary Phase (Days 1-180)

	CTLA4Ig (BMS 188667)			
	10 mg/kg	2 mg/kg	Placebo	Total
No. Treated, n	115	105	119	339
No. Discontinued, n (%)	17 (14.8)	25 (23.8)	41 (34.5)	83 (24.5)
Adverse Event	3 (2.6)	7 (6.7)	9 (7.6)	19 (5.6)
Lack of Efficacy	12 (10.4)	16 (15.2)	28 (23.5)	56 (16.5)
Withdrawal of Consent	2 (1.7)	2 (1.9)	4 (3.4)	8 (2.4)
Completed 180 Days of Therapy, n (%)	98 (85.2)	80 (76.2)	78 (65.5)	256 (75.5)

Cumulative Discontinuations (Days 1-360)

A total of 235 subjects (69.3% of those randomized) completed both phases of the study; 104 subjects discontinued by Day 360 (Table 4). The same general pattern in disontinuations noted in the primary phase (2-fold higher incidence with placebo compared with 10 mg/kg CTLA4Ig group) was also observed overall (Days 1-360). This included the overall discontinuation rate, discontinuations due to a lack of efficacy and discontinuations due to an AE.

TABLE 4

Reasons for Discontinuation: Both Phases (Days 1-360)

	CTLA4Ig (BMS-188667)			
	10 mg/kg	2 mg/kg	Placebo	Total
No. Treated, n	115	105	119	339
No. Discontinued, n (%)	25 (21.7)	31 (29.5)	48 (40.3)	104 (30.7)
Adverse Event	5 (4.3)[b]	9 (8.6)	11 (9.2)	25 (7.4)
Death	0	1 (1.0)	0	1 (0.3)
Lost to Follow-up	1 (0.9)	2 (1.9)	0	3 (0.9)
Other[a]	1 (0.9)	0	1 (0.8)	2 (0.6)
Lack of Efficacy	13 (11.3)	17 (16.2)	30 (25.2)	60 (17.7)
Withdrawal of Consent	5 (4.3)	2 (1.9)	6 (5.0)	13 (3.8)
Completed 360 Days of Therapy, n (%)	90 (78.3)	74 (70.5)	71 (59.7)	235 (69.3)

[a] Other reasons for discontinuation were related to compliance

[b] Subject IM101100-32-5 in the 10 mg/kg CTLA4Ig group reported an AE that was recorded as having resulted in discontinuation from the study; however, this subject was not included in this table.

A Kaplan-Meier plot of the cumulative proportion of subjects who discontinued for any reason during the first 12 months is presented in Figure 69; the cumulative proportion of subjects who discontinued due to lack of efficacy in presented in Figure 70. Note that in both graphs after approximately 30 days of therapy, discontinuation rates with placebo were consistently higher compared with both CTLA4Ig (BMS-188667) groups. Additionally, after approximately 150 days of therapy, discontinuation rates for 2 mg/kg CTLA4Ig group were higher than those for 10 mg/kg.

Demography and Subject Characteristics

Overall, baseline demographic characteristics and baseline clinical RA characteristics were generally comparable across the three treatment groups and were typical of relatively advanced RA encountered in clinical practice (Table 5 and Table 6). The majority of subjects were white females approximately 55 years old with a mean duration of RA of approximately 9 to 10 years, a relatively large number of

active joints (approximately 29 tender and 21 swollen joints) and visual analogue scores (VAS) approximately 59-65 mm (100 mm scale).

TABLE 5

Baseline Demographic Characteristics

	CTLA4Ig (BMS-188667)		
	10 mg/kg	2 mg/kg	Placebo
No. Randomized	115	105	119
Age (yrs)			
Mean ± SD (Range)	55.8±12.5 (17, 83)	54.4±11.3 (23, 80)	54.7±12.0 (23, 80)
Weight (kg)			
Mean ± SD (Range)	77.8±18.6 (40.1, 144.0)	78.7±21.4 (48.4, 186.8)	79.9±17.6 (44.0, 140.0)
Gender			
Males, n (%) Females, n (%)	29 (25) 86 (75)	39 (37) 66 (63)	40 (34) 79 (66)
Race			
White, n (%) Black, n (%) Other, n (%)	100 (87) 6 (5) 9 (8)	91 (87) 0 14 (13)	104 (87) 3 (3) 12 (10)
Duration of RA (yrs)			
Mean ± SD (Range)	n=114[a] 9.7±9.8 (0, 38)	n=105 9.7±8.1 (0, 36)	n=117[a] 8.9±8.3 (0, 41)

Duration of RA was not reported for 3 subjects.

Although not a component of the ACR criteria, duration of morning stiffness was also assessed and was nearly 2 hours in each of the three groups. Positive results for RF at baseline were also assessed, and the CTLA4Ig (BMS-188667) treatment groups had higher percentages of subjects who tested positive for RF (86% for both the 10 mg/kg and 2 mg/kg CTLA4Ig groups compared to 76% for the placebo group).

TABLE 6

Baseline Clinical Rheumatoid Arthritis Characteristics

	CTLA4Ig (BMS-188667)		
Characteristic	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)

Characteristic	CTLA4Ig (BMS-188667) 10 mg/kg (n=115)	CTLA4Ig (BMS-188667) 2 mg/kg (n=105)	Placebo (n=119)
Tender Joints, n	115	105	119
Mean ± SD	30.8 ± 12.2	28.2 ± 12.0	29.2 ± 13.0
Range	11.0, 66.0	3.0, 62.0	4.0, 68.0
Swollen Joints, n	115	105	119
Mean ± SD	21.3 ± 8.4	20.2 ± 8.9	21.8 ± 8.8
Range	9.0, 54.0	4.0, 48.0	8.0, 64.0
Pain (VAS 100 mm), n	113	104	119
Mean ± SD	62.1 ± 21.4	64.3 ± 22.3	65.2 ± 22.1
Range	0.0, 99.0	8.0, 100.0	3.0, 95.0
Physical Function (MHAQ 0-3), n	115	105	119
Mean ± SD	1.0 ± 0.5	1.0 ± 0.5	1.0 ± 0.6
Range	0.0, 2.5	0.0, 2.5	0.0, 2.3
Subject Global Assess (VAS 100 mm), n	113	105	119
Mean ± SD	60.1 ± 20.7	59.4 ± 23.7	62.8 ± 21.6
Range	10.0, 100.0	8.0, 99.0	4.0, 94.0
MD Global Assess (VAS 100 mm), n	113	105	119
Mean ± SD	62.1 ± 14.8	61.0 ± 16.7	63.3 ± 15.5
Range	20.0, 98.0	8.0, 95.0	18.0, 93.0
CRP (mg/dL), n	112	99	115
Mean ± SD	2.9 ± 2.8	3.2 ± 2.5	3.2 ± 3.2
Range	0.2, 19.9	0.2, 10.8	0.2, 20.9
Morning Stiffness (in minutes), n	115	103	119
Mean ± SD	97.9 ± 63.1	104.1 ± 63.9	106.0 ± 64.2
Range	0.0, 180.0	0.0, 180.0	0.0, 180.0
Rheumatoid Factor (IU/mL), n	99	90	90
% Positive	86%	86%	76%

Baseline demographics and RA characteristics of the overall population of subjects who had at least one dose of study drug and discontinued due to lack of efficacy were generally comparable to the entire study population, however, a greater proportion of subjects in this subpopulation had been diagnosed with RA for >10 years (45%) compared to the overall study population (34%).

Medical History Findings and Prior Medications

Medical history findings for subjects in this study were consistent with relatively advanced RA and were generally similar among treatment groups. The most frequently occurring findings (in >40% of the subjects) were musculoskeletal findings (not including RA symptoms; 59.3%), gastrointestinal findings (45.1%), and genitourinary findings (42.2%). Other important medical history findings included

cardiovascular disease in approximately 39% of subjects in all treatment groups and endocrine/metabolic findings in approximately 29% of all subjects.

Overall use of MTX, systemic (non-topical) corticosteroids, DMARDs and biologic RA medications prior to entering the study was generally comparable across the three treatment groups (Table 7). All subjects were to have received prior treatment with rheumatic medications, including MTX, to be eligible for the study. Prior treatment with MTX was not recorded for 4 subjects. Systemic (non-topical) corticosteroid use prior to randomization was comparable among the three treatment groups, with slightly more subjects in the 2 mg/kg CTLA4Ig and placebo groups taking systemic (non-topical) corticosteroids (~67-68%) compared to subjects in the 10 mg/kg CTLA4Ig group (60.0%). Use of other DMARDs and biologic RA medications prior to entering the study varied from 0 to 12% across treatment groups with no overall predominance in any treatment group. Mean dosing of MTX and of systemic (non-topical) corticosteroids on Day 1 were comparable among all three treatment groups (~15-16 mg/wk, ~6-7 mgs/day, respectively.

TABLE 7

Summary of Rheumatic Medications Prior to Enrollment

Prior Rheumatic Medication, n (%)[a]	CTLA4Ig (BMS-188667) 10 mg/kg (n=115)	CTLA4Ig (BMS-188667) 2 mg/kg (n=105)	Placebo (n=119)
No. Subjects on Prior Medications	114 (99.1)	103 (98.1)	118 (99.2)
Methotrexate[b]	114 (99.1)	103 (98.1)	118 (99.2)
Systemic (non-topical) corticosteroids	69 (60.0)	71 (67.6)	80 (67.2)
Other DMARDs	19 (16.5)	19 (18.1)	25 (21.0)
Sulfasalazine	9 (7.8)	2 (1.9)	10 (8.4)
Hydroxychloroquine	8 (7.0)	6 (5.7)	14 (11.8)
Cyclosporine	2 (1.7)	4 (3.8)	4 (3.4)
Infliximab	2 (1.7)	2 (1.9)	2 (1.7)
Etanercept	1 (0.9)	4 (3.8)	1 (0.8)
Chloroquine	1 (0.9)	0	0
Leflunomide	0	2 (1.9)	2 (1.7)

[a] Categories of prior rheumatic medications were not mutually exclusive.

[b] Administration of MTX was not recorded for 4 subjects

Study Therapy

Of the three treatment groups, the 10 mg/kg CTLA4Ig group had the longest mean duration of exposure for both study phases and the placebo group had the shortest mean duration of exposure for both study phases (Day 180: 163 days, 156 days, 140 days; Day 360: 286 days, 268 days, and 234 days; 10 mg/kg, 2 mg/kg, and placebo, respectively).

At Day 180 (end of the primary phase), the proportion of subjects receiving infusions was higher in the 10 mg/kg CTLA4Ig group (85%) compared with the 2 mg/kg CTLA4Ig group (79%) and the placebo group (66%) (Table 8). At Day 330 (day of last scheduled infusion in the secondary phase), the proportion of subjects receiving infusions was also higher in the 10 mg/kg CTLA4Ig group (78%) compared with the 2 mg/kg CTLA4Ig group (70%) and the placebo group (59%).

TABLE 8

Subjects Who Received Infusions on Given Study Days

	Number (%) of Subjects		
	CTLA4Ig (BMS-188667)		
Day	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
1	115 (100)	105 (100)	119 (100)
15	114 (99)	104 (99)	117 (98)
30	113 (98)	101 (96)	111 (93)
60	108 (94)	97 (92)	103 (87)
90	106 (92)	94 (90)	94 (79)
120	100 (87)	86 (82)	83 (70)
150	98 (85)	83 (79)	81 (68)
180	**98 (85)**	**83 (79)**	**78 (66)**
210	94 (82)	80 (86)	78 (66)
240	95 (83)	78 (74)	76 (64)
270	93 (81)	77 (73)	73 (61)
300	90 (78)	74 (70)	72 (61)
330	**90 (78)**	**73 (70)**	**70 (59)**

Methotrexate

Subjects were to have been treated with a "stable" dose of MTX (10-30 mg weekly) for at least 6 months, for 28 days prior to Day 1. With the exception of 4 subjects , all subjects received between 10 and 30 mg of MTX weekly in addition to CTLA4Ig (BMS-188667) during the primary phase (Day 1-180). During the

secondary phase (Day 181-360), the dose of MTX could have been adjusted provided it remained between 10 and 30 mg weekly.

Measurements of Treatment Compliance

During the primary phase, the number of missed infusions of study drug was ≤2 at any time point (Table 9). During the secondary phase, subjects in the placebo group appeared to have missed slightly fewer infusions than subjects in the CTLA4Ig (BMS-188667) groups. However, more placebo than CTLA4Ig (BMS-188667) subjects discontinued by these later time points (see supra).

TABLE 9

Number of Missed Infusions of Study Drug

	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Day 1	0	0	0
Day 15	1	1	0
Day 30	0	1	1
Day 60	0	0	0
Day 90	0	0	0
Day 120	0	1	2
Day 150	1	2	0
Day 180	**1**	**0**	**1**
Day 210	4	0	0
Day 240	1	2	1
Day 270	0	2	0
Day 300	1	1	0
Day 330	**0**	**0**	**0**

Concomitant Therapy

Systemic (non-topical) corticosteroid use was generally comparable among the three groups during screening/enrollment (58-67%) and during the primary phase of the study (67-71%), Tables 10 and 11, respectively. While corticosteroid use decreased in all three treatment groups by Day 360, more subjects in the 10 mg/kg CTLA4Ig group took systemic (non-topical) corticosteroids (63.5%) compared to the other two treatment groups (53.3% and 45.4% for the 2 mg/kg CTLA4Ig and placebo

groups, respectively). Several subjects (CTLA4Ig: 0-3%, placebo: 0-10%) received DMARDs other than MTX during screening/enrollment.

TABLE 10

Summary of Rheumatic Concomitant Medications During Screening/Enrollment

Rheumatic Medication, n (%)[a]	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
No. Subjects on Prior Medications	114 (99.1)	103 (98.1)	118 (99.2)
Methotrexate	114 (99.1)	103 (98.1)	118 (99.2)
Systemic (non-topical) corticosteroids	67 (58.3)	70 (66.7)	75 (63.0)
Other DMARDs	5 (4.3)	6 (5.7)	14 (11.8)
Sulfasalazine	3 (2.6)	1 (1.0)	4 (3.4)
Hydroxychloroquine	2 (1.7)	3 (2.9)	12 (10.1)
Cyclosporine	1 (0.9)	1 (1.0)	2 (1.7)
Etanercept	0	1 (1.0)	0

[a] Drug categories were not mutually exclusive.

TABLE 11

Subjects Who Received Clinically Relevant Concomitant Medications During Both Study Phases

Medication[a]	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Systemic (non-topical) corticosteroids (Primary Phase)	77 (67.0)	71 (67.6)	85 (71.4)
Systemic (non-topical) corticosteroids (Secondary Phase)	73 (63.5)	56 (53.3)	54 (45.4)

[a] Drug categories were not mutually exclusive.

Note: Subject IM101100-83-3 (10 mg/kg CTLA4Ig) took mefloquine and subject IM101100-28-7 (placebo) took quinine between Days 1 and 180; subject IM101100-18-11 (10 mg/kg CTLA4Ig) took quinine between Days 181 and 360 as an antimalarial, and was not considered a significant protocol violation.

Efficacy Results

The CTLA4Ig (BMS-188667) 10 mg/kg group had superior efficacy compared to the placebo group at Day 180 and Day 360. For the 2 mg/kg CTLA4Ig

group, results for some efficacy parameters were significantly better compared to the placebo group, results for most other efficacy parameters were numerically higher compared to placebo.

ACR Responses at Day 180

Analysis of the primary efficacy variable for this study, ACR20 response rate at Day 180, showed that the 10 mg/kg CTLA4Ig group was significantly ($p<0.001$) more effective than placebo (Table 12, Figure 71A and Figure 71B).

The ACR50 and ACR70 responses at Day 180 for the 10 mg/kg CTLA4Ig group were also significantly higher compared to the placebo group (Table 12, Figure 71A and Figure 71B). The ACR50 and the ACR70 responses at Day 180 for the 2 mg/kg CTLA4Ig group were significantly higher compared to the placebo group. The ACR20 response at Day 180 for the 2 mg/kg CTLA4Ig group was slightly higher compared to the placebo group; however, no statistically significant differences were observed.

TABLE 12

ACR Responses at Day 180

	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
ACR 20			
n (%)	70 (60.9)	44 (41.9)	42 (35.3)
CI	25.6 (12.8, 38.4)	6.6 (-6.2, 19.4)	N/A
p-value	<0.001[a]	0.31	N/A
ACR 50			
n (%)	42 (36.5)	24 (22.9)	14 (11.8)
CI	24.8 (13.8, 35.7)	11.1 (1.2, 20.9)	N/A
p-value	<0.001[a]	0.027[a]	N/A
ACR 70			
n (%)	19 (16.5)	11 (10.5)	2 (1.7)
CI	14.8 (7.5, 22.2)	8.8 (2.7, 14.9)	N/A
p-value	<0.001[a]	0.005[a]	N/A

[a] Statistically significant difference for the comparison of BMS-188667 vs placebo.

ACR Responses at Day 360

At Day 360, ACR20, ACR50 and ACR70 responses for the 10 mg/kg CTLA4Ig group were significantly ($p<0.001$) higher compared to the placebo group (Table 13, Figure 72A and Figure 72B). Although the same response rates for the 2 mg/kg CTLA4Ig group were numerically higher compared to the placebo group, these differences were not statistically significant.

TABLE 13

ACR Responses at Day 360

	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
ACR 20			
N (%)	72 (62.6)	43 (41.0)	43 (36.1)
CI	26.5 (13.7, 39.3)	4.8 (-7.9, 17.6)	N/A
P-value	<0.001[a]	0.459	N/A
ACR 50			
N (%)	48 (41.7)	23 (21.9)	24 (20.2)
CI	21.6 (9.7, 33.4)	1.7 (-8.9, 12.4)	N/A
P-value	<0.001[a]	0.75	N/A
ACR 70			
N (%)	24 (20.9)	13 (12.4)	9 (7.6)
CI	13.3 (4.4, 22.2)	4.8 (-3.0, 12.6)	N/A
P-value	0.003[a]	0.227	N/A

[a] Statistically significant difference for the comparison of 10/mg/kg CTLA4Ig group vs placebo.

ACR Responses by Visit

For the comparison of the 10 mg/kg CTLA4Ig group to the placebo group, statistically significant improvements were observed for all three response rates (ACR 20, ACR 50, and ACR 70) by Day 90, and these values remained statistically significant at every time point up to and including Day 360 ($p\leq0.008$ for all three ACR response rates) (Figure 73A, Figure 73B, and Figure 73C). In fact, statistically significant improvements in ACR 50 and ACR 70 response for the 10 mg/kg CTLA4Ig group occurred as early as Day 30 ($p=0.039$ and $p=0.04$, respectively).

For the 2 mg/kg CTLA4Ig group, statistically significant improvements compared to placebo were observed in ACR 50 and ACR 70 responses at Day 180 ($p=0.027$ and $p=0.005$, respectively). At Day 360, improvements in ACR response

were slightly greater in the 2 mg/kg CTLA4Ig group compared to the placebo group; however, no statistically significant differences were observed.

After adjusting for visit using the Cochran-Mantel Haenszel test, a significant difference in ACR 20 response was observed for the 10 mg/kg CTLA4Ig group compared to the placebo group at both Day 180 and Day 360. No significant difference was observed between the 2 mg/kg CTLA4Ig and placebo groups at both timepoints. Similar results were obtained for ACR 50 response at both time points. For ACR 70 response at both time points, a significant difference was observed for both CTLA4Ig (BMS-188667) treatment groups compared to the placebo group.

Summary of Major Clinical Response

Major Clinical Response was defined as maintenance of an ACR 70 response over a continuous 6-month period. The percentages of subjects who achieved a Major Clinical Response at Day 360 were significantly higher in both the 10 mg/kg and 2 mg/kg CTLA4Ig groups (7.8% and 5.7%, respectively) when compared to the placebo group (0.8%; p=0.008 and 0.036, respectively) (Table 14).

TABLE 14

Summary of Major Clinical Response by Day 360

	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
No. Subjects with a Major Response	9 (7.8)	6 (5.7)	1 (0.8)
Diff (CI)	7.0 (1.8, 12.2)	4.9 (0.3, 9.4)	N/A
p-value	0.008[a]	0.036[a]	N/A

[a] Indicates a statisticall significant difference for the comparison of BMS-188667 vs placebo.

Mean Numeric ACR (ACR-N) and ACR-N Area Under the Curve (ACR-N-AUC)

Overall, mean numeric ACR (ACR-N) for all treatment groups increased over time during the first 6 months of the study (Figure 74). During the second 6 months, mean ACR-N increased slightly with 10 mg/kg CTLA4Ig, but remained relatively unchanged with 2 mg/kg CTLA4Ig and placebo. At each study visit, the ACR-N was

consistently higher for the 10 mg/kg CTLA4Ig group compared to the 2 mg/kg CTLA4Ig and placebo groups.

Compared to the placebo group, the differences in values for ACR-N AUC (area under the curve) for the 10 mg/kg CTLA4Ig group was significantly ($p<0.001$) higher by Day 360.

Percentage Improvement from Baseline at Day 180

For the 10 mg/kg CTLA4Ig group, improvements in each individual ACR component (tender and swollen joint counts, CRP, pain, subject global assessment, physician global assessment, and physical function) at Day 180 were statistically significant relative to improvements for the placebo group (Table 15).

For the 2 mg/kg CTLA4Ig group, statistically significant improvements compared to the placebo group were observed in physician global assessment and CRP at Day 180. Furthermore, CRP levels in the placebo group actually worsened at Day 180. Change from baseline in mean duration of morning stiffness was comparable among the three treatment groups at Day 180.

TABLE 15

Mean Percentage Improvement from Baseline at Day 180 (Individual Components of ACR Criteria)

	CTLA4Ig (BMS-188667)		
Component	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Tender Joints	n=114	n=104	n=118
Mean % Change	59.78*	43.15	31.88
Swollen Joints	n=114	n=104	n=118
Mean % Change	55.28*	45.34*	33.49
CRP	n=108	n=98	n=114
Mean % Change	31.79*	16.41*	-23.43
Pain	n=109	n=102	n=118
Mean % Change	46.19*	22.09*	8.20
Subject Global Assessment	n=111	n=103	n=118
Mean % Change	40.76*	9.07	17.48
MD Global Assessment	n=111	n=103	n=116
Mean % Change	51.91*	38.71*	25.14
Physical Function	n=107	n=98	n=110
Mean % Change	41.21*	21.63	13.71
Duration Morning Stiffness	n=98	n=82	n=80

Component	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Mean ± SD (minutes)	61.9 ± 55.4	60.8 ± 66.1	55.9 ± 66.2

* Indicates $p<0.05$ in comparison with placebo since 95% CIs did not include zero

Percentage Improvement from Baseline at Day 360

For the 10 mg/kg CTLA4Ig group, improvements in each individual ACR component (tender and swollen joint counts, CRP, pain, subject global assessment, physician global assessment, and physical function) at Day 360 were statistically significant relative to improvements for the placebo group. Mean percentage improvements from baseline to Day 360 are presented in Table 16 for all clinical parameters of the ACR criteria.

For the 2 mg/kg CTLA4Ig group, statistically significant improvements compared to the placebo group were observed in physician global assessment and CRP at Day 360. Furthermore, CRP levels in the placebo group actually worsened at Day 360. At Day 360, the CTLA4Ig (BMS-188667) treatment groups had greater changes from baseline in duration of morning stiffness compared to the placebo group.

TABLE 16

Mean Percentage Improvement from Baseline at Day 360 (Individual Components of ACR Criteria)

Component	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Tender Joints	n=115	n=105	n=119
Mean % Change	66.39*	43.54*	29.97
Swollen Joints	n=115	n=105	n=119
Mean % Change	59.74*	46.40	36.17
CRP	n=112	n=98	n=115
Mean % Change	27.59*	10.31*	-31.26
Pain	n=112	n=104	n=119
Mean % Change	44.93*	26.26	12.55
Subject Global Assessment	n=113	n=105	n=119
Mean % Change	41.01*	16.08	1.99

	CTLA4Ig (BMS-188667)		
Component	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
MD Global Assessment	n=113	n=105	n=119
Mean % Change	53.48*	37.87*	24.14
Physical Function	n=109	n=100	n=111
Mean % Change	42.32*	22.94	10.25
Duration Morning Stiffness	n=88	n=71	n=72
Mean ± SD	66.2 ± 59.5*	66.6 ± 72.2	49.7 ± 73.9

* Indicates $p<0.05$ in comparison with placebo since 95% CIs did not include zero

New Active Joints

The proportion of new active joints was determined using the validated 28-joint count (out of 68 total tender joints and out of 66 total swollen joints) proposed by Smollen et al (Smollen JS, Breedveld FC, Eberl G, Jones I et al. Validity and reliability of the twenty-eight-joint count for the assessment of RA activity. Arthritis & Rheum 1993; 38: 38-43). The proportion of new active joints (both tender and swollen) at Day 180 was lowest for subjects receiving 10 mg/kg CTLA4Ig (Figure 75).

At Day 180, the percentages of subjects reporting no new tender joints and no new swollen joints was highest in the 10 mg/kg CTLA4Ig group (Figure 76A, Figure 77A). The percentage of subjects who reported no new tender joints and no new swollen joints was approximately 59% and 52%, respectively, in the 10 mg/kg CTLA4Ig group; 38% and 44%, respectively, in the 2 mg/kg CTLA4Ig group; and 41% and 37%, respectively, in the placebo group.

The proportion of new active joints (both tender and swollen) at Day 360 was lowest for subjects receiving 10 mg/kg CTLA4Ig (Figure 78). This pattern for the proportion of new active joints mirrored the pattern seen at Day 180.

Similarly, at Day 360, the proportion of subjects reporting no new tender joints and no new swollen joints was highest in the 10 mg/kg CTLA4Ig group (Figure 76B, and Figure 77B). The percentage of subjects who reported no new tender and no new swollen joints was approximately 71% and 61%, respectively, in the 10 mg/kg CTLA4Ig group; 41% and 44%, respectively, in the 2 mg/kg CTLA4Ig group; and 42% for both counts in the placebo group.

Improvement in Clinical Parameters Among Subjects with an ACR Response

Among ACR 20, ACR 50, and ACR 70 responders, improvement in the core components of the ACR criteria were slightly greater for the two CTLA4Ig (BMS-188667) treatment groups compared to placebo.

The onset of action for subjects who received the 10 mg/kg CTLA4Ig dose occurred after approximately 15 days, with significant increases in ACR 20 improvement occurring at ≥Day 60 for ACR50 at ≥Day 90 for ACR70 at ≥Day 30 and in each instance, continuing until Day 360 (see Figure 73A, Figure 73B and Figure 73C).

Changes from Baseline for the Health Outcomes Short Form Questionnaire (SF-36)

The impact of CTLA4Ig (BMS-188667) on health-related quality of life was assessed using the Health Outcomes Short Form Questionnaire SF-36 (summary scores range from 0 to 100 with higher scores indicating a better quality of life). Analyses were performed on the LOCF (last observation carried forward) data set as well as the as the observed data set.

For the 10 mg/kg CTLA4Ig group, statistically significant improvement from baseline compared to the placebo group was observed in all four mental health and all four physical health domains of the SF-36 at Day 180, using the LOCF analysis (i.e., 95% CIs did not include 0) (Figures 79A, 79B). For the 2 mg/kg CTLA4Ig group, there were numerical improvements in the mental health or physical health domains compared to placebo at Day 180, however, these improvements were not statistically significant.

Results of analyses performed on the as-observed data set were similar to those observed for the LOCF data set except that the "role emotional" domain at Day 180 was not significantly improved (but was numerically improved) for the comparison between the 10 mg/kg CTLA4Ig and placebo groups using the as-observed data set.

The physical component and the mental health component summary measures at Day 180 are shown in Table 17.

TABLE 17

Mean Change from Baseline to Day 180 for the SF-36 (Physical and Mental Health Components)

	CTLA4Ig (BMS-188667)		
Summary Score	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Mental Health Component	n=115	n=103	n=118
Baseline Mean	44.52	43.06	41.75
Postbaseline Mean	48.69	45.59	44.04
Mean Change from Baseline	4.17	2.53	2.30
95% CI	(2.46, 5.88)	(0.39, 4.67)	(0.42, 4.17)
Physical Component	n=115	n=103	n=118
Baseline Mean	31.13	30.80	32.33
Postbaseline Mean	39.30	35.47	35.21
Mean Change from Baseline	8.16	4.67	2.88
95% CI	(6.33, 9.99)	(3.25, 6.09)	(1.54, 4.22)

Results of the Health Outcomes at Day 360 were similar to those seen at Day 180. For the 10 mg/kg CTLA4Ig group, statistically significant improvements from baseline compared to the placebo group were observed in all four mental and all four physical domains of the SF-36 at Day 360, using the LOCF analysis (i.e., 95% CIs did not include 0) (Figures 80A, and 80B). For the 2 mg/kg CTLA4Ig group, a statistically significant difference in three of four physical domains at Day 360 and one of four mental domains at Day 360 compared to the placebo group was observed.

Results of analyses performed on the as-observed data set were similar to those observed for the LOCF data set.

The physical component and mental health component summary measures at Day 360 is shown in Table 18.

TABLE 18

Mean Change from Baseline to Day 360 for the SF-36 (Summaries of Physical Component and Mental Health Component)

	CTLA4Ig (BMS-188667)		
Summary Score	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Mental Health Component	n=115	n=103	n=118
Baseline Mean	44.52	43.06	44.75
Postbaseline Mean	48.83	45.65	43.22

	CTLA4Ig (BMS-188667)		
Summary Score	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Mean Change from Baseline	4.31	2.59	1.47
95% CI	(2.64, 5.98)	(0.64, 4.55)	(-0.14, 3.08)
Physical Component	n=115	n=103	n=118
Baseline Mean	31.13	30.80	32.33
Postbaseline Mean	38.93	36.49	34.93
Mean Change from Baseline	7.79	5.69	2.60
95% CI	(5.90, 9.68)	(4.10, 7.28)	(1.09, 4.11)

Biomarker and Pharmacodynamic Data

There were significant improvements (decreases) in 5 of the 6 biomarker/pharmacodynamic (PD) parameters with 10 mg/kg CTLA4Ig at Day 180 (soluble IL-2r, rheumatoid factor (RF), ICAM-1, E-selectin and IL-6) and a numerical decrease in TNF-α (Table 19). There were significant improvements (decreases) in 3 of the 6 biomarker/PD parameters with 2 mg/kg CTLA4Ig at Day 180 (soluble IL-2r, RF and IL-6) and a numerical improvement in ICAM-1. There were no significant changes in any of the biomarker/PD parameters with placebo at Day 180. There appears to be a dose response relationship with the improvements (decreases) in biomarker/PD parameters.

TABLE 19

Pharmacodynamic Measures at Day 180

	CTLA4Ig (BMS-188667)		
Parameter	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Soluble IL-2r (Normal range : 640-2543 pg/mL)	n=95	n=84	n=76
Baseline Mean (±SD)	1426.19 ± 751.76	1396.82 ± 610.21	1429.13 ± 667.84
Postbaseline Mean (±SD)	1112.62 ± 699.68	1261.31 ± 473.66	1470.03 ± 637.75
Mean Change	-316.23	-135.51	43.59
95% CI	(-417.73, -214.72)	(-241.48, -29.53)	(-71.24, 158.43)
Rheumatoid Factor (Normal Range: 0-20 IU/mL)	n=95	n=84	n=74
Baseline Mean (±SD)	289.71 ± 401.95	256.19 ± 307.92	196.11 ± 265.48
Postbaseline Mean (±SD)	185.43 ± 269.52	227.82 ± 276.27	204.36 ± 320.09
Mean Change	-104.27	-28.12	-0.62
95% CI	(-151.53, -57.01)	(-52.13, -4.11)	(-31.67, 30.43)
ICAM-1	n=95	n=82	n=75
Baseline Mean (±SD)	404.89 ± 137.72	393.47 ± 150.85	387.33 ± 230.93
Postbaseline Mean (±SD)	364.74 ± 109.47	387.25 ± 142.73	386.17 ± 163.82
Mean Change	-40.42	-6.22	1.09
95% CI	(-58.06, -22.78)	(-27.49, 15.05)	(-31.88, 34.05)
E-selectin	n=89	n=80	n=71
Baseline Mean (±SD)	68.07 ± 32.93	67.32 ± 37.13	68.23 ± 43.09
Postbaseline Mean (±SD)	61.01 ± 31.53	67.86± 40.20	67.37 ± 35.66
Mean Change	-8.41	0.54	-0.68
95% CI	(-13.24, -3.58)	(-5.95, 7.03)	(-6.87, 5.51)
Serum IL-6 (Normal Range: 0.3-14.8 pg/mL)	n=86	n=74	n=69
Baseline Mean (±SD)	28.47 ± 38.28	31.75 ± 42.29	21.20 ± 26.51
Postbaseline Mean (±SD)	9.25 ± 15.85	16.00 ± 22.13	23.98 ± 37.92
Mean Change	-20.30	-16.10	1.98
95% CI	(-27.55, -13.06)	(-24.20, -8.00)	(-7.21, 11.17)
TNFα (1.2-8.0 pg/mL)	n=84	n=74	n=69
Baseline Mean (±SD)	11.17 ± 23.72	7.51 ± 13.25	13.12 ± 23.20
Postbaseline Mean (±SD)	7.57 ± 7.90	6.20 ± 4.48	9.59 ± 11.21
Mean Change	-3.66	-1.21	-3.54
95% CI	(-8.62, 1.30)	(-4.32, 1.90)	(-7.82, 0.75)

Overall, the pattern in the changes in biomarker/PD data at Day 360 were similar to that seen at Day 180. There were significant improvements (decreases) in 5 of the 6 biomarker/PD parameters with 10 mg/kg CTLA4Ig at Day 360 (soluble IL-2r, RF, ICAM-1, E-selectin and IL-6) and a numerical, but not statistically significant improvement observed for TNF-α (Table 20). There was a significant improvement (decrease) in IL-6 only with 2 mg/kg CTLA4Ig at Day 360, however, numerical improvements were seen with RF and ICAM-1. There were no significant changes in

any of the biomarker/PD parameters with placebo at Day 360. As seen with Day 180 data, it appeared that all of the improvements (decreases) in biomarker/PD parameters occurred in a dose response manner.

A comparison of the postbaseline means for the biomarker/PD parameters at Day 180 to those at Day 360 reveals important trends. For the 10 mg/kg CTLA4Ig group, all biomarkers/PD measures continued to decrease, with the exception of soluble IL-2r which increased slightly. For the 2 mg/kg CTLA4Ig group, mean values for 3 of the biomarkers/PD parameters either decreased slightly (ICAM-1, serum IL-6) or remained relatively constant (E-selectin) and mean values for the other 3 biomarkers/PD measures increased slightly (soluble IL-2r, RF, TNF α). For the placebo group, mean values for all of the biomarkers/PD parameters increased slightly at Day 360, with the exception of TNF α which remained relatively unchanged.

TABLE 20

Pharmacodynamic Measures at Day 360

CTLA4Ig (BMS-188667)

Measure	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
Soluble IL-2r (Normal range : 640-2543 pg/mL)	n=68	n=56	n=55
Baseline Mean (±SD)	1372.10 ± 770.11	1373.86 ± 567.75	1459.93 ± 695.07
Postbaseline Mean (±SD)	1185.51 ± 638.95	1413.84 ± 452.50	1666.59 ± 611.97
Mean Change	-194.31	39.99	206.22
95% CI	(-305.67, -82.96)	(-69.87, 149.84)	(35.88, 376.56)
Rheumatoid Factor (Normal Range: 0-20 IU/mL)	n=69	n=55	n=58
Baseline Mean (±SD)	261.43 ± 333.58	258.42 ± 318.65	179.12 ± 207.72
Postbaseline Mean (±SD)	143.13 ± 180.80	236.61 ± 287.36	206.42 ± 256.27
Mean Change	-118.30	-25.64	20.90
95% CI	(-175.19, -61.42)	(-58.50, 7.23)	(-10.72, 52.51)
ICAM-1	n=77	n=68	n=64
Baseline Mean (±SD)	406.44 ± 145.22	393.41 ± 132.97	405.67 ± 245.16
Postbaseline Mean (±SD)	354.90 ± 111.40	380.42 ± 113.20	405.07 ± 194.15
Mean Change	-55.15	-12.98	1.47
95% CI	(-74.80, -35.49)	(-35.36, 9.39)	(-26.41, 29.35)
E-selectin	n=75	n=68	n=62
Baseline Mean (±SD)	68.84 ± 34.38	66.75 ± 37.10	69.72 ± 44.38
Postbaseline Mean (±SD)	58.77 ± 26.61	67.58± 31.50	71.90 ± 47.43
Mean Change	-10.89	0.83	2.34
95% CI	(-15.70, -6.08)	(-5.62, 7.28)	(-4.53, 9.20)
Serum IL-6 (Normal Range: 0.3-14.8 pg/mL)	n=56	n=47	n=48
Baseline Mean (±SD)	27.68 ± 38.56	27.19 ± 32.45	17.27 ± 22.47
Postbaseline Mean (±SD)	7.64 ± 14.21	13.93 ± 19.00	17.72 ± 29.76
Mean Change	-20.88	-12.72	-0.19
95% CI	(-31.56, -10.19)	(-22.49, -2.94)	(-7.55, 7.18)
TNFα (1.2-8.0 pg/mL)	n=61	n=48	n=50
Baseline Mean (±SD)	9.71 ± 22.80	6.27 ± 3.62	10.81 ± 21.24
Postbaseline Mean (±SD)	6.67 ± 4.80	7.18 ± 8.14	9.36 ± 26.43
Mean Change	-3.02	1.08	-1.41
95% CI	(-8.70, 2.67)	(-1.26, 3.42)	(-5.14, 2.33)

The data are shown graphically for these biomarker/PD measures, as well as for changes in CRP levels, in Figures 81 through 87.

In order to assess the integrity of the planned analyses, all subjects who received study medication and discontinued the study for any reason were considered ACR non-responders at all scheduled study visits subsequent to discontinuation.

Results of these analyses (Table 21) were consistent with the efficacy results already presented. The proportion of subjects who received 10 mg/kg CTLA4Ig and achieved an ACR 20, ACR 50, or ACR 70 response at Day 180 was significantly ($p<0.001$) higher compared to the proportion of subjects who received placebo. For the 2 mg/kg CTLA4Ig group, a significantly ($p\leq0.009$) higher proportion of subjects achieved either an ACR 50 or ACR 70 response.

TABLE 21

ACR Response at Day 180 (Non-Completer Equals Non-Responder)

	CTLA4Ig (BMS-188667)		
	10 mg/kg (n=115)	2 mg/kg (n=105)	Placebo (n=119)
ACR 20, n (%)	67 (58.3)	41 (39.0)	38 (31.9)
Diff (CI)	26.3 (13.6, 39.1)	7.1 (-5.4, 19.7)	N/A
p-value	<0.001[a]	0.266	N/A
ACR 50, n (%)	41 (35.7)	24 (22.9)	12 (10.1)
Diff (CI)	25.6 (14.8, 36.3)	12.8 (3.1, 22.4)	N/A
p-value	<0.001[a]	0.009[a]	N/A
ACR 70, n(%)	19 (16.5)	11 (10.5)	2 (1.7)
Diff (CI)	14.8 (7.5, 22.2)	8.8 (2.7, 14.9)	N/A
p-value	<0.001[a]	0.005[a]	N/A

[a] Indicates a statistically significant difference for the comparison of BMS-188667 vs placebo.

In addition, all primary efficacy analyses were performed on the WOCF (worst observation carried forward) data set. ACR responses based on the WOCF data set were slightly lower than those reported in Table 13 and were comparable to those presented in Table 21. These findings confirm the consistency of ACR response rates in the CTLA4Ig (BMS-188667) treatment groups.

The dosages of anti-rheumatic concomitant medications were to be collected to assess the need for these medications at 6 and 12 months; however, the available data were inadequate to perform these analyses. Only baseline values for mean dose of methotrexate and systemic (non-topical) corticosteroids are provided.

Efficacy Conclusions

CTLA4Ig (BMS-188667) administered at 10 mg/kg (+ MTX) had superior efficacy compared to placebo (+ MTX) at Day 180 and Day 360. For the following efficacy parameters, administration of 10 mg/kg CTLA4Ig was significantly better than placebo:

- Primary efficacy variable: ACR20 response at Day 180 ($p<0.001$)
- ACR50 and ACR70 responses at Day 180 ($p<0.001$)
- ACR20, ACR50 and ACR70 responses at Day 360 ($p\leq0.003$)
- Statistically significant differences in ACR50 and ACR70 responses observed by Day 30 ($p=0.039$ and $p=0.04$), statistically significant differences in all 3 response rates (ACR 20, ACR50 and ACR70) observed by Day 90; these values remained statistically significant at every timepoint up to and including Day 360 ($p\leq0.008$)
- Proportions of subjects who achieved a Major Clinical Response (maintenance of an ACR 70 response over a continuous 6-month period) at Day 360 ($p=0.008$)
- Mean numeric ACR-AUC by Day 360 ($p<0.001$)
- Mean percentage improvements in each individual ACR component at Day 180 and Day 360 ($p<0.05$, 95% CIs did not include 0)
- Improvements in all four mental and all four physical domains of the Health Outcomes evaluation (SF-36) at both Day 180 and Day 360 ($p<0.05$, 95% CIs did not include 0)

In addition to the above statistically significant differences, the 10 mg/kg CTLA4Ig group had a lower number of new active joints and a higher number of subjects reporting no new active tender and swollen joints compared with the placebo group at Day 180 and at Day 360.

Significant improvement with 10 mg/kg CTLA4Ig compared with placebo was seen in nearly all measured pharmacodynamic paramenters (soluble IL12r, RF, ICAM-1, E-selectin and IL-6) and numerical improvement in TNF-α up to 1 year.

For the 2 mg/kg CTLA4Ig group, some efficacy parameters were significantly better compared to the placebo group:

- ACR50 response at Day 180 (p=0.027)
- ACR70 response at Day 180 (p=0.005)
- Statistically significant differences in ACR70 observed by Day 60 (p=0.032) and statistically significant differences in ACR 50 and ACR 70 at Day 180 (p=0.027 and p=0.005)
- Proportions of subjects who achieved a Major Clinical Response (maintenance of an ACR 70 response over a continuous 6-month period) at Day 360 (p=0.036)
- Mean percentage improvements in some of the individual ACR component at Day 180 and Day 360 ($p<0.05$, 95% CIs did not include 0)

For many other efficacy parameters, 2 mg/kg CTLA4Ig was numerically better than placebo.

Safety Results

Overall, the safety profile of CTLA4Ig (BMS-188667) was similar to placebo. There were no major safety problems.

Clinical Laboratory Evaluation

Overall, no new safety issues emerged from the evaluation of mean changes in laboratory values. Mean values for hemoglobin, WBCs, neutrophils, platelets, ALT, AST, GGT and total protein were within the normal range at baseline and remained within the normal range during the study. In general, results of the laboratory tests did not reveal consistent out-of range values or abnormal trends that could be attributed to study medication.

Vital Signs, Physical Findings, and Observations Related to Safety

On each day of study drug administration, vital signs (body temperature, heart rate, and seated blood pressure) were monitored pre-dose and at 15, 30, 45, 60, 75, 90 and 120 minutes post-infusion. Overall, mean values for all vital sign parameters

were within normal range and stable throughout the 360-day study period for all treatment groups.

EXAMPLE 8

While established animal models that mimic acute coronary syndrome in humans do not exist, the use of CTLA4Ig or L104EA29YIg to inhibit atherosclerosis in animal models may be possible. Using murine CTLA4Ig, the ability to suppress atherosclerosis in apolipoprotein E null (apoE -/-) mice maintained on a high fat diet and with and without a stimulus to promote inflammation (i.e., infectious agents, cytokine administration) is explored. It is anticipated that CTLA4Ig may afford reduction of atherosclerotic plaque formation in this animal model as compared with mice receiving placebo (sham) injections.

EXAMPLE 9

The potential use of CTLA4Ig or L104EA29YIg is further explored in several patient populations.

Patients presenting to the hospital with unstable angina or non-ST-segment elevation myocardial infarction (non-STEMI) and evidence of underlying inflammation (i.e., elevated hsCRP) are randomized to receive either placebo or CTLA4Ig/L104EA29YIg. Patients receive a background of standard medical or interventional therapies as ethically mandated and consistent with the most recent American Heart Association / American College of Cardiology Guidelines. The primary endpoint would be a clinical composite reflecting a reduction in subsequent patient morbidity and mortality and/or a redcution of subsequent risk reduction as measred by hsCRP (or other infalmmatory marker[s]).

WHAT IS CLAIMED IS:

1. Use of a soluble CTLA4 mutant molecule comprising an extracellular domain of a CTLA4 molecule, or portion thereof which binds a B7-1 or B7-2 antigen expressed on activated B cells, wherein the extracellular domain of the CTLA4 mutant 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 and comprises a mutation in the extracellular domain of a CTLA4 molecule in the manufacture of a medicament for treating a cardiovascular disease comprising administering to a subject in need thereof an effective amount of the CTLA4 mutant molecule, wherein the cardiovascular disease is selected from the group consisting of: atherosclerosis; coronary heart disease (CHD); restensosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); myocardial infarction; acute myocardial infarction (AMI), including first or recurrent myocardial infarction, non-Q wave myocardial infarction, non-ST-segment elevation myocardial infarction and ST-segment elevation myocardial infarction.

2. The use of Claim 1, wherein said cardiovascular disease is associated with at least one marker of inflammation selected from the group consisting of CRP, hsCRP, IL-10, CD40L, sCD40L, IL-6, sICAM-1, TNF-α, white blood cell count, fibrinogen, and serum amyloid A.

3. The use of Claim 2, wherein said marker of inflammation is selected from the group consisting of CRP, hsCRP, IL-6, and TNF-α.

4. The use of Claim 1, wherein the effective amount the soluble CTLA4 molecule is about 0.1 to 100 mg/kg weight of the subject, about 0.5 to 100 mg/kg weight of a subject 0.5 to 5 mg/kg weight of a subject, about 5 to 10 mg/kg weight of a subject, about 10 to 15 mg/kg weight of a subject, about 15 to 20 mg/kg weight of a

subject, about 20 to 25 mg/kg weight of a subject, about 25 to 30 mg/kg weight of a subject, about 30 to 35 mg/kg weight of a subject, about 35 to 40 mg/kg weight of a subject, about 40 to 45 mg/kg of a subject, about 45 to 50 mg/kg weight of a subject, about 50 to 55 mg/kg weight of a subject, about 55 to 60 mg/kg weight of a subject, about 60 to 65 mg/kg weight of a subject, about 65 to 70 mg/kg weight of a subject, about 70 to 75 mg/kg weight of a subject, about 75 to 80 mg/kg weight of a subject, about 80 to 85 mg/kg weight of a subject, about 85 to 90 mg/kg weight of a subject, about 90 to 95 mg/kg weight of a subject, about 95 to 100 mg/kg weight of a subject, about 2 to 10 mg/kg weight of a subject, about 0.1 to 4 mg/kg weight of a subject, about 0.1 to 0.5 mg/kg weight of a subject, about 0.5 to 1.0 mg/kg weight of a subject, about 1.0 to 1.5 mg/kg weight of a subject, about 1.5 to 2.0 mg/kg weight of a subject, about 2.0 to 2.5 mg/kg weight of a subject, about 2.5 to 3.0 mg/kg weight of a subject, about 3.0 to 3.5 mg/kg weight of a subject, about 3.5 to 4.0 mg/kg weight of a subject, about 4.0 to 4.5 mg/kg weight of a subject, about 4.5 to 5.0 mg/kg weight of a subject, about 5.0 to 5.5 mg/kg weight of a subject, about 5.5 to 6.0 mg/kg weight of a subject, about 6.0 to 6.5 mg/kg weight of a subject, about 6.5 to 7.0 mg/kg weight of a subject, about 7.0 to 7.5 mg/kg weight of a subject, about 7.5 to 8.0 mg/kg weight of a subject, about 8.0 to 8.5 mg/kg weight of a subject, about 8.5 to 9.0 mg/kg weight of a subject, about 9.0 to 9.5 mg/kg weight of a subject, about 9.5 to 10.0 mg/kg weight of a subject, about 0.1 to 2 mg/kg weight of a subject, about 2 to 4 mg/kg weight of a subject, about 4 to 6 mg/kg weight of a subject, about 6 to 8 mg/kg weight of a subject, about 8 to 10 mg/kg weight of a subject, about 10 to 12 mg/kg weight of a subject, about 12 to 14 mg/kg weight of a subject, about 14 to 16 mg/kg weight of a subject, about 16 to 18 mg/kg weight of a subject, about 18 to 20 mg/kg weight of a subject, about 0.5 mg/kg weight of the subject, 2 mg/kg weight of the subject, 10 mg/kg weight of the subject, about 0.5 mg/kg to 100 weight of the subject, about 0.5 to 10 mg/kg weight of a subject, about 0.1 to 20 mg/kg weight of a subject , about 500 mg for a subject weighing less than 60 kg, 750 mg for a subject weighing between 60-100 kg or 1000 mg for a subject weighing more than 100 kg.

5. The use of Claim 1, wherein the soluble CTLA4 mutant molecule is a CTLA4 mutant fusion molecule.

6. The use of Claim 5, wherein the CTLA4 mutant fusion molecule comprises an extracellular domain of a CTLA4 molecule which binds a B7-1 or B7-2 antigen expressed on activated B cells joined to a non-CTLA4 molecule.

7. The use of Claim 6, wherein the non- CTLA4 molecule comprises an amino acid sequence which alters the solubility or affinity of the soluble CTLA4 molecule.

8. The use of Claim 7, wherein the amino acid sequence which alters the solubility or affinity comprises an immunoglobulin moiety.

9. The use of Claim 8, wherein the immunoglubulin moiety comprises one or more mutations to alter effector function.

10. The use of Claim 8, wherein the immunoglobulin moiety comprises a hinge and any or all of the cysteine residues within the hinge are substituted with serine.

11. The use of Claim 8, wherein the immunoglubulin moiety is an immunoglubulin constant region or portion thereof.

12. The use of Claim 11, wherein the immunoglubulin constant region or portion thereof is mutated to alter effector function.

13. The use of Claim 11, wherein the immunoglubulin constant region comprises a hinge, CH2 and CH3 regions of an immunoglobulin molecule.

14. The use of Claim 11, wherein the immunoglubulin constant region or portion thereof is a human or monkey immunoglobulin constant region or portion therof.

15. The use of Claim 1, wherein the soluble CTLA4 mutant molecule comprises an extracellular domain of CTLA4,

a) wherein the extracellular domain of CTLA4 begins with methionine at position +1 or with alanine at position -1 and ends with aspartic acid at position +124 as shown in Figure 23, and

wherein at position +104 of the extracellular domain of CTLA4, leucine is substituted with any other amino acid.

16. The use of Claim 1, wherein the soluble CTLA4 mutant molecule comprises an extracellular domain of CTLA4,

a) wherein the extracellular domain of CTLA4 begins with methionine at position +1 or with alanine at position -1 and ends with aspartic acid at position +124 as shown in Figure 23,

b) wherein at position +104 of the extracellular domain of CTLA4, leucine is substituted with glutamic acid, and

c) wherein at position +29 of the extracellular domain of CTLA4, alanine is substituted with tyrosine.

17. The use of Claim 1, wherein the soluble CTLA4 mutant molecule is L104EA29YIg as shown in Figure 19 beginning with methionine at position +1 or with alanine at position -1 and ending with lysine at position +357 as shown in Figure 19.

18. The use of Claim 17, wherein said cardiovascular disease is associated with at least one marker of inflammation selected from the group consisting of CRP, hsCRP, IL-10, CD40L, sCD40L, IL-6, sICAM-1, TNF-α, white blood cell count, fibrinogen, and serum amyloid A.