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Regulatory T Cells and Atherosclerosis

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Abstract

Atherosclerosis is a chronic autoimmune inflammatory disease. The involvement of both innate and adaptive immune responses in the pathogenesis of the disease has been well recognized. Tregs are an essential part of the immune system and have indispensable functions in maintaining immune system homeostasis, mediating peripheral tolerance, preventing autoimmune diseases, and suppressing inflammatory and proatherogenic immune response. Tregs carry out their immunosuppressive functions via several mechansims. One of the well-documented suppressive mechanisms of Tregs is the secretion of anti-inflammatory cytokines including IL-10, TGF-β, and IL-35. Studies have found that IL-10 and TGF-β have atheroprotective properties.

In addition, Tregs can suppress the activity of proatherogenic effector T cells, suggesting an atheroprotective role. In fact, fewer Tregs are found in atherogenic *ApoE-/-* mice comparing to wild-type mice, suggesting an uncontrolled balance between weakened Tregs and effector T cells in atherogenesis. Some clinical studies of autoimmune diseases also suggest that decreased Tregs numbers are associated with increased disease activity. The importance of Tregs in many autoimmune diseases and experimental atherosclerosis has been established in *in vivo* and *in vitro* studies. However, the roles of Tregs in atherosclerosis in the clinical setting remains to be further characterized.

Keywords: Regulatory T cells; Vascular inflammation; Atherosclerosis; Immune suppression

Introduction

Cardiovascular disease, a leading cause of mortality worldwide, is mainly caused by atherosclerosis. According to the Center for Disease Control and Prevention, heart disease caused almost 25% of deaths in United States of America [1]. Globally, the World Health Organization in 2008 reported that 30% of all deaths worldwide are related to cardiovascular event. It is estimated by 2030 almost 23.6 million people will die from cardiovascular diseases (CVDs). The American Heart Association defines atherosclerosis as a chronic inflammatory disease with immunological activity of medium and large-sized arteries, which may start in childhood and in some people progresses rapidily in their 30s or in their 50s. It is a chronic and progressive disease with a long asymptomatic phase characterized by the accumulation of white blood cells, cell debris, cholesterol, fatty acids, calcium, and fibrous tissue (plaque or atheromas) in the walls of arteries.

The immunoinflammatory response plays an important role in the development, progression, and complication of atherosclerotic disease [2-4]. Recent evidence suggests that the vascular inflammation in atherosclerosis is modulated by autoimmune responses against self-antigens such as oxidized low-density lipoprotein (ox-LDL) in the vascular wall [3,5,6]. However, the role of the immune system in the pathogenesis of atherosclerosis is more complex than in classic autoimmune diseases such as type 1 diabetes mellitus (T1DM), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [7]. The innate and adaptive immune systems are involved in the development of atherosclerosis, which is well-accepted and is not longer controversial [8,9].

CD4+CD25$^{+/high}$Foxp3+ regulatory T cells (Tregs) are a subpopulation of T cells, specialized in the suppression of pathogenic response from the immune system against self or foreign antigens [9]. The role of Treg cells in the suppression of the proatherogenic T cell reponse has been documented. The role of natural Tregs in experimental atherosclerosis was initially reported by Ait-Oufella et al. in 2006, demonstrating that depletion of peripheral Tregs by anti-CD25 monoclonal antibodies increased atherosclerotic lesion size and vulnerability in atherogenic mouse model apolipoprotein E gene deficient (*ApoE-/-)* mice [10]. Given the importance of Tregs in the suppression of the immune response in many autoimmune diseases and the fact that atherosclerosis is a chronic inflammatory disease with immunologic activity in every stage, Tregs are currently one of the most active topics in cardiovascular research. The suppressive arm of the adaptive immune system, which includes Treg cells, deserves specific attention because it can induce the downregulation or tolerance of the immune system leading to limitation of atherosclerotic plaque progression and complications in an antigen-specific and non-specific manner.

We notice that there are several excellent reviews that discussed Tregs and atherosclerosis [11-13]. However, due to the recent progress in this field [14-16], we believe that there is a need for us to analyze the most recent progress. In this review, in addition to brief discussion on the roles of Tregs in inhibiting other autoimmune diseases, we will focus on the cause-and-effect relationship between reduced/weakened Tregs and atherosclerosis development, the mechanisms of how Tregs modulate the immune response in the atherosclerotic process, and the clinical and therapeutic aspects of Tregs.

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Role of the Immune System in Atherosclerosis Development

For many years, atherosclerosis was described as a disease caused by lipid accumulation in the vessel wall. Currently, after extensive research, atherosclerosis is accepted as a multifactorial chronic inflammatory disease with immunological involvement [17]. The role of the immune system in the development of atherosclerosis has been studied extensively, and a large body of evidence supports an important role of the adaptive immune system in atherosclerosis [18]. Two decades ago, T lymphocytes were found to be present in human atherosclerotic plaque. Since then, research in this field has focused on the functional importance of these cells in atherogenesis. Of note, the immune system not only has an immune effector function in removing foreign antigenic substances and endogenous metabolic stresses but also has an immune tolerance function towards self-antigens and an immunesuppressive function in controlling pathogenic immune responses.

The majority of T lymphocytes in atherosclerotic lesions are $CD4^+$ T-helper cells with phenotypic characteristics of the type 1 proinflammatory T-helper (Th1) subset, which plays an important role in atherogenesis in humans and mice [19]. T cells within atherosclerotic lesions have a preferential expression of a limited number of T cell receptor (TCR) variable gene segments, which suggest a limited group of auto-antigens is responsible for eliciting specific clonal T cell reponse in atherosclerosis [20]. Treg cells are a subpopulation of T cells specialized in the suppression of pathogenic response against self or foreign antigens driven by proatherogenic effector T helper cells [9]. The newly characterized immune paradigm, immune effectors versus immune suppressors, suggests that an imbalance between the pathogenic immunity and weakened regulatory/suppressive immune response plays a major role in the development of atherosclerosis.

The retention of cholesterol in the subendothelial region of the artery is the main pathogenic event that contributes to atherosclerosis developmet [17]. Cholesterol and triglycerides are insoluble in plasma and they are transported by LDL, which is normally associated with apolipoprotein B 100 (APOB-100). The interaction of positively charged APOB with negatively charged proteoglycans leads to the retention of APOB-linked lipoproteins in the arterial vessel wall [21]. These sequestered lipoproteins are susceptible to modification by oxidation, enzymatic cleavage, and aggregation, which become immunogenic and elicit the immune response [22]. For example, Ox-LDL induces an autoimmune response by eliciting anti-ox-LDL IgG generation in atherosclerotic lesions in mouse models and in humans [23]. Oxidation of LDL is responsible for generation of reactive aldehydes and truncated lipids by cleaving the fatty acid double bonds in phospholipids, triglycerides, and cholesterol esters [24]. Modified phospholipids can activate natural killer T cells (NKT), macrophages and endothelial cells [17,25].

The innate immune responses may result from inflammatory signaling through a large protein family known as pathogen-associated molecular pattern recognition receptors (PRR), which includes toll like receptors (TLRs) and nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), etc. [26]. These receptors can recognize endogenous metabolic stress-related danger signals such as cholesterol crystals and ox-LDL, leading to the assembly of the inflammatory sensor caspase-1-activating protein complexes termed inflammasomes. Activation of proinflammatory caspase-1 is essential for maturation and secretion of proinflamamtory cytokines IL-18 and IL-1β. In response to exogenous and endogenous danger signals mediated by PRRs, endothelial cells may become activated. The components of ox-LDL can bind to TLRs and trigger an intracellular signaling cascade and transcription factor nuclear factor-κB (NF-κB) activation, which leads to the upregulated expression of genes encoding for proinflammatory molecules such as cytokines, chemokines, eicosanoids, proteinases, and T cell co-stimulatory molecules. For example, upon activation, endothelial cells upregulate adhesion molecules including vascular cell-adhesion molecule 1 (VCAM-1), E-selectin, and intercellular adhesion molecule 1 (ICAM-1), as well as the secretion of proinflammatory cytokines and chemokines [27]. Of note, activated endothelial cells play essential roles in recruiting monocytes, macrophages, T cells, dendritic cells and other immune cells into the artery wall. In addition, innate immune response generated in macrophages begins when macrophages try to clean up cholesterol deposits in arteries. Upon loaded with cholesterol, macrophages form foam cells and fatty steaks, which drive the initiation of atherosclerosis [17]. Foam cells linen the arterial wall, trigger inflammatory response and become one of the major components of the growing atherosclerotic plaque. Macrophages and dendritic cells generate free oxygen radicals, proteases, complement factors and cytokines, which enhance the innate immune response. A fibrous cap is compose mainly of collagen that covered the lesion, and the shoulder region consists of activated T cells, macrophages, and mast cells [17].

Besides the innate immune system, the role of the adaptive immune system in the development of atherosclerosis has also been described in the literature. T cells recognize specific autoantigens that are produced in relative abundance in hyperlipidemic environments including ox-LDL, heat shock protein 60/65 (HSP60/65), β2-glycoprotein or ApoB100-derived peptides [2]. They are called autoantigens because they are the targets of an innate or adaptive mediated immune response despite being parts of normal tissue constituents. T cells become activated when macrophage or dendritic cells (DCs) present these autoantigens on their major histocompatibility complexes (MHCs) to them in plaque lesions or lymphoid tissues [12]. In more details, at the early stages of atherosclerotic development, DCs accumulate within the intima of arteries that experience disturbed blood flow (as a proatherogenic factor), through a mechanism involving VCAM-1 and CX3C chemokine receptor 1 (CX3CR1; also termed fractalkine receptor or G-protein coupled receptor 13 (GPR13)) [28,29]. The continued accumulation of dendritic cells during lesion development is correlated with lesion progression and inflammation, and their clustering with T cells within the lesions suggests an important role of DCs in the modulation of T cell adaptive immunity. Scavenging of modified lipids and other potential antigenic structures within the lesions induce DC maturation and migration to secondary lymphoid organs, where they promote the generation of antigen-specific pathogenic and/or regulatory T cells. These antigen-specific T cells migrate to the atherosclerotic lesion and could be reactivated and modulate the immunoinflammatory response. The survival and maintenance of Tregs would be more readily compromised compared with T effector cells, leading to an imbalance in T cell adaptive immunity.

The activated Th1 cells produce proinflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α), and membrane CD40-ligand, which amplify the immune response through activation of macrophages, vascular smooth muscle cells, and endothelial cells [30]. Interleukin-12 (IL-12) production by DCs and monocytes/macrophages play a critical role in Th1 differentiation, upregualtion of IFN-γ, and downregulation of IL-4 and IL-5 expresssion in T cells [12].

Th2 cells are rarely detected in atherosclerotic lesion but their induction is promoted in a severe hyperlipidemic environment [12]. Th2 cells secrete numerous cytokines including IL-4, IL-5, IL10, IL-13, and IL-33. The role of Th2 in atherosclerosis remains to be controversial. In mouse model resistant to atherosclerosis, a Th-2 bias has been shown to protect against early fatty streak development [31]. Moreover, in LDL receptor deficient (*LDLR-/-)* mice, deficiency of IL-4 did not affect lesion development [32], but others reported a decrease in atherosclerotic lesion formation [33]. IL-5 and IL-33 appear to have anti-atherogenic properties. IL-33 can reduce atherosclerosis development in *ApoE-/-* on a high fat diet via the induction of IL-5 and ox-LDL antibodies [34].

Th17 cells secrete IL-17, which have been associated with proinflammatory response and atherosclerosis development [23]. On the contrary, Mallat et al. have suggested that IL-17 is atheroprotective [35,36]. An imbalance between Th17/Tregs in patient with acute coronary syndrome (ACS) has been reported, suggesting a potential role of Th17 in plaque destabilization [37]. Futher studies are needed to determine the role of IL-17 in atherosclerosis development and progression.

An unusual subset of T cells characterized by the lack of CD28 (costimultory molecule that binds CD80/CD86 on the surface of APCs), termed CD4+CD28 null T cells, have been showed in the peripheral blood of patient with coronary artery disease and in patient with unstable angina [38]. These cells are characterized by the production of high levels of INF-γ and TNF-α. However, the lack of these cells in mice prevents us from further characterizing this subset in mice.

Regulatory T Cells

The recognition of regulatory T cells, originally termed suppressor T cells, resulted from experiments performed in the 1960s and 1970s by Gerson and Kondo, which described the induction of suppressor T cells capable of down-regulation of antigen-specific T-cell responses [39]. Due to the lack of molecular markers, research on suppressor T cells was stopped. However in 1995, Sagakuchi et al. identified CD25 as a surface phenotypic marker for suppressive CD4 cell in mice [40]. Since then, suppressive T cells have been called regulatory T cells. Later, the discovery of Foxp3 as a specific transcrition factor and marker of natural regulatory T cells and adaptive/induced regulatory T cells gave a molecular anchor to the population of Tregs [41]. The identification of these molecular markers led to an increase in interest in regulatory T cells during the last decade, which has identified Tregs as a plausible theraupetic choice for several autoimmune diseases such as colitis, LES, RA, T1DM, and atherosclerosis among others.

Originally, the high expression of CD4 and CD25 surface markers was used to identify Tregs (CD4+CD25+ cells). However, because CD25+ has been found in other non-Treg T cells, the measurement of the intracellular expression of Foxp3 transcription factor allowed for more specific Tregs's analysis (CD4+CD25+Foxp3+ cells). Since Foxp3 is also expressed in effector T cells, negative expression of CD127 is often used as another marker [42], since CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. Several additional markers have been described, such as CTLA-4 (cytotoxic T-lymphocyte associated molecule-4), glucocorticoid-induced TNF receptor (GITR), CD39, and CD45RA, however the functional significance of this expression remains to be defined.

Currently, several immunologic products exist that simplify the identification, isolation, and characterization of Treg cells using fluorescent antibodies for CD4+, CD25+, Foxp3+ and CD127-. Moreover, the isolation of mRNA for cDNA synthesis is used to analyze FoxP3 expression in Tregs using a quantitative real-time PCR [43]. Tregs are caracterized for the secretion of IL-10, IL-35 and transforming growth factor- β (TGF-β). Furthermore, enzyme–linked immunoabsorbent assay (ELISA) and Western blot have been used for the detection of Tregs. Also, the cytokines can be measured using a cytokine secretion assay [44]. A new method to more accurately analyze and monitor Treg cells has been described in the literature based on DNA methylation analysis. Only in Treg cells, but not in any other cell type, including activated effector T cells, a certain region within the Foxp3 gene (Treg-specific-demthylated region, TSDR) is found demethylated, which allows for the monitoring of Treg cells through PCR reaction or other DNA-based analysis methods [45].

Tregs have indispensable functions in maintaining immune homeostasis. They are essential in mediating peripheral tolerance, preventing autoimmune diseases, and suppressing inflammatory responses. Immune tolerance is defined as a function of the immune system in maintaining immunological unresponsiveness to self-antigens and suppressing an exaggerated immune response, which could lead to autoimmune diseases and atherosclerosis [11]. There are two types of tolerance: central tolerance is the elimination of self-reactive T cells within the thymus through a process termed negative selection, and peripheral tolerance is the elimination of self-reactive T cells outside of the thymus such as immunosuppressive activity of Tregs [19].

There are two classes of $CD4+CD25^{high}$ Tregs cells, naturally occurring Tregs (nTreg), which comprise 5-10 % of murine and human CD4+ cells, and adaptive/induced Treg cells (iTreg). Naturally occurring Treg are matured within the thymus and express Foxp3 transcription factor. Experimental evidence indicates that nTregs exist without peripheral antigenic stimulation [9,11,46]. On the other hand, adaptive Tregs are generated in the periphery from CD4+CD25- T cell population and are induced in response to particular antigenic stimulation and cytokines. Once Tregs are activated, they can suppress other T cells by different mechanisms, which will be discussed in detail within other section of this review.

Naturally occurring treg cells

Naturally occurring Treg cells are developed in the thymus and are characterized by the expression of CD4, CD25 high and transcriptional factor FOXP3 [47]. Initially identified by their co-expression of CD4 and CD25 cell surface markers, subsequent reports have used other cell surface markers such as CD103, CD62L, lymphocyte activation gene 3 protein (LAG 3), C-C chemokine receptor type 5 (CCR5), neurophilin, the activation antigens glucocorticoid-induced tumor necrosis factor receptor (GITR), and cytotoxic T-lymphocyte protein 4 (CTLA-4, also known as CD152), as well as the lack of certain cell surface markers such as CD127 (the α chain of the IL-7 receptor) to identify Tregs [48]. They recognize specific self-antigen and prevent autoimmunity by the inhibition of pathogenic lymphocytes. The role of natural Tregs in experimental atherosclerosis was initially reported in 2006 by Ait-Oufella et al., showing an increased in atherosclerotic lesion size and vulnerability in *ApoE-/-* mice depleted of peripheral Treg cells [10].

Naturally Tregs express the transcription factor Foxp3, which is a member of the Fork-head/wingled-helix family of transcription factors and a master regulator of Treg development and function in the thymus. Foxp3 was first identified by Brunkow et al. in 2001 as a defective gene in the mouse strain scurfy, an X chromosome-

linked recessive lymphoproliferative disease. Scurfy is lethal in hemizygous males, which exhibit hyperactivation of CD4+ T cells and overproduction of proinflammatory cytokines within a month after birth. In humans, mutation of the Foxp3 gene leads to the development of immune dyregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX), in which multiple autoimmune diseases such as diabetes mellitus, allergy, and inflammatory bowel disease are present [41]. Fully-matured Foxp3+ natural Tregs exit the thymus and migrate to the secondary lymphoid organs where they suppress the proliferation of tissue-specific autoimmune T cells and their differentiation into Th1,Th2, and Th17 lineage *in vivo* [15]. Naturally Tregs inhibit polyclonal T cell activation and the function of B cells, macrophages, and DCs [15]. Naturally Tregs exert their suppressive function especially via cell-cell contact, membrane-bound TGF-β, IL-10, CTLA-4 or deprive T effector cell of the IL-2 necessary for their proliferation [49,50]. IL-35 is a newly identified mechanism of Tregs suppression demonstrated by Collison et al. in 2007. Our recent report showed that IL-35 is a novel responsive anti-inflammatory cytokine expressed by natural Tregs but not by resting or activated effector T cells [51].

Adaptive Treg cells

Adaptive or inducible Tregs (iTregs) are induced in the periphery from a CD4+CD25- T cell precursors, which acquire the expression of CD25 (interleukin-2 receptor α chain; IL-2Rα). Tregs are developed from naïve CD4+ T cells in the lymphoid tissues in response to specific antigens in the presence of transforming growth factor-β1 (TGF-β1), IL-10, and IL-4, while in the absence of pro-inflammatory cytokines such as IFN-γ, IL-1, IL-6, and IL-12. This antigen presentation in the absence of danger signals is referred to as tolerogenic, which is essential for the suppression of undesired immune reactivity against non-harmful materials such as airborne particles, commensal bacteria, and foods. In addition, iTregs depend on IL-2 for development and survival. In an atherosclerotic setting, iTregs may exert their suppressive function on foam-cell formation in atherosclerotic lesions. Furthermore, iTregs may be able to redirect macrophage differentiation toward an anti-

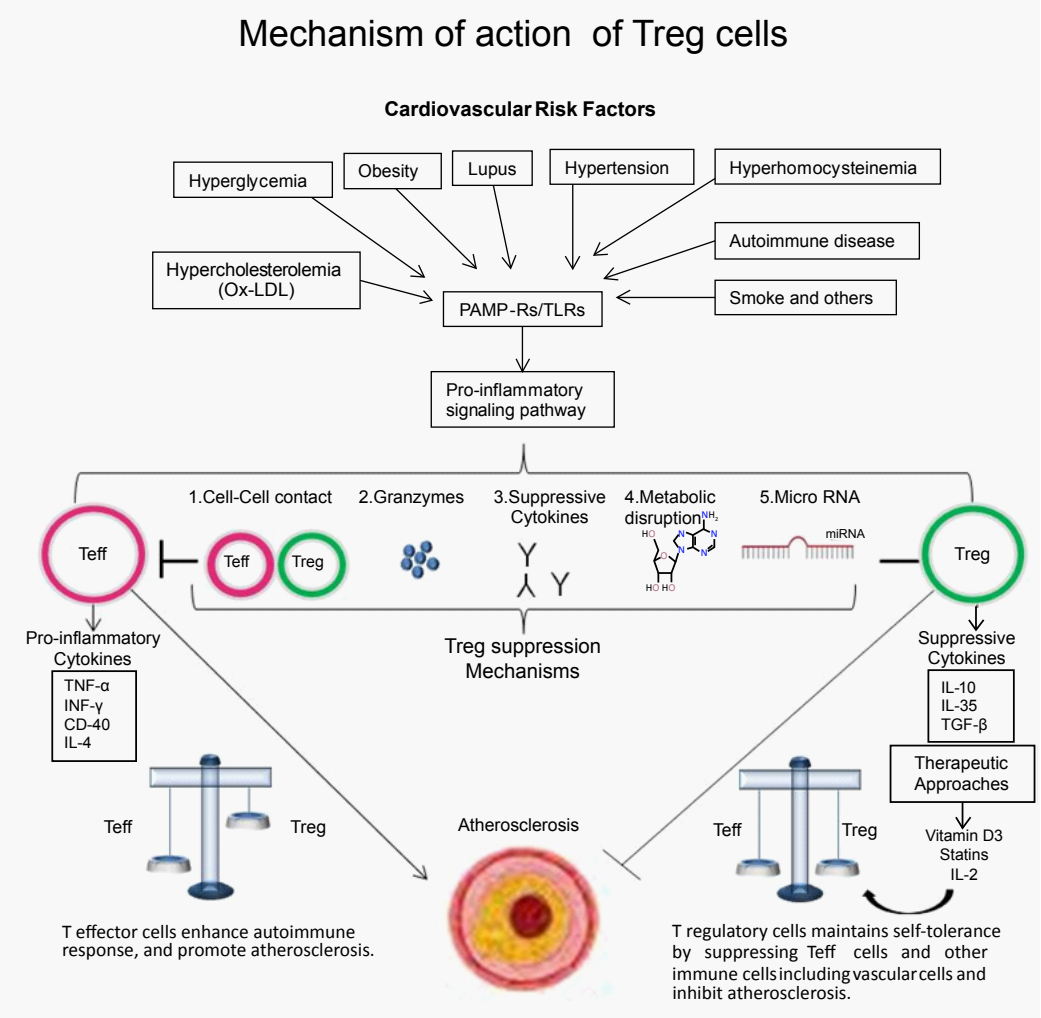

Figure 1: The schematic representation of our working model. Several cardiovascular risk factors induce pro-inflammatory signaling pathway inducing T cells activation. T effector cell activation promotes pro-inflammatory cytokines leading to break-down of immune tolerance and finally autoimmune disease. In contrast, enhance Tregs expansion and function inhibits Atherosclerosis by maintaining immune tolerance. Suppression of Teff and others immune cells by Treg is proposed in 5 mechanisms: Cell-Cell contact, Granzymes, Suppressive Cytokines, Adenosine and micro RNA. Vitamin D3, Statins and IL-2 are new therapeutic targets that promote Treg survival and homeostasis in order to maintain the balance in the immune system.

inflammatory cytokine-producing type 2 macrophage phenotype (M2) rather than proinflammatory type 1 macrophages (M1 phenotype) [40].

Different subsets of iTregs have been reported including T regulatory cell type 1 (Tr1) and T helper type 3 (Th3) [52]. Tr1 cells are CD25-FOXP3- characterized by the secretion of large amount of IL-10, some IL-5 and IFN-γ with or without TGF-β, IL-2 or IL-4 [53]. Tr1 can control the activation of naïve and memory T cells *in vivo* and *in vitro*, and suppress the immune response from Th-1 and Th-2 to pathogens, tumors and alloantigens [54]. The capacity of DCs to induce T cell proliferation is strongly reduced by the supernatant of activated Tr1 [55], suggesting that Tr1 suppression is mediated by secreted cytokines. It has been shown that Th3 cells produce high amounts of TGF-β when induced by oral tolerance in mucosal tissue in an antigen-specific manner [15]. CD4+LAP+ (latency associated peptide) Tregs have been identified recently as another Treg subtype whose suppression is mediated by TGF-β in immune diseases including experimental autoimmune encephalitis (EAE), autoimmune diabetes, lupus, collagen-induced arthritis, type II diabetes, and atherosclerosis in mice [15,56].

Mechanisms of Action of Regulatory T Cells

Several mechanisms underlying how Tregs fulfill their suppressive functions to maintain immune homeostasis have been described. The five characterized mechanisms of Tregs suppression described in Figure 1 include:

Cell-Cell contact

Cell-cell contact is an important means of Treg-mediated suppression through membrane-tethered TGF-β. It has been shown that through membrane-tethered TGF-β, Tregs can delay the progression of diabetes by suppressing the infiltration of T cells into pancreatic islets [57]. Another important molecule in Treg-mediated cell-cell contact inhibition is cytotoxic T-lymphocyte antigen 4 (CTLA-4), a co-inhibitory molecule that is constitutively expressed by Tregs. Upon Treg activation, CTLA-4 is exposed on the cell membrane [58]. Blockade or deficiency of CTLA-4 in mice causes autoimmunity, which can be rescued by Tregs, suggesting the importance of CTLA-4 in peripheral tolerance [59,60]. In addition, CTLA-4 has been shown to be involved in the direct interaction of Tregs with APCs. Tregs are shown to downregulate the expression of co-stimulatory molecules CD80/CD86 by APCs in a CTLA-4-dependent mechanism. The direct effect of Tregs on antigen-presenting cells (APCs) thus can indirectly influence the activation of T effector cells. Moreover, Treg-mediated suppression of T effector cells via APCs is decreased in CTLA-4-deficient Tregs [61,62]. Previous studies using Transwell culture plates suggested that Treg were unable to suppress responder T effector cell proliferation when the cells are separated by the membrane. Another data suggest that is not only the function of Treg that is contact-dependent, but also the induction of suppression. This data demonstrated that Treg cells regulate the immune response via cell-cell contact between Tregs and any other inflammatory cells, induce the suppression of immune response and decrease the development of atherosclerosis.

Granzyme-Perforin

Tregs can mediate cytolysis and induce apoptosis of effector T cells via secretion of a family of serine proteases termed granzymes. Tregs were shown to have high expression of granzyme-B. Tregs from mice lacking granzyme-B have reduced suppressive activity [63]. Furthermore, Tregs can also suppress B cell and NK cell functions via granzyme-B-dependent and perforin-(creating transmembrane pores that act as ion channels in the target cell)-dependent cell death [64,65]. In addition to granzyme-B, tumor necrosis factor related apoptosis inducing death receptor 5 (TRAIL-DR5) pathways and galectin-1 have also been implicated in Treg-mediated T-cell apoptosis [66,67].

Supresssive cytokines

TGF-β, IL-10 and newly-characterized IL-35 are immunosuppressive cytokines produced by Tregs [51,68,69]. TGF-β and IL-10 have been extensively studied whereas the role of IL-35 in Treg-mediated suppression has been uncovered in recent years. Upon stimulation, CD4+CD25+ Tregs can produce high levels of TGF-β and IL-10 [68,69]. However, other types of cells also have the capacity to produce these two cytokines [70,71]. The protective role of IL-10 and TGF-β in atherosclerotic disease has been suggested in in vitro and *in vivo* studies in animals [72,73].

IL-10 deficient mice showed an increased susceptibility in atherosclerosic size lesion and *in vivo* transfer of murine IL-10 reduced size lesion [72]. IL-10 is decreased in patient with vulnerable coronary plaques [74].

TGF-β has been suggested to be important in controlling T cell-mediated autoimmunity by the regulation of T cell proliferation, differentiation, and survival [75]. This function may be explained by the fact that TGF-β induces a Treg phenotype in CD4+CD25-T cells through induction of Tregs-specific transcription factor Foxp3 and the down-regulation of inhibitory Smad 7 protein [76,77]. Also, TGF-β can induce the development of T helper cell 17 (Th17), which are linked to Treg development and function [77,78]. TGF-β-dependent mechanisms are involved in Treg regulation of airway allergic responses [79]. Moreover, not only does Treg-mediated suppression of T effector cells require TGF-β, but also the development and proliferation of Tregs also needs TGF-β [80].

IL-35 is a novel responsive anti-inflamatory cytokine that may be specifically produced by Tregs and is required for maximal suppressive activity and increase following the contact with T effector cells [13]. IL-35 has been shown to inhibit T cell proliferation, but its effect on other cell types has yet to be elucidated [51].

Metabolic disruption of target cells

Tregs also mediates suppression via metabolic disruption of target cells. One of the proposed mechanisms is via the consumption of IL-2. IL-2 is important not only in promoting Treg survival and proliferation but is also required by other T cells. Therefore, Tregs may induce T effector cell apoptosis by *consuming*/depleting the IL-2, which T effector cells need for survival and proliferation [81]. In atherosclerosis, the administration of IL-2 has been found to expand Treg population and thereby attenuate initial atherosclerotic lesion development and stabilize lesions, suggesting that Tregs have higher affinity to IL-2 than other T cells [82]. Another metabolic disruptive mechanism involves the expression of CD39 and CD73 ectoenzymes, which hydrolyze extracellular ATP to generate pericelluar adenosine [83,84]. Adenosine can suppress T effector cells via adenosine receptor 2A activation and enhance Treg generation by inducing TGF-β secretion and inhibiting IL-6 expression [85]. In addition, Tregs, that are CD39-deficient, were shown to have reduced suppressive function. Futhermore, blocking ectonucleotidase activity in Tregs expressing CD39 reduces their ability to suppress T effector cell proliferation [86,87]. Meanwhile, ligation of adenosine to the adenosine receptor 2A increases intracellular cAMP levels, which has been linked to suppression of cellular proliferation

and differentiation, and inhibition of cytokine gene expression in lymphocytes [88]. In addition to increasing cAMP via adenosine generation, Tregs can enhance the levels of cAMP in target cells via direct delivery of cAMP into target cells through gap junctions [89].

microRNA-155

A recent report showed that inhibition of microRNA-155 (miR-155) in conventional CD4+ T helper cells strengthens nTreg-mediated suppression; where as overexpression of mature miR-155 in CD4+ T helper cell renders these cells to be unresponsive to nTreg-mediated suppression. These results suggest that miR-155 plays a crucial role in nTreg–mediated immune tolerance by regulating the susceptibility of conventional human as well as murine effecctor CD4+ T helper cells to nTreg-mediated suppression [90].

Effects of Cytokines on Tregs

The mechanisms of how cytokines, other than immunosuppressive cytokines, affect the homeostasis and immunosuppressive functions of Tregs have been reviewed extensively and are not completely understood. Most cytokines have a positive stimulating or supportive effect although some cytokines down-regulate the function of Tregs.

Common chain (γc) cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 bind to multimeric receptors that share the common γ chain. The γ chain is a critical part of the cytokine receptors and confers the ability of the receptors to activate mitogen-activated protein kinase (MAP kinase) and phosphoinositide-3 kinase (PI3 Kinase) signaling, leading to antiapoptotic and proliferation signals in lymphocytes. IL-2, IL-2 receptor α chain (IL2Rα, CD25), and IL-2Rβ are required for nTregs proliferation and survival [77]. Also, IL-2 protects Treg cells from Dexamethasone-induced cell death and regulates Foxp3 expression in human, which involves the binding of signal transducer activation of transcription-3 (STAT-3) and STAT-5 proteins to a STAT binding site located in the first intron of Foxp3 gene. On the other hand, IL-7, in combination with self-antigen presentation, promotes proliferation and accumulation of Tregs in specific histological sites where the antigen is presented. Recent studies showed that immunoregulatory dendritic cells (iDC) express high levels of IL-7 and delay autoimmunity through the Treg-dependent pathway [91]. Meanwhile, IL-15 is a T cell growth factor and its receptor shares the IL-2Rβ chain for increasing the expression of Bcl-2 and improves Treg expansion.

IL-13 and IL-4 are IL-4 receptor α chain (IL-4Rα)-binding cytokines, which induce Foxp3-expressing cells from CD4+CD25- T cell precursors outside the thymus via the antigen presenting mechanism. Since IL-4 is an essential cytokine for differentiation of Th2 cells, the results suggest a relationship between the immunoregulatory capacity of Th2 and Tregs [77,92].

IL-6 is a proinflamatory cytokine induced by Toll-like receptors through the recognition of microbial products, which blocks the immunosuppressive effects of Tregs and allows the activation of pathogenic immune response [77,93].

Some data suggest that TNF-α and anti-TNF-α antibody modulate type I diabetes mellitus in non-obese mice (NOD), with the alteration of the function and number of Tregs [94].

Type I interferons (IFN-α and IFN-β) are produced by many cell types. IFN-α induces the expression of Foxp3, although the mechanism is not clear since IFN-α also enhances the induction of Th1 and Th2 responses. Furthermore, IFN-β can promote survival of Tregs similar to IL-2 [77].

Tregs in Vascular Inflammation

Tregs comprise 5%-10% of peripheral CD4+ cells and are responsible for suppressing the hyperactive response of the immune system in vascular cells. The suppressive effects of Tregs are not restricted to adaptive immune cells such as CD4+ T-cells, CD8+ T-cells, or B cells; they can also suppress innate immune cells including natural killer, monocytes, macrophages, dendritic cells, and neutrophils. Weakened Tregs tip the balance between Tregs and T effector cells in favor of the development of vascular inflammation.

Endothelial cells (ECs) play an important role in immunologic events and represent a highly heterogeneous population of cells with the ability to modulate the function of immune cells [95,96,65]. Several studies demonstrate that ECs play a major role in intial atherosclerosis onset by changing from a quiescent state into a proinflammatory state to support the inflammatory process [97,98]. During inflammation, ECs exhibit increased adhesiveness for leukocytes and are involved in leukocyte recruitment to the interstitium of the tissue [99]. Furthermore, as conditional antigen presenting cells, ECs can effectively present alloantigens to lymphocytes, leading to T-cell activation including Treg cells. The importance of ECs role in antigen presentation and immunogenicity in vascular inflammation and autoimmune response has been well-recognized.

The immunologic role of ECs is significantly upregulated when ECs are activated, allowing for the high expression of HLA-DR (a type of major histocompatibility complex class II, MHC class II molecule) and T cell costimulators, such as CD86 and CD80 [12]. He et al. showed that Tregs are able to induce down-regulation of CD86, an immune phenotypic marker on activated human umbilical vein endothelial cells (HUVECs), which supports previous reports that costimulatory molecule expression in ECs can provide supplementary signals for Tregs interacting with ECs. Tregs have shown a suppressive effect on the ox-LDL/lipopolysaccharide (LPS)-induced inflammatory response of HUVECs by reducing NF-κB pathway activation as well as by down-regulating the expression of vascular cell adhesion protein 1(VCAM-1), monocyte chemotactic protein 1 (MCP-1), and IL-6 [44].

Tregs have various interactions with other vascular cells. A recent report showed that lymphocyte homing to nasal-associated lymphoid tissue (NALT) is mediated by specific interactions between lymphocytes and endothelial cells. Compared with the homing of CD4+CD25- T effector cells, the homing of Tregs to NALT was less dependent on the L-selectin-peripheral node address but was partially dependent on P-selectin glycoprotein ligand 1 and CD44. In addition, detailed studies showed that CD39 wild-type Tregs but not CD39-deficient Treg cells prevented adhesion of leukocytes and T cells to the endothelium before trans-endothelial migration. Impaired adhesion of effector T cells to inflamed endothelium was induced by adenosine-mediated downregulation of E-selectin and P-selectin on the vascular endothelium [100]. Thus, adenosine release by Tregs is essential in blocking leukocyte adhesion to endothelium, providing a novel mechanism, by which Tregs mediate immune suppression *in vivo* [101].

The interaction among vascular cells and Tregs contributes to decreasing vascular disease development and progression. Tregs have an active mechanism to induce immune suppression in vascular cells. However, the interaction between Tregs cells and vascular cells needs futher investigation.

The Contributions of Regulatory T Cells (Tregs) to Atherosclerosis

T cell self-tolerance and homeostasis are established within the thymus through self-reactive T cell deletion, receptor complex editing, or rendering those mature T cells inactive against self-antigens. Aside from this, in the periphery, Tregs suppress the activities of other self-reactive effector T cells that have potentially escaped from the thymus or that are extra-thymically generated but have not gone through the negative selection for self-reactivity. Thus, Tregs capacity of immunosuppression led investigators to examine the plausible role of Tregs in atherosclerosis protection. Found within atherosclerotic lesions, Tregs account for 1-5% of the localized T cell population. This number is comparatively lower than that found in other chronic inflammatory diseases where Tregs can be as high as 25% of localized T cells [102]. Interestingly, *ApoE-/-* mice exhibit significantly lower numbers of Tregs within the spleen than their wild-type counterparts. The detailed mechanisms for the reduction of Tregs in atherosclerosis are poorly characterized. Our recent reports suggest that this may result from increased expression of pro-apoptotic protein Bax and decreased expression of the anti-apoptotic Bax-antagonist protein, translationally control tumor protein (TCTP) in Tregs [103-105]. Our results indicate that enhancement of Tregs' survival is a future therapeutic target for suppressing proatherogenic autoimmune and chronic inflammatory process.

As mentioned previously, has been showed that depletion of peripheral Tregs by anti-CD25 monoclonal antibodies increased atherosclerotic lesion size and vulnerability in proatherogenic *ApoE-/-*mice [10]. Moreover, the direct effects of Tregs on atherosclerosis development were evaluated through the adoptive transfer of Tregs into *ApoE-/-*mice [10,106,107]. *ApoE-/-* mice recipient of Tregs showed decreased size of atherosclerotic lesion compared with *ApoE-/-* mice treated with phosphate buffered saline or T effector cells [107].

Additionally, the irradiation-bone marrow transplantation model has been used to study the role of Tregs in atherosclerotic disease [10]. The interaction of costimulatory molecules CD80, CD86, and CD28 are required for the generation and homeostasis of CD4+CD25+ Tregs. The important role of T cell co-stimulation signals through the CD80/CD86 and CD28 pathways in the maintenance of the Treg pools was elucidated with the generation of chimeric mice (low density lipoprotein receptor gene knock-out *(Ldlr-/-)* mice, in which reconstitution with CD80/CD86-deficient and CD28-deficient bone marrow cells had a marked increase in atherosclerotic lesion size in compared with controls, suggesting the effect of CD80/CD86 and CD28 deficiency-induced Treg reduction in the progression of aterosclerosis [10]. Our results showed that CD28-promoted anti-apoptotic Bax-antagonist protein, TCTP, is critical for Treg survival [103-105]. Caveolin-1 plays an important role in the regulation of atherosclerosis and inflammation. Calveolin-1 deficiency resulted in increase in regulatoy T cells, decrease in CD4+ effector cells in lymphoid organ and decreased size of atherosclerotic plaque in *Cav1-/-ApoE-/-*mice receiving *Cav1-/-ApoE-/- or Cav+/+ ApoE-/+* bone marrow transplantation has been described [108].

Foxp3 expressing T cells were targeted using DCs electrophorated with mRNA encoding for Foxp3, which induces a cytotoxic T lymphocytes response against Foxp3 leading to Tregs apoptosis and augmentation of atherosclerosis in *Ldlr-/-* [49]. In addition to these cellular-based studies, both Tregs' hallmark cytokines, IL-10 and TGF-β, have anti-atherogenic effects. When the functionality of either of these cytokines is mitigated within atherosclerosis-prone mouse models, accelerated development of atherosclerotic lesions is witnessed in both *ApoE-/-* and *Ldlr-/-* mice. It has been shown that administration of type I clone Treg cells that produce high levels of IL-10 downregulates the pathogenic immune response and leads to a decrease in the development of atherosclerotic plaques and inflammation in the *ApoE-/-*model [72,73]. A clinical study showed that patients with clinically-stable atherosclerotic plaque have higher levels of Tregs and IL-10 than vulnerable patients with recurrent myocardial infarction [74]. Taken together, these reports clearly demonstrate that Tregs act in an atheroprotective fashion and therefore possess a strong therapeutic potential.

The ability to induce peripheral immunotolerance prior to exposure to specific antigens is an attractive therapeutic approach in the treatment of allergies, inflammation, autoimmunity, as well as atherosclerosis [109]. Thus far, the pursuit of garnering peripheral tolerance with Tregs has shown a degree of success. Independent studies that individually administering the pro-atherogenic autoantigens apolipoprotein B-100, heat shock protein 60, and ox-LDL into atherosclerosis prone mice as an immunotolerance treatment/vaccination led to the inhibition of atherosclerosis development. In all three instances, the protection garnered was attributed to either the augmentation of the Tregs population and the production of TGF-β and/or an increase in IL-10 [110-112]. The oral administration of atherosclerosis-related antigens such as ox-LDL and HSP60 increased the numbers of Foxp3 Tregs in several organs and decreased atherosclerosis development in *LDLr-/-* mice [110,111]. In fact, Treg population expansion has been seen with long-term subcutaneous injections of influenza peptide as a result of mature effector T cell differentiation into Tregs [113]. Furthermore, the atheroprotective effects bestowed upon the apolipoprotein B-100 treated mice were nullified with the depletion of Tregs from these mice [2]. Subcutaneous delivery of ApoB 100-derived peptides in mice reduces atherosclerosis development and progression through the promotion of antigen-specific Tregs [28]. These findings provide conclusive evidence that Tregs play a critical role in the establishment of anti-atherogenic antigen-specific immune tolerance.

Aside from being the underlying cellular cause for the pro-atherogenic antigen toleration, Tregs have also been attributed as the basis for the atheroprotective effects associated with other treatments. The measles virus is known to suppress the immune system, therefore expectedly, *ApoE-/-* mice inoculated with the virus show a significant reduction in atherosclerosis [114]. Mechanistically, it was determined that this protection was also accompanied with an increase in Treg numbers [96]. Similarly, it has been reported that the active form of vitamin D3 can also drive immune tolerance. In fact, the administration of vitamin D to *ApoE-/-* mice resulted in delayed atherogenesis. Again, this protection was associated with an induction of Tregs as well as tolerogenic dendritic cells [115]. Meanwhile, Simvastatin treatment resulted in increased expression of Foxp3 and accumulation of Tregs in atherosclerotic lesions of *ApoE-/-* mice. Futhermore, simvastatin increased the numbers of Tregs and repaired inhibitory properties in patients with acute coronary syndrome (ACS) [116]. These findings poignantly suggest that expansion of Treg numbers can be therapeutically useful in combating proatherogenic inflammation and autoimmunity.

Foxp3 is considered the most specific marker for Tregs, even though it is expressed at lower levels on effector T cells. For the purpose of using Tregs in a clinical setting it is necessary to find an independent molecular marker for Tregs in order to be differentiated from effector T cells. Further investigations are needed into Treg molecular markers

before the development of therapeutic approaches using Tregs in the treatment for autoimmune diseases and atherosclerosis.

The levels of circulating CD4+CD25+ Tregs range from 0.6%-7.9% in healthy humans. Based on these reports we cannot generalize that Tregs are reduced in the periphery of patients with any autoimmune disease [117]. Additionally, specific measurements are needed to determine Treg levels at the site of inflammation. Many investigators have shown an increase in Treg numbers at inflammatory sites compared to peripheral blood. Despite the depletion of CD25+ cells in mice, activated T cells are still incapable of inducing autoimmunity without the administration of other adjuvants. This observation supports the multifactorial pathogenesis of autoimmune diseases.

Acute coronary syndrome (ACS) is a cardiological term that encompasses unstable angina, non ST-elevation myocardiac infarction (MI) (alternatively described as non Q-wave MI, often referred to as non-STEMI), and ST-elevation MI (alternatively described as Q-wave MI, often referred to as STEMI). Mor et al. demonstrated that patients with ACS have dysfunctional Tregs, and decreased levels of Tregs [107]. They also found high CD25+ Treg levels in patients with ST-elevation ACS compared with decreased levels in non St-elevation ACS patients [118]. This study also demonstrated that there is no correlation between circulatory Treg levels and the thickness of the carotid artery.

The immunosuppressive capacity of Tregs in autoimmune disease and atherosclerosis has been demonstrated in several studies. The mucosal route (nasal and oral) for the induction of immune tolerance via administration of autoantigens in animals has been used as a therapeutic approach in treating atherosclerosis and has been shown to reduce atherosclerosis development experimentally in animal models [8]. However, further investigation is needed to establish the clinical importance of Tregs in atherosclerotic disease as well as the therapeutic potential of Tregs in treating the disease.

Conclusion

The role of the immune system in atherosclerosis has been demonstrated. The characterization of two aspects of immune responses, immune effectors versus immune suppressors, suggest a novel concept that an imbalance between the pathogenic immunity and weakened regulatory/suppressive immune response plays a major role in the development of atherosclerosis. Tregs are critical for the mainteinance of immune homeostasis and their importance in atherosclerosis has been established. Depletion of peripheral Tregs by anti-CD25 monoclonal antibodies increased atherosclerotic lesion size and vulnerability in *ApoE*-/-mice. CD4+CD25^{high}Foxp3+ nTregs has a potent immunosuppression in the regulation of autoimmunity and inflammation in atherosclerotic disease. The levels of circulating CD4+CD25+ Tregs range from 0.6%-7.9% in healthy humans. The suppressive activity of Tregs is enhanced in cells with the highest CD25 levels expression (CD4+CD25^{high}). Patients with acute coronary syndrome have dysfunctional and decreased levels of Tregs. Moreover, patients with clinically stable atherosclerotic plaque reported higher levels of Tregs and IL-10 than patients with recurrent myocardial infarction. Furthermore, the interaction among vascular cells and Tregs decrease the development and progression of atherosclerosis. Tregs have a role in the immune suppression in vascular cells and several mechanisms of regulatory/suppressive function of Tregs have been described but have not yet been clearly elucidated. The interaction between Tregs and vascular cells is less well-known and needs futher investigation before considering Tregs as a therapeutic option for atherosclerotic disease.

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