Review

Regulatory T Cells in Atherogenesis

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Atherosclerosis is believed to be an inflammatory condition of the arterial wall. It has become apparent that various types of cells of innate and adaptive immunity participate in atherogenesis. T cells are of particular interest because they mediate pathogenic immune responses involved in the acceleration of atherosclerosis. Recent studies from several independent groups indicated that subsets of regulatory T cells (Tregs) actively mediate immunologic tolerance and inhibit atherosclerosis development or progression through the down-regulation of effector T-cell responses. It is likely that there is an imbalance between pathogenic effector T cells and Tregs under atherosclerotic conditions. Recent evidence suggests that in addition to the thymus, gut-associated lymphoid tissues are the main sites for the generation of several subsets of peripherally inducible Tregs. This indicates that intervention in the gut environment to promote an endogenous regulatory immune response may serve as a possible therapeutic approach to suppress atherosclerotic diseases. In this review, we discuss not only the possible role of Tregs in the prevention of atherosclerosis, but also promising strategies to prevent or cure atherosclerotic diseases by promoting an endogenous regulatory immune response, particularly by oral immune modulation.


Key words; Atherosclerosis, Immune system, Inflammation, Regulatory T cells, Oral immune modulation

Introduction

Atherosclerosis is an inflammatory condition of the arterial wall, which is regulated by cells of innate and adaptive immunity\(^1-4\). This causes unstable plaques and thrombotic occlusion of the artery, leading to severe clinical events, including acute coronary syndrome (ACS) and stroke, which account for approximately a quarter of deaths in Japan.

Both animal and human studies have shown that hypercholesterolemia is associated with the accumulation and retention of low-density lipoprotein (LDL) in the arterial intima, which is considered to be an initial inflammatory response in the artery wall in atherogenesis. LDL is prone to oxidative and enzymatic modifications, leading to the up-regulation of leukocyte adhesion molecules in endothelial cells. Through the activated endothelium, monocytes enter the subendothelial space or intima and differentiate into macrophages. These processes are critical for the initiation of atherosclerosis both in humans and animals. Modified LDL particles are taken up by scavenger receptors on macrophages, which results in intracellular cholesterol accumulation and transformation of monocytes into foam cells. These differentiated macrophages also express toll-like receptors, which bind lipopolysaccharide, oxidized LDL (oxLDL), heat-shock protein (HSP) 65/60, and other ligands, inducing their activation and leading to the production of pro-inflammatory molecules. Numerous studies of human atherosclerotic lesions have provided important clues to the pathogenesis of atherosclerosis, including immune cells. Because of the limited elucidation of the molecular mechanisms of atherogenesis in human studies, two strains of genetically altered mice, apolipoprotein E-deficient (\(\text{ApoE}^{-/-}\)) and low-density lipoprotein receptor-deficient (\(\text{LDLR}^{-/-}\)) mice, have been used for many studies.

Atherosclerotic lesions in human and \(\text{ApoE}^{-/-}\) or
Th2-mediated immune responses in atherosclerosis remains controversial depending on the produced cytokines or animal models used and needs further investigations. Th17 cells, which have been recently identified, play a highly pathogenic role by producing inflammatory cytokine interleukin (IL)-17. There are several isoforms of IL-17, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Th17 cells are known to produce IL-17A and IL-17F, and the former is reported to play the most critical role in mediating autoimmune diseases. Experimental evidence suggests that pathogenic immune responses caused by this subset massively contribute to the progression of several autoimmune diseases in mice. Several studies suggest a proatherogenic role for Th17 cells in hyperlipidemic mice, whereas experimental data from another group demonstrated a protective role of IL-17 in atherosclerosis. A recent study used a genetic deletion of IL-17A and showed that IL-17A is involved in systemic and vascular inflammation, but did not alter plaque burden in experimental atherosclerosis. Thus, the role of Th17 cells in atherosclerotic diseases is still controversial.

Accumulating evidence suggests that several subsets of regulatory T cells (Tregs), which have been shown to maintain immunological unresponsiveness to self-antigens and suppress excessive immune responses, play a crucial role in maintaining immune balance in atherosclerosis. LDLR−/− mice have both CD4+ and CD8+ T cells, and the former population is predominant. After antigen presentation by antigen-presenting cells (APCs) such as macrophages or dendritic cells (DCs) to naïve T cells, adaptive T-cell-mediated immune responses occur. CD4+ T-cell clones derived from atherosclerotic plaques, which are restricted by major histocompatibility complex (MHC) class II molecules, have been shown to recognize oxLDL, HSP-60, and other antigens from microorganisms such as Chlamydia pneumonia, which are supposed to be candidate self-antigens for atherogenesis. Although there are many CD8+ T cells in atherosclerotic lesions, which recognize viral antigens presented on MHC class I molecules, little is known about their roles in atherogenesis. It is now well known that in addition to macrophage activation, CD4+ T-cell-mediated pathogenic immune responses play an important role in atherogenesis in humans and mice. Depending on stimulation by several cytokines or regulation by specific transcription factors, naïve CD4+ T cells can differentiate into T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17) lineage. Regulatory T cells (Tregs) suppress polyclonal T cell activation and the differentiation of naïve T cells into Th1, Th2, and Th17 lineage and down-regulate APC function.
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kines in atherosclerosis, IL-10 and transforming growth factor (TGF)-β, play a key role in the suppressive function or differentiation of several subsets of Tregs, suggesting that the protective effects of these responses23), inhibit atherosclerosis development or progression through the down-regulation of activated T-cell responses (Fig. 2A and 2B)24-27. It has been demonstrated that two major anti-inflammatory cytokines in atherosclerosis, IL-10 and transforming growth factor (TGF)-β, play a key role in the suppressive function or differentiation of several subsets of Tregs, suggesting that the protective effects of these

Fig. 2. Several Subsets of Tregs May Play a Protective Role in Atherogenesis: a Hypothesis.

Several subsets of Tregs can differentiate from naïve T cells in gut-associated lymphoid tissues, as well as in the thymus. A, CD25+Foxp3+ Tregs may inhibit APC function, possibly in a cell-contact dependent manner such as down-regulation of CD80/86 expression on APCs by CTLA-4 or through the production of TGF-β, which may lead to anergy or apoptosis of effector T cells, suppression of their responses, and attenuation of atherosclerosis development. APC, antigen-presenting cell; Foxp3, forkhead box P3. B, Peripherally generated Tregs such as Tr1 cells and CD4+LAP+ Tregs down-regulate effector T cell and macrophage function through the production of IL-10 or TGF-β, respectively, leading to reduction of atherosclerosis. LAP, latency-associated peptide.
cytokines in atherogenesis are partly due to the modulation of Treg functions\textsuperscript{28, 29}. Deficiency of Treg function or development has been shown to be a primary cause of some autoimmune diseases in humans and animals\textsuperscript{30-32}. Similarly, the balance between effector T cells and Tregs may be important for the control of atherosclerotic diseases\textsuperscript{28}. In consideration of this, we believe that promotion of an endogenous regulatory immune response has therapeutic potential to suppress atherosclerotic disease.

In this review, we discuss the current knowledge of Treg generation and function, the proposed specific roles of Tregs in atherosclerosis (summarized in Table 1), and the strategies to prevent or cure atherosclerotic diseases by promoting an endogenous regulatory immune response, particularly by oral immune modulation.

**Generation and Function of Regulatory T Cells**

Sakaguchi et al. first reported that naturally arising Tregs constitutively express high levels of CD25 (IL-2 receptor α-chain) molecule, and that depletion of the CD4\textsuperscript{+}CD25\textsuperscript{+} T cell population, which constitutes 5-10% of peripheral CD4\textsuperscript{+} T cells in normal naïve mice and humans, elicits autoimmune similar to its human counterparts\textsuperscript{30}. Firm evidence has shown that Tregs, which are produced by normal thymus, play a crucial role in dominant suppression of pathogenic immune responses and maintenance of self-tolerance and immune homeostasis\textsuperscript{33-35}. In addition, natural Tregs specifically express transcription factor Foxp3 (forkhead box P3), which is essential for their development and function and is currently the most reliable molecular marker for them\textsuperscript{33-35}. Mutations of human gene FOXP3 cause the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), in which autoimmune diseases such as type 1 diabetes, allergy and inflammatory bowel disease can be observed at high frequency\textsuperscript{32}. Foxp3\textsuperscript{+} natural Tregs suppress the proliferation of tissue-specific autoimmune T cells and their differentiation into Th1, Th2, and Th17 lineage in vivo. Moreover, they inhibit polyclonal T cell activation and the function of other types of lymphoid cells, including B cells, macrophages and DCs (Fig. 1)\textsuperscript{36}.

Several lines of evidence have suggested that Treg-mediated suppression includes multiple mechanisms, such as secretion of immunosuppressive soluble factors (IL-10, IL-35, TGF-β, granzyme, and perforin); deprivation of IL-2 by Tregs, which is necessary for the maintenance of responder T cells; cell-contact-dependent suppression, and functional modification or killing of APCs, which may operate synergistically and in a complementary manner\textsuperscript{36-38}.

Recent evidence has demonstrated that Foxp3\textsuperscript{+} Tregs can differentiate from naïve T cells in the periphery under certain conditions, as well as in the thymus\textsuperscript{39, 40}. Peripherally generated Tregs are called adaptive or inducible Tregs (iTreg) and are reported to have similar immunological properties with thymus-derived Tregs. In addition to Foxp3\textsuperscript{+} iTregs, several kinds of iTregs, including T regulatory type 1 (Tr1) or T helper type 3 (Th3) cells, have been reported. Tr1 cells without Foxp3 expression were demonstrated in Peyer’s patches of mice fed a low dose of β-lactoglobulin and produced immunomodulatory cytokine IL-10\textsuperscript{41}. Th3 cells are induced in mucosal tissues during oral tolerance and produce high amounts of TGF-β in an antigen-specific manner\textsuperscript{41, 42}. Also, it is reported that some Th3 cells are TGF-β-induced Foxp3\textsuperscript{+} iTregs. Recently, CD4\textsuperscript{+}LAP\textsuperscript{+} (latency-associated peptide) Treg has been identified as a new subset of Tregs that suppresses autoimmune diseases in mice\textsuperscript{43-47}. This Treg population is linked to inflammatory bowel disease in mice\textsuperscript{48}. Recent studies from other groups and our group have consistently shown that oral or nasal anti-CD3 antibody is biologically active and induces CD4\textsuperscript{+}LAP\textsuperscript{+} Tregs that suppress experimental autoimmune encephalitis (EAE)\textsuperscript{43}, autoimmune diabetes\textsuperscript{44}, lupus\textsuperscript{45, 46}, collagen-induced arthritis\textsuperscript{47}, type 2 diabetes\textsuperscript{49}, and atherosclerosis\textsuperscript{27} in a TGF-β-dependent fashion (Fig. 2B).

**Protective Roles of Treg Cells in Experimental Atherosclerosis**

Based on the role of the immunoregulatory functions of Tregs described above, it can be speculated that antigen-specific Tregs can be produced in response to a number of altered self antigens including LDL, oxidized epitopes on apoptotic cells and HSP 65/60 to dampen inflammation within the atherosclerotic plaques\textsuperscript{8, 50}. However, at the same time, much more activated effector T cells, which have been presented such self-antigens by APCs in the plaques, can be seen in atherosclerotic lesions and regional lymph nodes. Under atherosclerotic conditions, there might be an imbalance between effector T cell and Treg responses both in the atherosclerotic lesions and in systemic lymphoid organs\textsuperscript{28}. Tregs control pathological immune responses in an antigen-specific manner. Importantly, once they are activated after antigen presentation by APCs, they exert suppressive function regardless of their antigen specificity\textsuperscript{29}; therefore, in addition to antigen-specific Tregs, polyclonal activated Tregs may be also important for the suppression of pathogenic immune responses in atherosclerosis,
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was associated with a significant increase of atherosclerotic lesions. Moreover, they also demonstrated that transfer of CD28−/− splenocytes to ApoE−/−Rag-2−/− mice with lymphocyte deficiency accelerates atherosclerosis development compared with the transfer of wild-type splenocytes.

Adoptive transfer of natural CD4+CD25+ Tregs reduces atherosclerosis development in ApoE−/− mice. Peripherally generated Tregs such as Tr1 cells play a protective role in the development of atherosclerosis in ApoE−/− mice through the production of IL-10. Oral tolerance induction to oxidized LDL or heat-shock protein-60 in LDLR−/− mice attenuates atherosclerosis in both early and advanced stages, in association with increased numbers of CD25+Foxp3+ Tregs in the MLNs and spleen, and increased expression of Treg-associated molecules in atherosclerotic lesions.

Table 1. Current Knowledge of Possible Roles of Tregs in Atherogenesis

<table>
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<th>Description</th>
<th>References</th>
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<tr>
<td>Treg deficiency due to reconstitution with CD80−/−/CD86−/− or CD28−/− bone marrow in LDLR−/− mice is associated with the significant increase of atherosclerotic lesions.</td>
<td>24</td>
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<tr>
<td>Transfer of CD28−/− splenocytes (Treg deficiency) to ApoE−/−Rag-2−/− mice with lymphocyte deficiency accelerates atherosclerosis development compared with the transfer of wild-type splenocytes.</td>
<td>24</td>
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<tr>
<td>Adoptive transfer of natural CD4+CD25+ Tregs reduces atherosclerosis development in ApoE−/− mice.</td>
<td>26</td>
</tr>
<tr>
<td>Peripherally generated Tregs such as Tr1 cells play a protective role in the development of atherosclerosis in ApoE−/− mice through the production of IL-10.</td>
<td>61</td>
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<tr>
<td>Oral tolerance induction to oxidized LDL or heat-shock protein-60 in LDLR−/− mice attenuates atherosclerosis in both early and advanced stages, in association with increased numbers of CD25+Foxp3+ Tregs in the MLNs and spleen, and increased expression of Treg-associated molecules in atherosclerotic lesions.</td>
<td>62, 64</td>
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<td>Intravenously administered FcR-non-binding CD3-specific antibody induces Foxp3+ Tregs producing TGF-β and inhibits atherosclerosis development and progression in LDLR−/− mice.</td>
<td>73</td>
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<tr>
<td>Oral anti-CD3 antibody treatment induces CD4+LAP+ Tregs and CD4+CD25+Foxp3+ Tregs in the MLNs and spleen and inhibits atherosclerotic plaque formation in ApoE−/− mice through a TGF-β-dependent mechanism.</td>
<td>27</td>
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What Is Unknown?

Previous studies do not provide direct evidence of the atheroprotective role of endogenous Foxp3+ Tregs, and further studies with more sophisticated methods will be needed to clarify this issue. Although several mechanisms of Treg-mediated immune suppression have been proposed, the core mechanism of Treg-mediated regulation in atherosclerosis remains unclear.

It remains unclear whether, in addition to polyclonal activated Tregs, antigen-specific Tregs are important for the suppression of pathogenic immune responses in atherosclerosis, because we still do not know which antigen is the most critical for disease initiation and progression.

Although recent studies suggest that Tregs migrate to atherosclerotic lesions, where antigen presentation may occur as well as in lymphoid organs, the actual roles of intraplaque Tregs in atherogenesis have not been clarified.

In the clinical setting, it is unknown whether impaired function or reduced numbers of Tregs may contribute to the progression of atherosclerotic diseases in humans.

Foxp3, forkhead box P3; MLNs, mesenteric lymph nodes.

where we still do not know which antigen is the most critical for disease initiation and progression.

Recently, Ait-Oufella H et al. demonstrated that endogenous CD4+CD25+ natural Tregs play a protective role in atherogenesis in mice. 24 It is known that the generation and homeostasis of Tregs needs interactions between the costimulatory molecules CD80/CD86 and CD28. 51) The authors investigated the effect of CD80/CD86 or CD28 deficiency on the development of atherosclerosis in LDLR−/− mice using the irradiation/bone marrow transplantation model. They found that Treg deficiency due to reconstitution with CD80−/−/CD86−/− or CD28−/− bone marrow was associated with a significant increase of atherosclerotic lesions. Moreover, they also demonstrated that transfer of CD28−/− splenocytes to ApoE−/−Rag-2−/− mice, which are deficient in lymphocytes, accelerated atherosclerosis development compared with the transfer of wild-type splenocytes. Another group supports this idea, showing that adoptive transfer of natural CD4+CD25+ Tregs reduces atherosclerosis development in ApoE−/− mice. 26 Collectively, these data suggest a crucial role for endogenous Tregs in the control of atherosclerosis (Fig.2A). However, these in vivo studies described above do not provide direct evidence for the atheroprotective role of Tregs, because Ait-
Oufella H et al. used a bone marrow transplantation model with CD80$^{-/-}$/CD86$^{-/-}$ or CD28$^{-/-}$ bone marrow, where deficiency of CD80/CD86 or CD28 could affect the function of not only Tregs but also other effector T cells$^{39}$. In addition, Tregs are reported to have constitutively high expression of CD25, whereas this molecule is upregulated upon activation of effector T cells$^{52}$, suggesting the possibility that the previous studies described above could not evaluate the exact effects of Treg deficiency on the development of atherosclerosis. It is now well known that natural Tregs specifically express Foxp3, which is a master regulator of T reg development and function and therefore is currently the most reliable molecular marker for natural Tregs$^{23, 33}$. The question of whether Foxp3$^+$ Tregs play a critical role in the suppression of atherogenesis will be directly addressed by analyzing bacterial artificial chromosome transgenic mice expressing a diphtheria toxin receptor (DTR) under the control of the foxp3 gene locus$^{53}$ or foxp3$^{DTR}$ knock-in mice$^{54}$, allowing selective and efficient depletion of Foxp3$^+$ Tregs by diphtheria toxin injection.

Tregs may play a protective role against vascular dysfunction not only in hypercholesterolemia but also in hypertension. A recent study demonstrated that adoptive transfer of Tregs had a protective effect on the cardiac damage caused by angiotensin II, a major mediator of hypertension and cardiac damage, although it had no impact on angiotensin II–induced arterial hypertension$^{55}$. Notably, recent accumulating evidence clearly suggested that the pathogenic T cell response contributes to hypertensive disease and vascular dysfunction in mouse models$^{56}$. Thus, further studies are needed to identify the role for Tregs in the prevention of hypertension and consequent reduction of atherosclerosis.

**Strategies for Enhancing Treg-Mediated Immune Regulation in Atherosclerosis**

Recent studies have established that T-cell costimulatory or coinhibitory pathways may contribute to naïve T cell activation and enhancement of effector and memory T cell responses or negative regulation of T-cell responses, respectively. It has now become evident that these pathways play crucial roles not only in modulating naïve and effector T cell function but also in Treg generation and function, although the precise roles of these pathways in Treg function remain to be determined$^{51}$. A recent study documented that inducible costimulatory molecule (ICOS), which is a CD28 family member involved in the costimulatory pathway, plays a pivotal role in the regulation of atherosclerosis through the modulation of Treg responses$^{35}$. One of the important coinhibitory molecules is cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which is a CD28 family member expressed only on activated T cells including Foxp3$^+$ Tregs. B7 family molecules, such as B7-1 (CD80) and B7-2 (CD86), which are mainly expressed on macrophages and DCs, bind to CTLA-4, possibly resulting in suppression of T-cell activation, although the precise molecular mechanisms for this negative regulation are still not well-defined$^{57}$. Interestingly, CTLA-4 is induced on conventional T cells only after activation, whereas Foxp3$^+$ Tregs constitutively express this molecule. Moreover, CTLA-4-dependent suppression is supposed to be a key mechanism for Treg-mediated suppression$^{58, 59}$. One mechanism for CTLA-4-dependent suppression is reported to be down-modulation of APC function by reducing their CD80 or CD86 expression$^{58, 59}$. Taken together, CTLA-4 is considered to be a possible therapeutic target for atherosclerotic disease via promotion of Treg suppressor function, as well as down-regulation of effector T cell function. Thus, blockade of costimulatory pathways or enhancement of coinhibitory pathways could be effective treatments for atherosclerotic disease through modulation of Treg function as well as effector T cell function$^{60}$; however, it is possible that blockade of the costimulatory pathway may impair both effector T cell and Treg function. The effects of the modulation of costimulatory or coinhibitory pathways on each T cell subset should be clearly defined before this approach is applied to clinical use.

In addition to natural Foxp3$^+$ Tregs produced in the thymus, it is reported that peripherally generated Tregs such as Tr1 and Th3 cells play a protective role in the development of atherosclerosis in mice through the production of IL-10 or TGF-β, respectively (Fig. 2B)$^{61, 62}$. Mucosal tolerance induction has been shown to inhibit various autoimmune diseases in mice, partly through the induction of several types of adaptive Tregs$^{63}$; therefore, this implies that mucosal tolerance induction might be an effective way to treat atherosclerosis. For example, it was reported that oral tolerance induction to oxLDL or HSP-60 in LDLR$^{-/-}$ mice attenuated atherosclerosis in both early and advanced stages, in association with increased numbers of CD25$^+$ Foxp3$^+$ Tregs, increased production of TGF-β or IL-10 in the mesenteric lymph nodes (MLNs) and spleen, and increased expression of Treg-associated molecules in atherosclerotic lesions$^{62, 64}$. Recent evidence suggests that modulation of Treg-mediated immune responses, mainly via increasing their number, represents a novel therapeutic approach against atherosclerosis. Most previous stud-
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Table 2. Blood Treg Count in Patients with Coronary Artery Disease

<table>
<thead>
<tr>
<th>Reference</th>
<th>Treg definition</th>
<th>SA</th>
<th>MI</th>
<th>UA</th>
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<tbody>
<tr>
<td>Mor et al. 2006</td>
<td>CD4(^+)CD25(^{high})</td>
<td>not changed</td>
<td>decreased</td>
<td>not determined</td>
</tr>
<tr>
<td>Ammirati et al. 2010</td>
<td>CD4(^+)CD25(^{high})CD127(^{low})</td>
<td>not changed</td>
<td>increased</td>
<td>decreased</td>
</tr>
</tbody>
</table>

SA, stable angina; MI, myocardial infarction; UA, unstable angina.

ies have focused on searching for methods to increase the number of Tregs, whereas strategies for enhancing their suppressive function have not been extensively examined so far. As described before, in the fields of immunology, mechanisms of Treg-mediated suppression have been actively investigated and have attracted much attention\(^ {36-38} \). Although several mechanisms have been proposed, it remains unclear to what extent each mechanism contributes to suppression. It is possible that the dominant mechanism may vary depending on the situation or subset of Tregs. It is tempting to investigate the core mechanism of Treg-mediated immune suppression in atherosclerosis if it exists.

Interestingly, a recent in vitro study using a co-culture system demonstrated that human CD25\(^{high}\)Foxp3\(^{+}\) Tregs may inhibit proinflammatory activation in human umbilical vein endothelial cells via the cell-contact-dependent pathway or production of soluble factors such as IL-10 and TGF-\(\beta\)\(^ {65} \). The same group also reported that co-culture with mouse Tregs markedly suppressed macrophage foam-cell formation through the same pathways\(^ {66} \). Since it is well known that endothelial dysfunction and macrophage foam-cell formation play key roles in the initiation and progression of various forms of atherosclerotic diseases, these results support the idea that Tregs are closely involved in the protection against atherosclerosis. The elucidation of core Treg-mediated suppressive mechanisms will contribute to the development of more effective therapeutic tools for restricting atherosclerosis based on the modulation of Treg-mediated immune regulation.

Role of Tregs in Clinical Atherosclerotic Disease

It has now become evident that Tregs play protective roles in atherogenesis in several experimental mouse models. Before translating this idea into the clinical setting, to ascertain whether impaired function or reduced numbers of Tregs may contribute to the progression of atherosclerotic diseases in humans, extensive clinical studies are required. Mor A et al. demonstrated that patients with ACS exhibited a reduced number and compromised functional properties of CD25\(^{+}\) Tregs\(^ {67} \). In contrast, there is a recent clinical report showing that Treg levels were significantly increased in the periphery of ST-elevation ACS patients, whereas their numbers were significantly decreased in non-ST-elevation ACS patients (Table 2)\(^ {68} \). This study also demonstrated that circulating Treg levels correlated neither with the intima-media thickness of the common carotid artery nor with its progression, indicating that peripheral Treg levels may not be a useful marker for evaluation of the severity of atherosclerosis. Thus, the role of Tregs in atherosclerotic diseases, including ACS, remains unclear and needs further clarification.

Assuming that Tregs migrate into regional lymph nodes, where self-antigens are presented, are activated by DCs, and also migrate into inflamed tissues to dampen local inflammation, it is possible that Treg numbers in the peripheral blood may not reflect their systemic status. Where can Tregs be found and work in the body under physiological and pathological conditions? A recent report demonstrated that Tregs in the circulation infiltrate the periphery, traffic to draining lymph nodes, and then recirculate back to the inflamed tissues to suppress pathogenic immune responses\(^ {69} \). Therefore, the dynamics of Treg trafficking and localization under atherosclerotic condition should be examined to establish the clinical importance of Tregs for the prevention of atherosclerotic diseases.

CD4\(^{+}\)LAP\(^{+}\) Tregs and Oral Anti-CD3 Antibody Therapy in Autoimmunity

Administration of FcR-non-binding anti-CD3 monoclonal antibody has been shown to induce long-lasting immune tolerance through the induction of TGF-\(\beta\)-producing CD4\(^{+}\)CD25\(^{+}\) Tregs and to be an effective treatment for autoimmune diabetes in mice and humans\(^ {70,71} \), and acute transplant rejection in humans\(^ {72} \). Notably, it has been shown that intravenously administered FcR-non-binding CD3-specific antibody induces a regulatory immune response and inhibits atherosclerosis development and progression\(^ {73} \). Interestingly, as described before, recent studies have revealed that oral or nasal anti-CD3 antibody with or without the Fc portion is biologically active and induces CD4\(^{+}\)LAP\(^{+}\) Tregs that suppress several autoimmune diseases in mice, and that this therapy would not be expected to have side effects regardless of the presence of Fc portion\(^ {43-47} \). Based on the results provided by animal experiments, murine anti-CD3
monoclonal antibody (OKT3) was orally administered to healthy human subjects. Oral OKT3 administration exerted many favorable immunologic effects, such as enhanced T cell proliferation, suppressed Th1 and Th17 responses, increased TGF-β and IL-10 expression, and decreased IL-23 and IL-6 expression in DCs. The findings from this report demonstrated that oral anti-CD3 monoclonal antibody is safe and biologically active not only in mice but also in humans. Based on the efficacy and safety of this antibody treatment in several mouse studies and human healthy volunteers, a clinical study using oral OKT3 has been performed for the treatment of human autoimmune diseases, including nonalcoholic steatohepatitis and type 2 diabetes, and showed beneficial effects in these patients.

Given this background, we hypothesized that the induction of CD4+ LAP+ Tregs by oral anti-CD3 antibody treatment would inhibit atherosclerosis in ApoE−/− mice.

**Oral Immune Modulation by Anti-CD3 Antibody Could Be Used as a Promising Therapeutic Approach for Atherosclerosis**

We fed 6-week-old male ApoE−/− mice 5 μg hamster anti-CD3-specific antibody or 5 μg hamster IgG by gastric intubation once a day for 5 consecutive days. Two days after the last feeding of anti-CD3 antibody, we found a significant increase in LAP+ Tregs and Foxp3+ Tregs in the CD4+ T cell population in MLNs of anti-CD3-treated mice. We next analyzed the cytokine and chemokine production profile from spleen lymphocytes on day 7 after treatment, which showed suppressed Th1 and Th2 immune responses in anti-CD3-treated mice. We also observed that TGF-β is the only cytokine with anti-inflammatory properties that is enhanced by anti-CD3 antibody treatment. Six-week-old ApoE−/− mice on a standard diet were given anti-CD3 antibody or control hamster IgG orally on 5 consecutive days, and atherosclerosis was assessed at 16 weeks of age. Oral administration of anti-CD3 antibody significantly reduced atherosclerotic lesion formation and the accumulation of macrophages and CD4+ T cells in plaques compared with controls. Neutralization of TGF-β in vivo using anti-TGF-β neutralizing antibody abrogated the preventive effect of oral anti-CD3 antibody, suggesting that TGF-β plays a crucial role in the inhibition of atherosclerosis development by oral anti-CD3 antibody. It is likely that CD4+ LAP+ Tregs and CD4+ Foxp3+ Tregs induced by oral anti-CD3 antibody reach mesenteric lymph nodes and other lymphoid organs such as the spleen through the bloodstream, where they may suppress pathogenic immune responses through the production of TGF-β, leading to the reduction of atherosclerotic plaque formation and inflammatory cell recruitment into plaque (Fig. 3).

Recent studies suggest that antigen presentation may occur within atherosclerotic plaque in addition to the lymphoid organs, and that Tregs migrate to atherosclerotic lesions to suppress local immune responses, although the actual roles of intraplaque Tregs in atherogenesis have not been clarified yet. By immunohistochemical study using anti-Foxp3 antibody, we found an increase in the number of natural Tregs within the plaque of anti-CD3-treated mice. These data indicate that oral anti-CD3 antibody treatment could inhibit the migration of effector T cells into the lesion selectively and relatively increase the proportion of natural Tregs in atherosclerotic plaque that might suppress pathogenic T cell immune responses or macrophage activation as well as in the lymphoid organs. It is reported that Tregs express several kinds of homing receptors whose expression plays a role in controlling their migration into inflamed tissues or infectious sites. Although we did not examine the expression patterns of homing receptors in Tregs after oral anti-CD3 antibody administration, it is possible that some alterations of such receptor expression may contribute to Treg accumulation in atherosclerotic plaque. If Treg migration into atherosclerotic lesions is important to suppress local immune reactions, manipulation of Treg trafficking and localization by modulating their homing receptors could be useful to treat atherosclerosis.

It has been reported that immunosuppressive effects by intravenous FcR-binding anti-CD3 antibody may involve mechanisms such as the depletion of pathogenic T cells from the bloodstream or lymphoid organs and modulation of the T-cell receptor on T cells. Different from the case of intravenous FcR-binding anti-CD3 antibody administration, we did not observe T-cell receptor down-modulation or depletion of T cells; however, comparing parenteral FcR-non-binding anti-CD3 antibody with oral FcR-binding or non-binding anti-CD3 antibody, there might be some common mechanisms underlying the suppression of autoimmunity, because both appear to work mainly by inducing TGF-β-producing Tregs rather than eliminating pathogenic T cells. One possibility to understand this similarity is that after oral administration, FcR-binding anti-CD3 antibody might lose its Fc portion in the gut and consequently produce F(ab)2 (FcR-non-binding anti-CD3 antibody), a small amount of which might enter the bloodstream, leading to the induction of Tregs.

TGF-β has a broad spectrum of functions such
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Intriguingly, in addition to an increased number of LAP\(^+\) Tregs, we found a significant increase in Foxp3\(^+\) Tregs in MLNs of anti-CD3-treated mice. This induction of Foxp3\(^+\) Tregs was not observed in previous studies by other groups\(^{43, 44}\). Recent studies suggest that differentiation of CD4\(^+\) Foxp3\(^+\) Tregs from naïve T cells may be facilitated by dendritic cells in gut-associated lymphoid tissue in the presence of TGF-\(\beta\)\(^{39, 40}\) and that retinoic acid produced by dendritic cells plays an important role in their differentiation\(^{82-84}\). In consideration of this, it is highly possible that up-regulated TGF-\(\beta\) secretion in the gut from CD4\(^+\) LAP\(^+\) Tregs could lead to a further increase in the number of not only CD4\(^+\) LAP\(^+\) Tregs but also CD4\(^+\) Foxp3\(^+\) Tregs, as well as enhancement of their suppressive function. In addition to the lymphoid organs, Tregs may migrate to atherosclerotic plaques and dampen local immune responses, resulting in the attenuation of atherosclerotic lesion formation.

as immune suppressive or promoting properties depending on the situation. TGF-\(\beta\) has been attracting much attention as a potent anti-atherosclerotic cytokine\(^{77}\). TGF-\(\beta\), which is produced by many types of cells in atherosclerotic plaques, suppresses pathogenic immune responses such as the recruitment of inflammatory cells into plaques or foam cell formation, and increases collagen biosynthesis\(^{78, 79}\). To investigate the intrinsic function of TGF-\(\beta\) in T cells, several groups have used transgenic approaches to block TGF-\(\beta\) signaling in T cells by expressing dominant negative TGF-\(\beta\) receptors, and found that atherosclerosis-prone mice such as ApoE\(^{-/-}\) mice or LDLR\(^{-/-}\) mice with specific deletion of TGF-\(\beta\) signaling in T cells show markedly accelerated plaque formation and inflammatory cell infiltration, associated with the enhancement of both Th1 and Th2 immune responses\(^{80, 81}\). These studies demonstrate that TGF-\(\beta\) signaling in T cells is indispensable for the regulation of atherosclerotic disease progression. In consideration of this, our data suggest that increased TGF-\(\beta\) production from T cells, possibly from CD4\(^+\) LAP\(^+\) Tregs, may be a key mechanism for the preventive effect of oral anti-CD3 antibody on atherosclerosis through the down-regulation of both Th1 and Th2 immune responses.

Intriguingly, in addition to an increased number of LAP\(^+\) Tregs, we found a significant increase in Foxp3\(^+\) Tregs in MLNs of anti-CD3-treated mice. This induction of Foxp3\(^+\) Tregs was not observed in previous studies by other groups\(^{43, 44}\). Recent studies suggest that differentiation of CD4\(^+\) Foxp3\(^+\) Tregs from naïve T cells may be facilitated by dendritic cells in gut-associated lymphoid tissue in the presence of TGF-\(\beta\)\(^{39, 40}\), and that retinoic acid produced by dendritic cells plays an important role in their differentiation\(^{82-84}\). In consideration of this, it is highly possible that up-regulated TGF-\(\beta\) secretion in the gut from anti-CD3-induced CD4\(^+\) LAP\(^+\) Tregs could lead to the induction of Foxp3\(^+\) Tregs. Interestingly, several recent lines of evidence indicate a reciprocal relationship between Tregs and Th17 cells\(^{85, 86}\). It has been reported that TGF-\(\beta\) stimulation leads to the differentiation of naïve T cells to Th17 cells or CD4\(^+\) Foxp3\(^+\) Tregs in mice in the presence of absence of IL-6, respectively\(^{87}\). Our data indicate a shift from Th17
cells to CD4⁺Foxp3⁺ Tregs in oral anti-CD3-treated mice, which might partially affect the athero-protective effects of oral anti-CD3, although whether Th17 cells accelerates atherosclerosis as well as other autoimmune diseases remains unclear so far.

Mucosal tolerance induction has been shown to inhibit various autoimmune diseases in mice and one of the most important mechanisms for this efficacy is believed to be the induction of various Tregs. In this context, low doses of oral antigen loading may play a critical role in increasing the number of self-antigen-specific Tregs. Similarly, autoimmune diseases have been shown to be suppressed by only low doses of oral anti-CD3 antibody, not by high doses, associated with an increase in LAP⁺ Tregs, although there is no evidence of antigen specificity with oral anti-CD3 antibody. Ochi et al. proposed the idea that, as observed in mucosal tolerance induction, low doses of oral anti-CD3 antibody administration may result in the induction of Tregs by delivering a weak signal to T cells. Based on the idea that the immunological effects of oral anti-CD3 antibody do not seem to be antigen specific, this could be applied for the treatment of a wide range of autoimmunity diseases, including atherosclerosis, in which many candidate antigens can elicit pathogenic immune responses. Although further elucidation of the cellular and molecular mechanisms underlying the induction of various Tregs by oral anti-CD3 antibody administration is needed, we believe that modulation of the Treg response via the intestinal approach may represent an attractive strategy for the treatment and prevention of atherosclerosis.

Concluding Remarks

It has now become evident that several types of Tregs are essential for the regulation of pathogenic T cell immune responses in atherogenesis, at least in mice. Enhancement of an endogenous regulatory immune response could be a novel therapeutic approach against atherosclerotic disease; however, further clinical studies are required to examine whether this could also apply to humans. We are interested in reagents, antibodies or novel methods that selectively affect Treg function or development but have less effect on effector T cell function. In consideration of the clinical applications, this seems to be quite important because therapies that might affect both Treg and effector T cell function could activate excessive immune responses, as observed in intravenous injection of anti-CD3 antibody or super-agonist anti-CD28 antibody. As another approach to enhance endogenous regulatory immune responses, cell-based therapy such as transfer of ex vivo expanded Tregs into patients with autoimmune disease or graft-versus-host disease after bone marrow transplantation is currently under investigation. If cellular therapy is successful for the treatment of such inflammatory diseases, this could be a possible therapeutic approach to treat atherosclerosis in humans; however, several key issues need to be addressed before their clinical use. Such cell therapy may come into clinical use if very pure Tregs can be separated and kept stable after expansion upon antigenic stimulation. Importantly, we should be aware of severe adverse effects, which have not been observed in animal experiments, before applying new methods to promote regulatory immune responses in the clinical setting.

Acknowledgments

We thank Drs Shimon Sakaguchi and Tomoyuki Yamaguchi (Laboratory of Experimental Immunology, WPI Immunology Frontier Research Center, Osaka University) for valuable discussions.

Sources of Funding

This work was supported by a Grant-in Aid for Scientific Research in Japan and Research Grants from Jinsenkai Medical Foundation, Kanae Medical Foundation, Suzuken Memorial Foundation, Uehara Medical Foundation, Medical Research Fund of Hyogo Medical Association, Cardiovascular Research Fund, Hyogo Science and Technology Association, and Takeda Science Foundation.

Disclosures

None.

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