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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 L104EA29WIIg), 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.

5 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
10 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
15 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
20 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
25 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
30 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

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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

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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.

5 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; 10 (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 15 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**

20 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).

25 **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)	↑	↑

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	Atherosclerosis/ Unstable Angina	Rheumatoid Arthritis
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
Cartilage antigens		Reported

5 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.

10 (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

15 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
20 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); TNF α , 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 linked 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.

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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

5 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-
10 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.

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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
20 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
25 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
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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

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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

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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 histocompatibility (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, TGF β). 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

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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 cholesterol 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 lesions 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 between 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 apolipoprotein 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 activated *in situ* is strengthened by the finding of CD80 and CD86 colocalization within both early and advanced atheroma. Second, the data

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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.

10 *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 L104EA29Wlg (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 IgC γ 1 (IgCgamma1), IgC γ 2 (IgCgamma2), IgC γ 3 (IgCgamma3), IgC γ 4 (IgCgamma4), IgC μ (IgCmu), IgC α 1 (IgCalpha1), IgC α 2 (IgCalpha2), IgC δ (IgCdelta) or IgC ϵ (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 C γ 1,

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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:

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Double-site mutants	Codon change
L104EG105FIg	Phenylalanine TTC
L104EG105WIIg	Tryptophan TGG
L104EG105LIIg	Leucine CTT
L104ES25RIg	Arginine CGG
L104ET30GIIg	Glycine GGG
L104ET30NIIg	Asparagine AAT
L104EA29YIIg	Tyrosine TAT
L104EA29LIIg	Leucine TTG
L104EA29TIIg	Threonine ACT
L104EA29WIIg	Tryptophan TGG

Further still, the invention provides mutant molecules having the extracellular domain of CTLA4 comprising three mutations, fused to an IgC γ 1 (IgC γ 1) 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
L104EA29YS25KIIg	Lysine AAA
L104EA29YS25KIIg	Lysine AAG
L104EA29YS25NIIg	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