Treating Atherosclerosis With Regulatory T Cells

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Abstract—Regulatory T cells (Tregs) play an important role in the regulation of T-cell–mediated immune responses through suppression of T-cell proliferation and secretion of inhibitory cytokines, such as interleukin-10 and transforming growth factor-β. Impaired Treg numbers and function have been associated with numerous diseases, and an imbalance between proinflammatory/proatherogenic cells and Tregs promotes atherosclerotic disease. Restoration of this balance by inducing Tregs has great therapeutic potential to prevent cardiovascular disease. In addition to suppressing differentiation and function of effector T cells, Tregs have been shown to induce anti-inflammatory macrophages, inhibit foam cell formation and to influence cholesterol metabolism. Furthermore, Tregs suppress immune responses of endothelial cells and innate lymphoid cells. In this review, we focus on the recent knowledge on Treg subsets, their activity and function in atherosclerosis, and discuss promising strategies to use Tregs as a therapeutic tool to prevent cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2015;35:280-287. DOI: 10.1161/ATVBAHA.114.303568.)

Key Words: atherosclerosis ■ immune system

Regulatory T cells (Tregs) form an important T-cell subclass that provides protection against autoimmunity and may be used for treatment of autoimmune-like disorders, such as atherosclerosis.1 Various subsets of Tregs exist, but the best-characterized are CD4+Foxp3+CD25hiCD127lo cells that comprise 5% to 10% of the CD4+ T cells in human blood, lymphoid tissue, and epithelial barrier tissues.2 A significant fraction of these CD4+ Tregs develops in the thymus and are called natural or thymic Tregs. The transcription factor Helios is reported to be exclusively expressed in thymic Tregs although this has been disputed.3 Tregs exert their immunosuppressive function mainly through secretion of the inhibitory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β, and cell–cell contact, mediated by membrane-bound TGF-β, cytotoxic T lymphocyte-associated antigen (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor family-related protein.4,5 In addition to the natural Tregs, CD4+ Tregs differentiate from naïve CD4+ T cells in secondary lymphoid organs and are called adaptive or peripheral Tregs, which include CD4+Foxp3+CD25hiCD127lo cells with a similar phenotype to natural Treg, as well as IL-10–producing T regulatory type 1 cells, TGF-β–producing T helper-3 cells, and CD8+Foxp3+ Tregs.6–11

The importance of Tregs in modulation of immune responses in atherosclerosis has been demonstrated in several studies in mice where Tregs were partially or entirely depleted. LDLr−/− mice lacking CD28 or CD80/CD86, costimulatory molecules that are essential for Treg development and homeostasis, show decreased Treg numbers associated with an increase in atherosclerosis,12 and treatment of ApoE−/− mice with a Treg depleting CD25-specific antibody (PC61) aggravates lesion development.12 The contribution of Foxp3+ Tregs to atherosclerosis development was first elucidated by a partial depletion of Foxp3+ Tregs using a dendritic cell (DC)–based vaccination that provoked cytotoxic T-cell responses against Foxp3-expressing cells leading to enhanced atherosclerosis.13 Recently, Klingenberg et al.14 showed that a specific depletion of Foxp3+ Tregs using DEREG/LDLr−/− mice increases atherosclerosis development 2.1-fold.

The focus of this review will be on the development of experimental therapies to increase the frequency of Tregs to reduce atherosclerosis and on their potency as a new immune-therapy to treat cardiovascular disease.

Frequency and Characterization of Tregs in Atherosclerosis

Tregs have been found in both mouse and human atherosclerotic lesions15,16 and most studies show that Treg numbers are reduced in hypercholesterolemic mice and in patients with cardiovascular when compared with healthy controls (Table).

Low Numbers of Tregs in Atherosclerosis

ApoE−/− mice have reduced numbers of Tregs, identified as either CD4+CD25+ cells or CD25+Foxp3+ cells, in lymphoid organs compared with C57BL/6 mice and younger ApoE−/− mice that have no evident atherosclerotic lesions.17 When fed a high-fat diet, ApoE−/− mice showed a reduction in CD4+CD25+Foxp3+ cells compared with mice fed a regular...
diet.\textsuperscript{19} In \textit{LDLR}\textsuperscript{−/−} mice, circulating and lesional CD4+Foxp3+ Tregs peak 4 weeks after initiation of a high-fat diet, but these numbers subsequently decline, resulting in an accumulation of effector T cells that contribute to disease progression.\textsuperscript{18} Similarly, low levels of circulating human Tregs are associated with increased lesion vulnerability.\textsuperscript{24} Interpretation of studies may be complicated because the term ACS often comprises different patient groups, including those having unstable angina, non–ST-segment–elevation myocardial infarction (MI) and ST-segment–elevation MI. Moreover, control groups vary in different studies, including either healthy individuals or patients with chronic stable angina/chest pain syndrome. Overall, in most of these studies, patients with unstable angina and non–ST-segment–elevation MI show reduced peripheral Tregs in the blood when compared with healthy individuals or patients with stable angina.

Originally, Tregs were characterized as CD4+CD25\textsuperscript{high} cells that express Foxp3 to maintain their suppressive capacity and as shown in the Table numerous studies validate their association with the development of ACS.\textsuperscript{20,21,23,25,27} Foxp3 is, however, also transiently upregulated in activated effector human T cells.\textsuperscript{33} Therefore, additional markers for the characterization of Tregs are required for accurate identification, including CD127, inducible T-cell costimulator (ICOS), latency-associated peptide (LAP), and glycoprotein A repetitions predominant (GARP). CD127 (IL-7 receptor) is downregulated on Tregs and inversely associated with Foxp3 expression in PBMCs isolated from healthy individuals.\textsuperscript{28} Ammirati et al\textsuperscript{21} found no correlation between circulating CD4+CD25+CD127(low) Tregs and Foxp3 expression and in mice ICOS deficiency accelerates atherosclerosis. Recently, reduced circulating CD4+LAP\textsuperscript{+} Tregs have been found in patients with ACS compared with patients with stable angina and control patients.\textsuperscript{29,30} Moreover, CD4+LAP\textsuperscript{+} Tregs from patients with ACS show a defect in their suppressive capacity when compared with those from control groups.\textsuperscript{31} GARP associates with latent TGF-β/LAP on Tregs and regulates the bioavailability and activation of TGF-β.\textsuperscript{32} In humans, GARP is only expressed on activated Tregs, whereas in mice GARP has also been found on some resting Tregs.\textsuperscript{33} Reduced circulating CD4+GARP\textsuperscript{+} Tregs and CD4+CD25+GARP\textsuperscript{+} Tregs are seen in patients with ACS compared with those with stable angina/chest pressure syndrome and healthy individuals.\textsuperscript{31,32} In addition, CD4+CD25+GARP\textsuperscript{+} Tregs isolated from patients with ACS showed a reduced ability to suppress effector T cells.

Possible Mechanisms Underlying the Low Frequency of Tregs in Atherosclerosis

Several mechanisms underlying the inverse correlation between Tregs and atherosclerosis progression have been explored. Possibly, survival of Tregs is impaired because Zhang et al\textsuperscript{28} observed increased apoptosis in Tregs of patients with non–ST-segment–elevated ACS compared with Tregs of patients with chronic stable angina/chest pain syndrome. Tregs from patients with non–ST-segment–elevated ACS contain lower mRNA levels of the antiapoptotic gene Bcl-2 and higher levels of the proapoptotic gene Bak. Moreover, they showed that oxLDL-induced apoptosis of Tregs and in light of elevated oxLDL levels in patients with non–ST-segment–elevation ACS, which may suggest that oxLDL is involved in the Treg defect in patients with cardiovascular disease. Previously, Mor et al\textsuperscript{20} already observed that oxLDL can reduce numbers of CD4+CD25+ Tregs in vitro, partially because of apoptosis induction. It was also found that oxLDL dose dependently increased methylation of the Treg-specific demethylated region within the Foxp3 gene, thereby reducing Foxp3 expression in PBMCs isolated from healthy individuals.\textsuperscript{37,38} Most interestingly, reduced Treg levels defined as demethylation at the Foxp3 demethylated region are observed in patients with ACS, and this was associated with the severity of ACS. Another possibility that might explain reduced Treg numbers in patients with cardiovascular was proposed by Zhang et al,\textsuperscript{28} who found that non–ST-segment–elevation ACS patients have impaired thymic Treg output determined by lower circulating CD45RO+CD45RA+CD3\textsuperscript{+} Tregs compared with patients with chronic stable angina/chest pain syndrome.

Function of Tregs in Atherosclerosis

In addition to lower numbers of Tregs in the circulation and atherosclerotic lesions, multiple studies report a dysfunction in their suppressive capacity during disease. Tregs from ApoE\textsuperscript{−/−} mice show hampered inhibition of effector T cells when compared with Tregs isolated from C57BL/6 mice\textsuperscript{17} and the suppressive function of human Tregs isolated from peripheral blood of patients with ACS is strongly decreased when compared with patients with stable angina and normal
Th1 cells and Tregs exists. Whereas circulating Th1 cells
patients with cardiovascular an inverse correlation between
Correspondingly, deficiency in T-bet or IFN-γ
ment of monocytes and T cells into the plaque, increase
stimulate the recruit-
via secretion of interferon (IFN)-γ
responses in atherosclerosis (Figure).
also reduced Foxp3 and CTLA-4 expression
In patients with non–ST-segment–elevated ACS compared with controls
↑ in patients with ST-segment–elevated acute MI compared with controls
↓ in patients having cardiovascular disease would benefit from
increased numbers of atheroprotective Tregs. Most research
that patients with cardiovascular disease would benefit from

Inhibition of Effector T Cells
Tregs show the ability to suppress proatherogenic effector
T cells in atherosclerosis. The majority of the pathogenic
CD4+ T cells in atherosclerosis are effector Th1 cells, which
via secretion of interferon (IFN)-γ stimulate the recruit-
ment of monocytes and T cells into the plaque, increase
lipid uptake by macrophages, and activate lesional APCs. Correspondingly, deficiency in T-bet or IFN-γ attenuates atherosclerosis. Multiple studies in mice have shown that inducing Tregs in atherosclerosis affects Th1 cells and in patients with cardiovascular an inverse correlation between Th1 cells and Tregs exists. Whereas circulating Th1 cells
are expanded in patients with stable angina, unstable angina, and acute MI compared with healthy individuals, Tregs are
reduced. IL-17–producing Th17 cells form another proinflammatory subset of effector CD4+ T cells although mouse studies have shown both Th17 cells or IL-17 may have either pro- or antiatherogenic effects. Nonetheless, a significant negative correlation between Th17 and Treg cell frequencies has been found in the circulation of patients with unstable carotid artery lesions. This imbalance between Th17 cells and Tregs has been confirmed, suggesting one of the mechanisms through which Tregs regulate atherosclerosis is the inhibition of Th17 cells. Interestingly, the ability of Treg to suppress Th1 or Th17 responses may require differential stimulation of the Treg by IFN-γ and IL-17 versus IL-10, respectively.

The inhibitory cytokines IL-10 and TGF-β strongly contribute to Treg-mediated suppression of effector T cells in atherosclerosis. In mice, IL-10–producing T regulatory type 1 cells reduce immune responses in ApoE-/- mice resulting in a decreased plaque size and inflammation as shown by lower
levels of IFN-γ. In addition, Tregs and serum IL-10 are decreased in vulnerable patients that have had recurrent cardiac events in comparison with stable patients. Deficiencies in total or T-cell–specific TGF-β signaling accelerate atherosclerosis and induce an unstable plaque phenotype in hypercholesterolemic mice. A relatively new cytokine associated with Treg-mediated effector T-cell suppression is IL-35. IL-35 consists of Epstein–Barr virus–induced gene 3 and IL-12 p35 (IL-12A), and both of these subunits are strongly coexpressed in human atherosclerotic lesions. Recently, it was shown that serum IL-35 is decreased in patients with acute MI, unstable angina, and stable angina compared with patients with chest pain syndrome, suggesting Treg-associated IL-35 can be a novel target to prevent atherosclerosis.

**Inhibition of DCs**

DCs are major contributors to the pathogenesis of atherosclerosis, in part, because of their essential role in activating T-cell activation. An inverse correlation between mature DCs expressing fascin and Tregs exists in atherosclerotic lesions; in vulnerable lesions, fascin-expressing DCs are increased, whereas Tregs are decreased when compared with patients with stable lesions. Tregs can inhibit DCs via not only their immunosuppressive cytokines, IL-10 and TGF-β, but also cell surface molecules, such as CTLA-4, programmed death-1, and their ligands programmed death-ligand (PD-L)/1,2, and lymphocyte activation gene-3. CTLA-4 expressed on Tregs binds to CD80/CD86 on DCs thereby blocking the ability of DC to activate naïve T cells. Increased mRNA levels of CTLA-4 have been associated with increased Tregs and reduced atherosclerosis in several studies. Moreover, treatment with a recombinant CTLA-4 Ig fusion protein, abatacept, reduced intimal thickening in a femoral artery cuff model in ApoE3*Leiden mice through reduced activation of IFN-γ–producing Th1 cells and elevated IL-10–producing Tregs. Signaling via coinhibitory PD-1 expressed on Tregs and PD-L1/2 on DCs also inhibits their activation. Mice deficient in either PD-1 or PD-L1/2 showed aggravated atherosclerosis, mediated by increased effector T-cell responses. Lympophcyte activation gene-3 is upregulated on activated Tregs and can bind with higher affinity than CD4 to major histocompatibility complex-II on the DC, which suppresses maturation and the immune-stimulatory capacity of DCs; however, its importance in atherosclerosis remains to be determined.

**Inhibition of Macrophage Inflammation and Foam Cell Formation**

Tregs can also exert atheroprotective effects by promoting differentiation of mouse M1 macrophages toward an anti-inflammatory M2 macrophage. Coculture of Tregs with monocytes from healthy individuals induced an M2 phenotype as illustrated by the surface expression of CD206 (mannose receptor), CD163 (hemoglobin scavenger receptor), elevated CCL18 (chemokine [C-C motif] ligand) production, and phagocytic activity. Moreover, in response to...
by lipopolysaccharide, these Treg-treated monocytes strongly reduced secretion of proinflammatory cytokines.

The transition of macrophages into foam cells is a hallmark of atherosclerosis. It has been shown that Tregs can impede this process by inhibiting lipid accumulation in peritoneal macrophages via the downregulation of scavenger receptor class A (SR-A) and CD36, but Tregs do not affect reverse cholesterol transport. In addition, lesional Tregs may suppress MCP-1 expression and monocyte recruitment into plaques, thereby reducing foam cell macrophage accumulation.

Enhancing Lesion Stability

Because M2 macrophages promote collagen synthesis, Tregs contribute to lesion stability by inducing M2 macrophages. In line with this finding, expansion of Tregs enhanced lesion stabilization in a regression model of atherosclerosis. In addition, Tregs dose dependently increase lesion stability, as measured by decreased macrophage and lipid content and increased smooth muscle cell and collagen content, and lower the incidence of lesion disruption in ApoE−/− mice. Moreover, Tregs inhibited the expression of inflammatory cytokines and the matrix metalloproteinase-2 and matrix metalloproteinase-9 and enhanced P4Hτ1 expression in atherosclerotic lesions.

Treg Effects on Cholesterol Metabolism

Overexpression of IL-10, a hallmark cytokine of Tregs, reduces very LDL and LDL levels in serum of LDLr−/− mice. Recent studies have revealed a direct role for Tregs in cholesterol metabolism because depletion of Tregs using DEREG mice significantly increases atherosclerosis associated with a 1.7-fold increase in plasma cholesterol levels. More specifically, very LDL levels were increased because the clearance of very LDL and chylomicron remnants was inhibited in the absence of Tregs. They found reduced expression of sortilin-1 in the liver and increased plasma enzyme activity of lipoprotein lipase, hepatic lipase, and phospholipid transfer protein in Treg-depleted mice. In addition, Treg expansion in a regression model of atherosclerosis significantly reduced cholesterol levels when compared with control mice.

Suppression of Endothelial Activation

Interestingly, adaptive Foxp3+ Tregs have also been shown to suppress tumor necrosis factor-α and IL-1β–mediated endothelial selectin expression and subsequently reduced leukocyte adhesiveness. In vivo, these adaptive Tregs inhibit acute inflammation in a peritonitis model via adherence to inflamed endothelium and secretion of inhibitory cytokines.

Treg Cell–Based Therapy

The usage of Tregs as a therapeutic agent shows great potential in the treatment of atherosclerosis. An early study showed that an adoptive transfer of CD4+CD25+ T cells in mice causes a reduction in atherosclerotic lesion development, and research is nowadays focused on the development of Foxp3+ T cells, either ex vivo or via expansion in vivo.

Adoptive Transfer of Tregs

A possible strategy to use Tregs for therapy is an adoptive transfer of ex vivo expanded Tregs. This procedure will require substantial numbers of Tregs, which can be achieved by in vivo isolation of naive or Foxp3+CD4+ T cells from peripheral blood and subsequent ex vivo Treg induction and expansion to obtain large numbers for therapy using appropriate cytokine mixtures. Trials on adoptive Treg therapy for Graft Versus Host Disease, transplantation, and autoimmunity have used varying numbers of Tregs, ranging from 5x10⁶ to 2.6x10⁹, injected once or twice. Treg-based therapy has proven to be effective, and no significant adverse effects have been reported. Most studies used polyclonal autologous Treg preparations although alloantigen-stimulated preparations have been used for allograft tolerance. There are insufficient data to assess how long the effects of transferred Tregs last. More data from these ongoing trials will be needed to inform the design of trials for atherosclerotic disease.

Induction of Tregs In Vivo

Alternatively, Tregs can be expanded in vivo. This can be achieved by targeting Treg via administration of an IL-2/anti–IL-2 immune complex. Administration of IL-2/anti–IL-2 complex to Western-type diet fed LDLr−/− mice significantly expanded IL-10 producing Tregs ≤10-fold in the circulation and several (lymphoid) organs. This expansion of Tregs potently suppressed effector T cells and reduced initial atherosclerotic lesion formation, whereas in combination with a vigorous lowering of blood lipid levels, it enhanced lesion stability in LDLr−/− mice with pre-existing lesions. Future research should reveal whether administration of this IL-2 complex would also be beneficial in patients with cardiovascular disease. Another frequently used method to induce Tregs is administration of antigens via a tolerogenic route. Administration of atherosclerosis relevant antigens, such as oxLDL, HSP60, β2-glycoprotein I, and ApoB100 peptide, via oral, nasal, and subcutaneous routes has been shown to suppress atherosclerosis in mice by increasing antigen-specific Tregs through the induction of tolerogenic DCs. Recent approaches also combine several peptide antigens derived from ApoB100 and HSP60 to induce a variety of antigen-specific Tregs. The induction of Tregs may be further improved by combining a relevant antigen with the cholera toxin B, as shown by the atheroprotective effect of a construct consisting of an apoB100 peptide combined with CTB. One may speculate that these approaches will in the near future form the basis for First-In-Humans clinical trials.

Induction of Tolerogenic DCs

A final approach to enhance the function and induction of Tregs may be to enhance the tolerogenic function of DCs. Oral and nasal routes of administration of antigens rely on the interaction of these antigens with tolerogenic DCs and subsequent induction of Tregs. This approach can be mimicked in vitro by incubating DCs with atherosclerosis-related antigens in the presence of IL-10 to induce a tolerogenic DC phenotype. Subsequent adoptive transfer of these antigen-loaded
DCs induces an atheroprotective effect via the induction of Tregs. Various other methods for inducing a tolerogenic DC phenotype have been described and are being applied to atherosclerosis studies in mice.

**Polyclonal Tregs Versus Antigen-Specific Tregs**

For potential Treg therapies based on adoptive transfers of Tregs, 3 kinds of Tregs can be used; general Tregs expanded ex vivo, antigen-specific Tregs expanded ex vivo, or induced Tregs differentiated from naïve CD4+ T cells in the presence of IL-2, αCD3/CD28, TGF-β both with or without retinoic acid (possibly antigen specific). The obvious advantage of induced Tregs is that a large number of cells can be produced with relative ease. However, maintaining stability of induced Tregs is a major complicating factor, and Blazar argued that induced Tregs have disappeared 14 days after infusion, which limits long-term effects. Nonspecific Tregs can be isolated from peripheral blood of patients and ex vivo expanded for maximally 3 rounds before they also lose their phenotype. The use of antigen-specific Tregs could be extremely effective in treating atherosclerosis. In addition to studies using oral or nasal administration of HSP60, Yang et al showed that adoptive transfer of HSP60-specific CD4<sup>+</sup>CD25<sup>+</sup> cells via in vitro induction through HSP60-loaded DCs inhibits atherosclerosis formation. Although several candidates of atherosclerosis-specific antigens, such as oxLDL, HSP60, and ApoB100, have been investigated, to date the dominant relevant antigens that are recognized by proatherogenic T cells are not known. This complicates the approach of using atheroantigen-specific Tregs. Moreover, antigen-specific Tregs studied in autoimmune diseases have a low frequency and, therefore, treatment with antigen-specific Tregs would also require repeated rounds of in vitro expansion to achieve a sufficient amount of cells and this often results in phenotype loss. Possibly Treg antigen-specificity can be achieved in vivo by, for example, combining oral tolerance induction against, eg, oxLDL with administration of low-dose IL-2 or an IL-2/anti-IL-2 complex that potently expands Tregs.

**Summary and Outlook**

Tregs are important regulators of immune responses and may hold great potential to be used as a therapeutic in atherosclerosis because enhanced Treg numbers are associated in experimental and by inducing tolerance induction against atherosclerosis-specific antigens leading to Tregs that inhibit atherosclerosis. It can be anticipated that improvement of these experimental therapies by enhancing the tolerogenic capacity of the antigen and enhancing the tolerogenic function of the DCs during tolerance induction will lead to a clinical application and a new therapy for atherosclerosis.

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**Disclosures**

None.

**References**


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