T-Cell Costimulation and Coinhibition in Atherosclerosis

Israel Gotsman, Arlene H. Sharpe, Andrew H. Lichtman

Abstract—Evidence from many human and rodent studies has established that T lymphocytes enhance inflammation in atherosclerotic plaques and contribute to lesion progression and remodeling. Recent work also indicates that regulatory T cells are important in limiting proatherogenic T-cell responses. Given the important role of T cells in atherosclerosis, there is a need to fully understand how proatherogenic T cells are activated and regulated. Antigen-dependent activation of naïve T cells, leading to clonal expansion and effector T-cell differentiation, and effector and memory T cells, is enhanced by signals provided by costimulatory molecules expressed by antigen presenting cells, which bind to receptors on the T cells. In addition, T-cell responses to antigen are negatively regulated by coinhibitory molecules expressed by antigen-presenting cells, which bind to receptors on T cells. Two major families of costimulatory molecules include the B7 and the tumor necrosis factor (TNF) families. These molecules bind to receptors on T cells belonging to the CD28 or TNF receptor families, respectively. The best-defined coinhibitors and their receptors belong to the B7 and CD28 families. Recent work has begun to define how these T-cell costimulatory and coinhibitory pathways influence atherosclerosis, largely in mouse models of the disease. Profound effects are attributable to molecules in both the B7/CD28 (B7-1/2, ICOS, and PD-L1/2) and the TNF/TNF receptor (CD40, OX40, and CD137) families. One emerging theme is that both pathogenic effector T-cell responses and regulatory T cells are influenced by overlapping sets of costimulators and coinhibitors. These complexities must be considered as immunotherapeutic approaches for atherosclerotic disease are developed. (Circ Res. 2008;103:1220-1231.)

Key Words: atherosclerosis ■ costimulation ■ coinhibition ■ T lymphocytes

The chronic inflammatory nature of atherosclerosis is now widely appreciated. The identification of molecular and cellular pathways that promote or inhibit arterial wall inflammation is a promising first step in development of immunomodulatory therapy for atherosclerosis. T-cell costimulatory and coinhibitory pathways are engaged at the same time as T-cell antigen recognition pathways and are of fundamental importance to the regulation of adaptive immunity and to diseases caused by dysregulated adaptive immune responses. In this review, we first provide a background on the role of T lymphocytes in atherosclerosis and the regulation of T-cell responses by costimulatory and coinhibitory molecules. We then review the evidence that T-cell costimulators and coinhibitors influence atherosclerotic disease.

T Lymphocytes and Atherosclerotic Lesions

The pathogenesis of atherosclerosis involves the deposition and modification of lipids within arterial walls, as well as a chronic inflammatory response to these modified lipids. The inflammatory response is mediated by components of the innate immune system, including macrophages and dendritic cells (DCs), and by components of the adaptive immune system, including T lymphocytes (Figure). T lymphocytes are present at all stages of lesion development, and mouse models of atherosclerosis, especially low-density lipoprotein receptor–deficient mice (Ldlr<sup>−/−</sup>) and apolipoprotein E–deficient mice (ApoE<sup>−/−</sup>), have established the pivotal role of T cells and their cytokines in the atherosclerotic process. In at least some studies with Ldlr<sup>−/−</sup> and ApoE<sup>−/−</sup> mice deficient for all lymphocytes (resulting from Rag or SCID gene mutations), there is a significant reduction of the atherosclerotic burden compared to immunocompetent controls, and transfer of CD4<sup>+</sup> T-cells into SCID ApoE<sup>−/−</sup> mice enhances atherosclerosis. The majority of T lymphocytes in mouse and human atherosclerotic lesions are CD4<sup>+</sup> T-helper cells that express the αβ T-cell antigen receptor (TCR) and have a T-helper (Th1) phenotype. Th1 cells are derived from naïve CD4<sup>+</sup> T-cell precursors by antigen and costimulators in the presence of certain cytokines, including interleukin (IL)-12 and interferon (IFN)γ. The defining feature of Th1 cells is their production of IFNγ, a proinflammatory cytokine that activates macrophages, as well as several other cell types. There is a significant presence of CD8<sup>+</sup> T cells in human lesions, but little is known about their specificities. In addition to αβ TCR T cells, there are smaller numbers of T cells expressing invariant TCRs, including γδ T cells and iNKT cells. Studies of iNKT-deficient mice support the role for these lipid-antigen specific cells in both promoting and limiting atherosclerosis.
Most T cells recognize peptides bound to cell surface major histocompatibility (MHC) molecules on antigen presenting cells (APCs); CD4⁺ T cells recognize peptides displayed by class II MHC, and CD8⁺ T cells recognize peptides displayed by class I MHC molecules. iNKT cells recognize lipids bound to the class I MHC–like CD1 molecules, and γδ T cells may recognize nonpeptide antigens. The relevant peptide antigen specificities of plaque CD4⁺ T cells have not been definitively established, but evidence implicates that many of these T cells recognize antigens generated by oxidative modification of low-density lipoproteins (ox-LDL). ox-LDL–specific T cells can be detected in atherosclerotic animals and patients and can be recovered from atherosclerotic lesions. ox-LDL–specific IgG antibodies, whose isotypes indicate T cell–dependent help of the B cell producing them, are also detectable in mice and humans with atherosclerosis. Moreover, adoptive transfer of ox-LDL antigen–specific T cells exacerbates atherosclerosis. A variety of studies of human and mouse lesions also indicate that T cells specific for heat shock protein (Hsp) 60/65 contribute to the inflammation in atherosclerotic arteries. In addition to endogenously generated autoantigens, T cells may recognize antigens produced by infectious organisms that reside in plaques. Perhaps the most extensively studied microbe putatively implicated in the pathogenesis of atherosclerosis is Chlamydia pneumoniae (CpN). T cells specific for CpN Hsp65 have been isolated...
from human lesions, and CpN Hsp65 has been detected in the majority of human lesions analyzed.\textsuperscript{17,18} Nonetheless, studies have failed to show that antibiotic therapy reduces atherosclerosis-related clinical events in patients at risk.\textsuperscript{16,18} Another candidate antigen that has also been implicated in the pathogenesis of atherosclerosis is β2-glycoprotein Ib (β2-GPI),\textsuperscript{19–21} which is expressed by platelets.

**Pathogenic Role of T Cells in Atherosclerosis**

Th1 cells are involved in 2 distinct pathophysiologically relevant phases of atherosclerotic disease. First, the T cells contribute, over many years, to the inflammatory processes that enhance accumulation of macrophage foam cells and smooth muscle cells, which comprise the bulk of the cellular mass of lesions. This pathogenic role of T cells in atherosclerosis appears to fit the description of a chronic Th1-mediated delayed type hypersensitivity (DTH) response. DTH responses involve cognate interaction of Th1 cells with antigen-presenting macrophages, and the bidirectional activation of each cell type. The 2 most important effector molecules produced by the activated Th1 cell in this context are membrane-bound CD40 ligand (CD40L), which binds to CD40 on the macrophage, and IFNγ, which binds to the IFNγ receptor on the macrophage. Signals from both CD40 and IFNγ receptor synergistically induce the expression of multiple proinflammatory genes in the macrophage. This type of activation promotes further inflammation by elaboration of cytokines such as IL-1 and tumor necrosis factor (TNF) and causes tissue destruction by upregulation of inducible nitric oxide synthase and phagocyte oxidase activity and the release of reactive oxygen species. Several laboratories have established that IFNγ has proatherogenic effects, as shown by increased lesion development in hypercholesterolemic mice given IFNγ\textsuperscript{22} and decreased lesion development in mice when IFNγ or its receptor are deficient.\textsuperscript{8,23} T-bet is a transcription factor required for Th1 differentiation. T-bet\textsuperscript{−/−} Ldlr\textsuperscript{−/−} mice develop less atherosclerosis than controls, and they have an impaired Th1 response to ox-LDL.\textsuperscript{24} A second pathogenic role of Th1 cells is their ability to stimulate the release of matrix-degrading enzymes, including matrix metalloproteinases, from lesional macrophages. These enzymes can reduce the collagen content of fibrous caps and render the plaques more likely to rupture and acutely precipitate intraluminal thrombus formation and ischemic damage of downstream tissues.\textsuperscript{25}

**Regulatory T Cells and Atherosclerosis**

Regulatory T cells (Tregs) are generally defined as T cells that actively suppress activation or effector function of other T cells, either by direct effects on these T cells or through effects on APCs. There are several different subsets of T cells defined operationally by T-cell suppressive functions and by cell surface molecule, cytokine, and transcription factor expression. An abundance of literature has established that 1 or more of these Treg subsets contribute to the maintenance of self-tolerance and to the regulation of immune responses. The best characterized Tregs are identified by a CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{lo} surface phenotype\textsuperscript{26} and expression of FoxP3, a forkhead family transcription factor. FoxP3 is a lineage-specification factor for these Tregs and has a crucial role in their suppressive function.\textsuperscript{27} These FoxP3\textsuperscript{+} Tregs comprise 5% to 10% of peripheral CD4\textsuperscript{+} T cells\textsuperscript{28} and are generated during thymic development, as well as in the periphery concomitantly with the generation of effector T cells from naïve T-cell precursors. Tregs are antigen-specific but in a permanent state of activation, enabling them to regulate other activated T cells by contact inhibition and/or through the secretion of antiinflammatory cytokines IL-10 and transforming growth factor (TGF)β.

Recent data suggest that Tregs are important in the regulation of proatherogenic T-cell responses.\textsuperscript{29} In mouse models, deficiency of Tregs is associated with increased atherogenesis and lesion inflammation.\textsuperscript{30–32} A large proportion of the T cells in the atherosclerotic plaque are Th1 cells secreting proinflammatory cytokines, and this is counterbalanced and suppressed by Tregs secreting antiinflammatory cytokines. Indeed, Th1 proinflammatory cytokines such as IL-12 and IFNγ have a key role in promoting atherogenesis,\textsuperscript{8,33} whereas the antiinflammatory cytokines produced by Tregs, IL-10, and TGFβ have been shown to attenuate atherosclerosis.\textsuperscript{34–37} Tregs may influence atherosclerosis by suppressing naïve T-cell activation in lymphoid tissues and also by suppressing effector T-cell activation in lesions, but the relative importance of these 2 sites of action is not known. It has become clear that a comprehensive understanding of the contribution of T-cell immunity to atherosclerosis must include elucidating how Tregs influence effector T cell responses to atherosclerosis-associated antigens.

**T-Cell Costimulation**

T cell–mediated immune responses begin when naïve T cells in lymphoid tissues are activated by antigen (eg, from a microbe) to proliferate and differentiate into effector T cells. The effector T cells may then cooperate with B cells in the lymphoid tissues to promote an antibody response, or they may migrate to peripheral tissue and interact with infected cells at these sites. A small number of T cells will survive as long-lived memory cells after an acute T-cell response wanes, and these cells may be reactivated by subsequent exposure to the same antigen. At each of these stages, the T cells are activated by the combination of 2 different types of signals, both delivered by APCs. Dendritic cells (DCs) are the APCs that initiate T-cell immune responses by transporting protein antigens from tissue sites to lymph nodes, processing the proteins into MHC-binding peptides, and displaying peptide–MHC complexes for recognition by naïve T cells that enter the lymph node from the circulation. The recognition of antigen by a naïve T cell generates signal 1. The second signal is generated by other proteins, called costimulatory molecules, which are expressed on the APC plasma membrane and bind to receptors on the T cells, simultaneously with the recognition of antigen (Figure). Costimulator expression on DCs is induced and/or upregulated by microbial products, such as bacterial lipopolysaccharide, which signal through innate pattern recognition receptors, including Toll-like receptors. Therefore, naïve T-cell activation will occur only when a DC displays an antigen and concurrently displays another molecule indicating infection or cell stress. In this way, costimulation serves to promote T-cell responses
only to appropriate antigens and limits unwanted responses to innocuous antigens including self-proteins.

Costimulatory pathways are not only necessary for naïve T-cell activation, but their absence at the time of presentation of antigen to a naïve T cell can lead to functional inactivation of the T cell, called anergy, or may cause death of the T cell by apoptosis (Figure). These consequences of costimulator deficiency are considered to be important for maintenance of self-tolerance, because the peptide antigens most likely to be presented in the absence of costimulation are derived from normal self-proteins. Blockade of costimulators is therefore a major investigational strategy for the therapeutic induction of tolerance to antigens that drive immune/inflammatory diseases.

In addition to naïve T-cell activation, costimulation of T cells can enhance responses of effector and memory T cells that are being reactivated by other types of APCs either in lymphoid tissues (eg, B cells) or in peripheral tissues (eg, macrophages). This concept first emerged when it was discovered that some costimulatory molecules are induced by various inflammatory stimuli on APCs other than DCs and that receptors for some of these costimulatory molecules are only expressed after T-cell activation, as discussed above. Although costimulation is now understood to regulate different stages of T-cell responses, the relative importance or the specific effects of costimulation do vary among different T cell subsets. For example, activation of naïve CD4+ T cells is stringently dependent on costimulation. Activation of effector CD8+ cytotoxic T lymphocytes is perhaps least dependent on costimulation as compared to other T-cell subsets.

Families of Costimulatory Molecules and Their Receptors

There are many different proteins with costimulatory activity (Table); the following discussion focuses on those with well-documented in vivo significance and/or those that have been studied in the context of atherosclerotic disease. The best-defined, and perhaps most biologically significant, costimulatory molecules belong to the B7 family.39 B7 family molecules bind to members of the CD28 family of receptors expressed on T cells. The prototypic members of the B7 family are B7-1 (CD80) and B7-2 (CD86), which are expressed by DCs, macrophages, B cells, and T cells. Both these proteins bind to CD28, which is constitutively expressed on virtually all mature murine T cells, 95% of human CD4+ T cells, and ~45% of human CD8+ T cells. CD28 signaling involves phosphorylation of a tyrosine in its cytoplasmic tail, binding of the Grb2 adapter protein, and activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway.39 This pathway, together with TCR signaling, promotes IL-2 gene expression and cellular proliferation, as well as expression of antiapoptotic genes. Although these effects are physiologically seen only with concomitant antigen–receptor signaling, superagonist anti-CD28 antibodies have caused unanticipated polyclonal T-cell activation in humans, presumably in the absence of TCR signaling.

Inducible costimulator (ICOS, CD278) is another CD28 family member and is expressed on recently activated and effector/memory T cells but is not present on resting naïve T cells.40 ICOS binds to ICOS ligand (CD275), which is expressed on bone marrow–derived APCs, as well as tissue cells such as endothelium. ICOS signaling involves phosphorylation of a tyrosine residue in the cytoplasmic tail, binding of the p85 subunit of PI3K and stimulation of the PI3K/AKT pathway. In contrast to CD28 signaling, Grb2 is not recruited, and IL-2 gene expression is not enhanced. ICOS is critical for T-dependent antibody responses to protein antigens.41 Some evidence suggests a special importance for ICOS in enhancing Th2 differentiation.42 The absence of ICOS impairs differentiation of Th2-mediated antiinflammatory responses with reduced IL-4 and IL-10 cytokine production43,44 and antibody isotype switching.45,46 ICOS has also been implicated in Treg function.46,47 ICOS deficiency has been shown to enhance helper T-cell responses in models of autoimmune diseases, including experimental autoimmune encephalitis40 and insulitis.46 This would suggest a prominent role for ICOS in vivo in the regulation of autoreactive T cells.

Several members of the TNF family of proteins also have T-cell costimulatory activity.48 These trimeric proteins are membrane bound ligands for trimeric TNF receptor (TNFR) family proteins that are expressed on T cells. On ligand binding, the cytoplasmic tails of TNFR family proteins recruit TRAF proteins, leading to activation of the nuclear factor κB and mitogen-activated protein kinase signaling pathways, which promote T cell survival, enhance cytokine production, and drive T cell mitotic activity. The costimulatory TNF/TNF family ligand–receptor pairs include CD70/CD27, OX40L (CD252)/OX40 (CD134), 4-1BB/CD137 (4-1BB), LIGHT/HVEM, CD30L/CD30, and GITRL/GITR (Table).

CD40L is a TNF family protein rapidly induced on T cells after initial activation. CD40L binds to CD40 on APCs, as well as other cell types. CD40 signaling in APCs upregulates expression of CD80 and CD86, thereby enhancing the ability of the APC to costimulate T cells. In this role, CD40 is not strictly acting as a costimulator but rather as an amplifier of B7 family costimulation. However, there is evidence, largely from in vitro studies, that TNF family members expressed on T cells can activate signaling pathways in the T cell, when they bind TNFRs on APCs. This phenomenon has been called “reverse signaling” and has been documented to enhance antigen-dependent T-cell proliferation and survival. CD40L, as well as LIGHT, TRANCE, CD30L, FasL, TNF, 4-1BBL, OX40, and CD70, can all participate in reverse signaling.39

In addition to B7/CD28 and TNF/TNFR family proteins, other proteins have costimulatory properties in vitro (eg, CD2/SLAM and TIM family members), but the contribution of these molecules to costimulation in vivo are only beginning to be elucidated. For example, CD2 on human T cells binds to LFA-3 (lymphocyte function–associated antigen 3), and transduces signals that enhance T cell proliferation and cytokine secretion.

B7/CD28 Family Coinhibitors

There are at least 4 pathways of negative regulation of T-cell responses that involve binding of B7 family molecules to CD28 family receptors on T cells (Table). The general importance of these negative regulatory, or coinhibitory,
pathways is evident from the autoimmune/inflammatory phenotypes of mice in which the pathways are genetically ablated. The outcome of a T-cell encounter with antigen can be viewed as an integration of both costimulatory and coinhibitory signals.

The first T-cell coinhibitory pathway to be discovered involves cytotoxic T-lymphocyte–associated antigen (CTLA)4, which is a CD28 family member expressed on the surface of T cells shortly after their activation.38 CTLA4 binds to B7-1 and B7-2 and inhibits T-cell activation through mechanisms that are not yet well understood. CTLA4 may sequester B7 ligands from CD28, compete for intracellular signaling molecules engaged by the CD28 pathway, or recruit tyrosine phosphatases, such as SHP2 (Src homology 2 do-

Table. T-Cell Costimulatory and Coinhibitory Molecules

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Expression</th>
<th>Ligand</th>
<th>Expression</th>
<th>Effects on Atherosclerosis</th>
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<td>CD28</td>
<td>T cells, constitutive</td>
<td>B7-1 (CD80), B7-2(CD86)</td>
<td>DCs, B cells, monocytes/macrophages, T cells</td>
<td>B7-1/B7-2/Ldlr KO: decreased,26 transplanted CD28 KO or B7-1/B7-2 KO bone marrow into Ldlr KO: increased39</td>
</tr>
<tr>
<td>ICOS (CD278)</td>
<td>Activated T cells, DCs</td>
<td>ICOSL (CD275)</td>
<td>B cells, monocytes, DCs, T cells, activated tissue cells</td>
<td>Immunized ApoE KO with ICOS-lg to generate anti-ICOS antibodies: increased,23 transplanted ICOS KO bone marrow into Ldlr KO: increased32</td>
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<tr>
<td><strong>Coinhibitory pathways (CD28/B7 families)</strong></td>
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<tr>
<td>CTLA4 (CD152)</td>
<td>Activated T cells</td>
<td>CD80, CD86</td>
<td>DCs, B cells, monocytes/macrophages, T cells</td>
<td>PD-L1/PD-L2/Ldlr KO: increased30</td>
</tr>
<tr>
<td>PD1 (CD279)</td>
<td>Activated T cells, activated B cells, activated DCs</td>
<td>PD-L1, (CD274) PD-L2 (CD273)</td>
<td>B cells, T cells, endothelial cells (PD-L1), activated monocytes, activated DCs, mast cells (PD-L1, PD-L2)</td>
<td>PD-L1/PD-L2/Ldlr KO: increased30</td>
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<td>?</td>
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<td>DCs, B cells, monocytes/macrophages</td>
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<tr>
<td>BTLA (CD272)</td>
<td>T cells, B cells, DCs, myeloid cells</td>
<td>HVEM (TNFR super family)</td>
<td>T cells, B cells, NK cells, DCs, myeloid cells, inducible in somatic tissues</td>
<td></td>
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<td><strong>Costimulatory pathways TNFR/TNF families</strong></td>
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<tr>
<td>4-1BB (CD137)</td>
<td>Activated T cells, activated B cells, activated DCs, endothelial cells</td>
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<td>Activated B cells, activated DCs, monocytes/macrophages, activated T cells</td>
<td>ApoE KO treated with agonist anti-CD137 mAb: increased106</td>
</tr>
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<td>OX40 (CD134)</td>
<td>Activated T cells, activated B cells, activated DCs</td>
<td>OX40L (CD252)</td>
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<td>OX40 KO C3H/He: decreased,69 OX40 transgenic over expression in C3H/He: increased,69 polymorphic human OX40 allele: increased MI risk69</td>
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<tr>
<td>CD27</td>
<td>T cells, activated B cells</td>
<td>CD70</td>
<td>Activated T cells, activated B cells, activated DCs, Activated monocytes/macrophages</td>
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<tr>
<td>CD30</td>
<td>Activated T cells, activated B cells, activated DCs</td>
<td>CD30L (CD153)</td>
<td>Activated T cells, activated B cells, activated monocytes/macrophages</td>
<td></td>
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<tr>
<td>CD40</td>
<td>B cells, DCs</td>
<td>CD40L (CD154)</td>
<td>Activated T cells, activated DCs</td>
<td>Treated Ldlr KO with anti-CD40L mAb: decreased,96,97 CD40/ApoE KO: decreased98</td>
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<tr>
<td>HVEM (TNFR super family)</td>
<td>T cells, B cells, NK cells, DCs, myeloid cells, inducible in somatic tissues</td>
<td>LIGHT (CD258)</td>
<td>Immature DCs, monocytes, activated T cells</td>
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<tr>
<td>GITR (TNFR super family)</td>
<td>Activated T cells, NK cells, PMNs, monocytes, macrophages, B cells, DCs, mast cells</td>
<td>GITRL</td>
<td>DC, activated monocytes/macrophages, B cells, NK cells, PMNs, endothelial cells, mast cells</td>
<td></td>
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<tr>
<td>BTLA (CD272)</td>
<td>T cells, B cells, DCs, myeloid cells</td>
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KO indicates knockout; mAb, monoclonal antibody; NK, natural killer; PMN, polymorphonuclear neutrophil.
main–containing phosphatase 2), which block TCR complex–mediated tyrosine kinase pathways.\textsuperscript{50}

A second T-cell coinhibitory pathway involves PD1 (CD279), a CD28 family member that binds to either of 2 B7 family proteins, programmed death ligand (PD-L)1 (B7-H1, CD274) or PD-L2 (B7-DC, CD273).\textsuperscript{51} Programmed death (PD)-1 expression is induced by various stimuli on a several cell types, including CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells, NK T cells, B cells, and activated monocytes.\textsuperscript{59} The 2 PD-1 ligands differ in their expression, with PD-L2 expression being much more restricted than PD-L1. PD-L2 is inducibly expressed on DCs, macrophages, B1 cells, and cultured bone marrow–derived mast cells. PD-L1 is expressed constitutively on T cells, B cells, DCs, macrophages, and bone marrow–derived mast cells. PD-L1 expression on these cells can be further upregulated on activation. PD-L1 is also expressed on a wide variety of nonhematopoietic cell types, including vascular endothelial cells, epithelial cells, muscle cells, and pancreatic islet cells.\textsuperscript{39} Importantly, this tissue expression of PD-L1 has been shown to limit T cell–mediated disease in a variety of experimental models, including murine autoimmune diabetes\textsuperscript{52} and cytotoxic T-lymphocyte (CTL)-mediated myocarditis.\textsuperscript{53} The pathways by which PD-1 exerts its inhibitory effects are incompletely understood. There is an immunoreceptor tyrosine-based switch motif (ITSM) and an immunoreceptor tyrosine-based inhibition motif (ITIM) in the PD-1 cytoplasmic tail. When PD-1 binds its ligands simultaneously with TCR binding antigen, the ITSM becomes phosphorylated. Protein tyrosine phosphatases (SHP2 and perhaps SHP1) may bind to the phosphorylated ITSM, and block kinase-dependent signals induced by TCR signaling.\textsuperscript{54} A role for the ITIM is not clear. PD-1 signaling can antagonize CD28-dependent expression of antiapoptotic genes. In addition to binding to PD-1, PD-L1 can also bind to B7-1 on T cells, resulting in inhibitory reverse signaling.\textsuperscript{55} Although there are reports suggesting that PD-L1 may have a stimulatory function,\textsuperscript{56} a receptor with such a function has yet to be found.

Two additional pathways in the B7/CD28 family also can provide coinhibitory signals, B7-H4 and BTLA/HVEM. B7-H4 is a B7 family member expressed on hematopoietically derived APCs, as well as other cell types. Its receptor on T cells is not yet known. Recombinant B7-H4-Ig fusion protein inhibits T-cell activation and cytokine production, and anti–B7-H4 monoclonal antibody exacerbates experimental autoimmune encephalitis.\textsuperscript{57} B- and T-lymphocyte attenuator (BTLA) is a CD28 family molecule expressed on activated T cells, and binds the TNFR family member herpesvirus entry mediator (HVEM). BTLA transduces inhibitory signals that block T-cell activation by antigen.\textsuperscript{58} The cytoplasmic tail of BTLA contains ITIMs that are phosphorylated on HVEM ligation and recruit SHP1 and SHP2. BTLA–/– mice have increased susceptibility to autoimmune and inflammatory diseases. As noted above, HVEM on T cells transduces costimulatory signals when it binds LIGHT; thus, similar to B7-1 and B7-2, HVEM can bind a stimulatory receptor and an inhibitory receptor.

Coinhibitory pathways appear to be required for regulation of T-cell responses at several stages, including the initial activation of naïve T cells and also effector and memory T-cell activation. The importance of coinhibition is illustrated by the upregulation of coinhibitory receptors by viruses that cause chronic infections. This appears to be a means of viral evasion of immune eradication. For example, PD-1 is highly expressed on virus-specific CTL in mice or humans with chronic viral infections, and blocking anti–PD-1 antibodies can reactivate virus specific CTL function and promote viral clearance.\textsuperscript{51}

**The Effect of Costimulatory and Coinhibitory Pathways on Treg Development and Function**

Although the emphasis of most of the original research regarding T-cell costimulatory and coinhibitory pathways has focused on their function in modulating activation of naïve and effector T lymphocytes, more recent work has established that these pathways also have profound effects on Treg development and function. Tregs express costimulatory and coinhibitory molecules, including B7-1/2, CD28, CTLA4, PD-1, PD-L1, ICOS, and OX40. The roles of these molecules in Treg biology are incompletely understood, but gene knockout and blocking studies in mice have provided compelling data supporting the importance of at least some of these proteins for Treg development and function. For example, the CD28/B7-1/B7-2 pathway is crucial for the homeostasis and survival of Tregs. Targeted mutations of B7-1/B7-2 or CD28 genes and CD28/B7-1/B7-2 pathway blockade by CTLA4-Ig cause a profound reduction in Treg numbers.\textsuperscript{59,60} B7-1 and/or B7-2 also may be important on effector T cells as the target of Treg suppressive function mediated by CTLA4.\textsuperscript{61} In addition, ICOS appears to be required for optimal Treg function.\textsuperscript{52,62,63} The costimulatory molecule OX40 inhibits the induction of Tregs and Treg suppressive function.\textsuperscript{64,65} The studies cited here and many others clearly indicate that the net effect of manipulating T-cell costimulation and coinhibition on atherosclerosis will reflect a balance of effects on both effector and Tregs.

**CD28 and B7-1/B7-2 Costimulatory Molecules in Atherosclerosis**

In light of the evidence discussed above that T cell–mediated immune responses influence atherosclerosis, the importance of T-cell costimulatory molecules in arterial disease becomes apparent. The first costimulatory molecules to be examined in the context of atherosclerosis were B7-1 and B7-2. These proteins were shown to be expressed in human\textsuperscript{66} and mouse\textsuperscript{67} atherosclerotic lesions. B7-1 and B7-2 expression was increased on splenic B cells\textsuperscript{67} in old versus young ApoE\textsuperscript{+/-} mice, and B7-1 was increased on splenic CD11c\textsuperscript{+} DCs from cholesterol diet–fed Ldlr\textsuperscript{-/-} mice compared to control diet–fed Ldlr\textsuperscript{-/-} mice.\textsuperscript{68} These results suggest that the systemic inflammatory responses to hypercholesterolemia activate APCs to express costimulatory molecules. However, I recent article has reported that the amount of B7-1 and B7-2 on DCs isolated from hypercholesterolemic ApoE\textsuperscript{-/-} mice after treatment with Toll-like receptor (TLR) ligands (lipopolysaccharide or CpG) was lower than the amount of B7-1 and B7-2 on DCs from similarly treated normocholesterolemic C57Bl/6 mice.\textsuperscript{69} Other data in that study indicated that DC maturation was impaired under...
hypercholesterolemia. These differing results suggest that modulation of costimulatory molecules expression may vary depending on the APC population, the type of the TLR ligands, or other stimuli, and perhaps the degree of hypercholesterolemia.

The contribution of B7-1 and B7-2 costimulation to proatherogenic immune response was more directly tested by analyzing lesions in cholesterol diet–fed B7-1+/B7-2−/− Ldlr−/− mice compared to Ldlr−/− controls. The absence of B7-1 and B7-2 reduced early diet-induced atherosclerotic lesion development in the Ldlr−/− mice.70 There was also less MHC II expression in the atherosclerotic lesions. CD4+ T cells from the B7-1+/B7-2−/−Ldlr−/− mice produced less IFNγ in response to the putative athero-antigen Hsp60 in vitro. These data are consistent with an important role for the B7/CD28 pathway in the development of atherosclerotic lesions through their role in priming of antigen-specific T cells. However, different results were obtained in another study that analyzed lesion development in irradiated bone marrow chimeric Ldlr−/− mice reconstituted with B7-1−/−/B7-2−/−, CD28−/−, or control bone marrow.30 In that study, B7-1/B7-2 or CD28 deficiency in the hematopoietic compartment resulted in more atherosclerotic lesion development. This result was attributed to markedly impaired Treg development in the chimeric mice, leading to enhanced proatherogenic effector T-cell responses. The opposing effects of elimination of the B7/CD28 costimulatory pathways on atherosclerosis in the 2 different studies cited above illustrate the complexities of these pathways, which influence the functions of both proinflammatory effector T cells and Treg suppression. In hematopoietically intact mice, B7-1/B7-2 deficiency manifests predominantly as an effector T-cell immunodeficiency71 and can reduce susceptibility to some autoimmune diseases, such as experimental allergic encephalomyelitis.72 These findings are consistent with the reduced proatherogenic T-cell responses in B7-1/B7-2−/−Ldlr−/− mice.70 Genetic deficiency or blockade of the B7/CD28 pathways results in a net enhancement of pathogenic effector T-cell responses attributable to reduced Treg numbers and function in mice with underlying genetic susceptibility to autoimmunity, such as the Nod mouse,60 or in mice that have reconstituted their immune system after lethal irradiation and bone marrow transplantation.30 We have found similar discrepancies between the influence of ICOS deficiency on atherosclerosis in hematopoietically unmanipulated mice versus bone marrow chimeras, which may be attributable to differences in the balance between effector T cells and Tregs, as discussed below. Differences in the observable net effect of costimulatory deficiency on atherogenesis, which depend on different underlying conditions of the host, pose challenges for the design of therapeutic strategies. Nonetheless, they affirm the fundamental importance of effector T-cell activation in the pathogenesis of atherosclerotic disease.

ICOS and ICOS Ligand Costimulatory Molecules in Atherosclerosis

Two published studies have addressed the influence of the ICOS costimulatory pathway on atherosclerosis. In the first study,73 ApoE−/− mice were immunized with ICOS–human Fc chimeric protein in adjuvant, which induced the production of anti-murine ICOS antibodies. Control mice were immunized with control IgFc. Immunization with the ICOS-Fc fusion protein increased atherosclerotic lesion formation. There was a 77% increase in aortic sinus fatty streak lesion formation in a 6-week study of chow-fed mice and a more modest increase in advanced aortic sinus lesion formation in an 8-week study with a high-fat diet. The findings suggested that the presence of blocking anti-ICOS antibody caused enhanced atherosclerosis. Limited immunohistochemical analyses did not detect increased macrophages or T cells in lesions, but a minor increase in IFNγ was found in the lesions of ICOS-Ig–immunized mice. The results of this study are consistent with the interpretation that ICOS exerts an antiatherogenic effect, but the experimental design leaves open the possibility that the induced anti-ICOS antibodies could act as agonists.

The influence of ICOS on atherosclerosis was also studied by the bone marrow chimeric approach in Ldlr−/− mice.32 Lethally irradiated Ldlr−/− mice were reconstituted with bone marrow from wild-type or ICOS−/− mice, and after hematopoietic reconstitution, the mice were fed a cholesterol-containing diet for 10 weeks. The results indicated that ICOS on bone marrow–derived cells had an atheroprotective influence and also limited atherosclerosis-associated immune responses. Mice transplanted with ICOS−/− marrow had a significant increase in the atherosclerotic burden compared to control mice transplanted with wild-type marrow. ICOS−/− mice also had increased lesional CD4+ T cells, macrophage, smooth muscle cell, and collagen content. In vitro–activated CD4+ T cells from ICOS−/− chimeras proliferated more and secreted more proinflammatory cytokines IFNγ and TNFα and less of the antiinflammatory cytokine TGFβ. These data support a suppressive effect of ICOS on atherogenesis.

Experimental evidence clearly shows that ICOS is a positive costimulatory molecule for CD4+ T cells.40 Therefore, it is paradoxical that ICOS deficiency increases immune responses in vivo. One possible explanation relates to the role of ICOS in the development and/or function of Th2 cells, which could downregulate Th1 responses.42 However, recent studies show that ICOS is also involved in Th1 differentiation.74,75 As mentioned above, ICOS also has been implicated in Treg function.46,47 Studies of ICOS−/− C57Bl/6 mice and irradiated ICOS−/− Ldlr−/− mice reconstituted with ICOS−/− bone marrow showed that FoxP3+ Tregs constitutively express high ICOS levels.32 In vitro data demonstrated that ICOS deficiency caused impaired Treg suppressive function, and in vivo data demonstrated that ICOS−/− mice had decreased numbers of FoxP3+ Tregs.32 Taken together, these data suggest that ICOS has a key role in controlling atherogenesis, through its effect on Treg responses. Interestingly, no significant difference in atherosclerotic lesion development was detected in hematopoietically unmanipulated ICOS−/− Ldlr−/− mice compared to Ldlr−/− control mice (I.G., A.H.S., and A.H.L., unpublished data, 2006).

We have mentioned 2 examples of differences in atherosclerosis and Treg development and function when a costimulatory deficiency (B7-CD28 or ICOS-ICOSL pathways) is
studied in gene knockout bone marrow recipients versus hematopoietically unmanipulated gene knockout mice. It should be pointed out that immunologic reconstitution is quite successful, and protective T-cell and humoral immune functions are achieved in mice and humans after lethal irradiation and bone marrow transplantation with genetically normal bone marrow. The mechanisms underlying variations in immune regulation observed when costimulator-deficient donor marrow is used require further study. These mechanisms may be clinically significant in the context of therapeutic bone marrow/hematopoietic stem cell transplant recipients.

**PD1 and PD-L1/PD-L2 Coinhibitory Molecules in Atherosclerosis**

The importance of the PD/PD-L pathway in the maintenance of T cell self-tolerance has been established in several animal models.51 The expression of PD-L1 and PD-L2 on DCs, and the wide distribution of PD-L1 on endothelium and other tissue cells, suggests that both initiation of T-cell responses in lymphoid tissues and effector T-cell responses in lesions may be regulated by PD-1 signaling. The influence of the PD-1/PD-L pathway on atherosclerotic disease was examined using PD-L1−/−PD-L2−/−Ldlr−/− triple knockout mice, generated by crossbreeding PD-L1−/−PD-L2−/− mice with Ldlr−/− mice. The extent and phenotype of diet-induced atherosclerosis and plaque antigen–specific cell-mediated responses was compared in PD-L1−/−PD-L2−/−Ldlr−/− and Ldlr−/− controls.68 In the absence of the PD-1 ligands, there was an exaggerated systemic immune response, including lymphadenopathy, increased numbers of activated T cells in lymphoid tissues, and elevated serum levels of the proinflammatory cytokine TNFα. This correlated with increased aortic atherosclerosis. After in vitro cholesterol loading, PD-L1−/−PD-L2−/− peritoneal macrophages and splenic DCs were more potent stimulators of CD4+ T-cell activation than were in vitro cholesterol-loaded cells from wild-type mice. APCs directly isolated from hypercholesterolemic PD-L1−/−PD-L2−/−Ldlr−/− mice stimulated stronger T-cell responses to ox-LDL than APCs from hypercholesterolemic Ldlr−/− mice. Thus, these findings demonstrate that PD-L1 and/or PD-L2 exert significant antitherogenic and antiinflammatory roles in hypercholesterolemic mice.

Immunohistochemical analysis of the atherosclerotic lesions in PD-L1−/−PD-L2−/−Ldlr−/− revealed an enhanced inflammatory phenotype with markedly increased numbers of T cells as well as increased macrophages and smooth muscle cells.68 Both CD4+ and CD8+ T cells were much more abundant in the lesions of PD-L1−/−PD-L2−/−Ldlr−/− than in the lesions of Ldlr−/− controls. CD8+ T cells are relatively rare in the plaques of Ldlr−/− mice. In human lesions, CD8+ T cells are usually present but are less numerous than CD4+ T cells. Therefore, the abundance of these cells in the lesions of the PD-L1−/−PD-L2−/−Ldlr mice may be an indication that CD8+ T cells specific for lesional antigens are normally under tight control by PD-1. These findings are of special interest in light of emerging evidence that chronic exposure to viral antigens can lead to upregulation of PD-1 on viral-specific CD8+ T cells and contribute to an exhausted phenotype in which T-cell proliferation and effector functions are impaired.76,77 Chronic exposure to atherosclerosis-related antigens also may lead to upregulated PD-1 expression and inhibition of CD8+ T cells specific for these antigens. Targeting PD-1 to enhance antiviral immunity, for example, in HIV infected patients, could have the complication of increased cardiovascular risks by activating proatherogenic T-cell responses. Analyses of polyclonal mixtures of CD8+ T cells from hypercholesterolemic Ldlr−/− mice have not revealed increased PD-1 expression compared to normcholesterolemic controls mice (A.H.L. and A.H.S. unpublished results, 2007). There are no tools such as peptide–MHC tetramers currently available to identify athero-antigen–specific CD8+ T cells and determine whether PD-1 is upregulated on these cells in hypercholesterolemic mice.

In the absence of infectious or other inflammatory challenges, PD-L1−/−PD-L2−/− mice do not show overt manifestations of dysregulated immunity. In the setting of an inflammatory challenge, the absence or blockade of PD-L1, PD-L2, or PD-1 results in enhanced T-cell responses and acceleration or exacerbation of disease.78–81 The disease phenotype of PD-L1−/−PD-L2−/−Ldlr mice supports the concept that hypercholesterolemia is a systemic inflammatory challenge.

The site where PD-L1 or PD-L2 may be inhibiting proatherogenic T-cell responses remains to be determined. APCs in lymphoid tissues are likely involved because hematopoietically derived APCs from spleen and paracortical lymph nodes were shown to be dependent on PD-L1/PD-L2 to limit T-cell activation.68 However, PD-L1 has also been shown to have an inhibitory function within tissue inflammatory sites.82,83 PD-L1 is expressed on microvascular endothelial cells and inhibits cytotoxic T-cell activation in vitro and in vivo.53 PD-L1 is expressed on DCs and macrophages in the neointima of atherosclerotic lesions but has not been observed on the aortic endothelium.68 Although PD-L1 may downregulate immune responses directly in the atherosclerotic tissue, this has yet to be established.

**OX40 and OX40 Ligand in Atherosclerosis**

The TNF family member OX40 and its ligand OX40L, a TNFR family member, provide T-cell costimulatory signals.85 OX40 is expressed on T cells, and OX40 ligand is expressed on APCs (and endothelium). This pathway may be particularly important for sustaining T-cell responses and enabling effective long-lasting T-cell responses.48 OX40 not only is important for the development and survival of memory CD4+ T cells but also inhibits T regulatory cell development and function.54,65,86 OX40-dependent costimulation enhances autoimmune diseases such as experimental autoimmune encephalitis.87

Several mouse and human studies have provided evidence that OX40 ligand is involved in promoting atherosclerotic disease and/or its complications. In a quantitative trait locus study to identify genes that render C57BL/6 mice more susceptible than C3H/He mice to diet-induced atherosclerosis, a locus on mouse chromosome 1 was identified that included the OX40 ligand gene, as well as 10 other known genes.88 OX40 ligand deficiency led to smaller lesions in...
high-fat diet–fed C3H/He mice compared to controls, and transgenic mice overexpressing OX40 ligand had larger atherosclerotic lesions than controls.89 Furthermore, a single nucleotide polymorphism in the OX40L gene Tnfsf4 was found to be more frequent in patients with myocardial infarction than controls.89 In a different study, blockade of OX40L in Ldlr−/− mice reduced atherosclerosis, and this was attributed to reduced IL-4–mediated Th2 isotype switching with decreased T cell–dependent anti–ox-LDL IgG responses and increased levels of anti–ox-LDL IgM.90 Overall, these findings support the concept that OX40-OX40L–dependent T-cell costimulation has an important role in promoting atherosclerotic disease.

CD40 and CD40L in Atherosclerosis
The CD40/CD40L pathway is not strictly a mediator of T-cell costimulation, and it functions mainly to activate APCs by the T cell. Nevertheless, this pathway has a significant contribution to the indirect activation of the T cell, because CD40 signaling in the APC upregulates costimulatory molecules and thus is also important in the activation of the T cell.91 CD40L and CD40 are expressed on numerous cell types within atherosclerotic lesions, including endothelial and smooth muscle cells, macrophages, and T lymphocytes.92 CD40L and CD40 interactions increase expression of proinflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases, and tissue factor, thus contributing to the recruitment and activation of inflammatory cells in atherosclerosis and to the instability of the atherosclerotic plaque.93 Importantly, platelets constitutively express CD40, and they express CD40L on activation.94 A new and active area of investigation addresses how CD40 signaling in platelets contributes to proatherogenic inflammatory processes.95 CD40L is important in vivo in the development of atherosclerosis. Deficiency of CD40L by antibody blockade or targeted mutations significantly reduces atherosclerosis, including the progression of established lesions.96–98 The relevant cell types that modulate atherosclerosis through CD40L expression is still under investigation. Transplantation of CD40L−/− bone marrow into Ldlr−/− mice did not significantly reduce atherosclerosis, suggesting that CD40L on nonhematopoietic cells mediate proatherogenic effects.99,100 It is possible, but not proven, that blockade or deficiencies in CD40/CD40L signaling may have antiatherogenic effects, at least in part, by reducing expression of B7-1, B7-2, and other T-cell costimulators.

CD137 and CD137 Ligand in Atherosclerosis
CD137 (4-1BB) is a TNF receptor family member that binds CD137 ligand (CD137L). Although the cellular distribution of both the molecules is wide, CD137 expression is induced on T cells by antigen recognition and acts as a costimulatory receptor when it binds CD137L expressed on APCs.101 Interest in CD137 as a potential therapeutic target was raised by studies in which administration of an agonistic anti-CD137 antibody ameliorated disease in mouse models of systemic lupus-like autoimmune disease102,103 and autoimmune arthritis.104 CD137 appears to have a particularly important role in controlling CD8+ T-cell responses.105 A recent study explored the relationship of the CD137/CD137L pathway in atherosclerosis.106 Significantly more CD137 mRNA was detected in human atherosclerotic arteries than normal arteries. CD137 protein was detected by immunofluorescence on T cells and endothelial cells in human atherosclerotic lesions. In vitro, CD137 was inducible on endothelial cells and vascular smooth muscle cells by proinflammatory cytokines, and crosslinking CD137 with trimeric rCD137L induced endothelial leukocyte adhesion molecule expression and reduced smooth muscle cell proliferation. When ApoE−/− mice were treated with agonist anti-CD137 antibody, this resulted in increased aortic atherosclerosis and a marked increase in lesional inflammatory cells and cytokines. Although the expression of CD137 and CD137L on multiple cell types present in atherosclerotic arteries complicates the study of mechanisms by which these molecules may influence atherosclerotic disease, T-cell costimulation is likely to play a significant role. In this regard, the CD137 agonist treatment resulted in abundant CD8+ T-cell infiltration into lesions, which are otherwise unusual in mouse lesions.106 As discussed earlier, lesional CD8+ T cells were also abundant in PD-L1/L2−/−Ldlr−/− mice. Together, these findings suggest opposing roles for CD137/CD137L and PD-1/PD-L pathways in controlling CD8+ T cells in atherosclerotic lesions. Because systemic lupus erythematosus predisposes to atherosclerotic disease, an important but unresolved question that arises is why agonist anti-CD137 antibody ameliorates lupus-like disease in mice but promotes atherosclerosis. Perhaps the opposing effects of the agonist antibody on different disease processes reflects differential effects on CD4+ and CD8+ T cells.

Conclusions and Therapeutic Implications
Costimulatory pathways are potentially excellent targets for therapeutic intervention in T cell–mediated diseases. Blockade of B7-CD28 interactions by CTLA4-Ig has already been established as an effective treatment for human autoimmune diseases including rheumatoid arthritis and psoriasis. Effector T cells play an important pathogenic role in development of atherosclerosis and in destabilizing advanced lesions. Therefore, inhibition of T-cell activation by targeting costimulatory pathways is a sensible approach for immune modulation of arterial disease. The influence of different costimulatory pathways in atherosclerosis is likely to vary depending on the stage of disease. For example, early lesion development is likely to be enhanced by priming of naïve CD4+ T cells in lymphoid tissues, which is highly dependent on CD28 signaling. In contrast, effector/memory T cells within lesions that can contribute to plaque instability may be more profoundly influenced by ICOS and PD-1 pathways. Thorough studies of the comparative roles of different costimulatory pathways in lymphoid tissues or in lesions, and at different stages of lesion progression and remodeling, have not been performed but are needed to rationally choose the best targets.

The importance of the balance of effector and Treg responses in atherosclerosis must be taken into account as such therapies are contemplated. Clearly, costimulatory blockade can impair both effector and Treg differentiation and function, and therefore modulation of these molecules...
could be a double-edged sword. Preclinical models also have limitations in predicting responses in humans. This was evident from the results of a limited trial of an agonist anti-CD28 monoclonal antibody. Although activation of CD28 by these antibodies in animals had a beneficial effect in reducing the immune response through its activation of Treg function, the antibody caused a hyperacute systemic inflammatory response syndrome caused by a proinflammatory cytokine storm that caused the subjects to become critically ill.\textsuperscript{107} Unanticipated effects of targeting costimulatory pathways may also arise because of the wide distribution of many of the cell surface molecules, beyond the T cells and APCs. This especially true of the TNF/TNFFR family pathways. For example, antibodies specific for CD40L could pose a risk for hemostasis/thrombosis complications because CD40L is expressed on platelets.\textsuperscript{108} There is also a reasonable likelihood example, antibodies specific for CD40L could pose a risk for hemostasis/thrombosis complications because CD40L is expressed on platelets.\textsuperscript{108} There is also a reasonable likelihood of the cell surface molecules, beyond the T cells and APCs.

The chronic nature of atherosclerotic disease also raises questions about the practicality of immune therapy directed at costimulatory pathways. As is the case for autoimmune diseases and allograft rejection, the ideal approach for immune therapy of atherosclerosis is to induce long-lasting specific T-cell tolerance to atherosclerosis-specific antigens, without global immunosuppression. One theoretical method of tolerance induction to such an antigen is delivery of a costimulatory blocking drug or a coinhibitory agonist drug, at the same time as immunization with the antigen. In this way, the T cells specific for the antigen would receive signal 1 (antigen) and no signal 2, or simultaneously signal 1 plus a coinhibitory signal. The first major hurdle that must be overcome to advance this approach is the identification of the relevant antigens. Although work is in progress to achieve a better molecular understanding of the relevant T-cell antigens, this remains 1 of the critical areas where accelerated research programs should be directed.

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T-Cell Costimulation and Coinhibition in Atherosclerosis
Israel Gotsman, Arlene H. Sharpe and Andrew H. Lichtman

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