CHAPTER 8

Regulatory T-Cells, FoxP3 and Atherosclerosis

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Abstract

Innate immune responses follow accumulation of modified lipids within the arterial wall thereby influencing atherosclerotic plaque progression. One of the mechanisms evolved in maintaining immunologic self-tolerance involves upregulation of regulatory T-cells, among which the $CD4^+CD25^+$ $FoxP3^+$ regulatory T-cells (Treg) are best characterized. The putative important role of Treg in the initiation of atherosclerotic lesions as well as in the progression towards unstable plaques leading to ischemic events, supported by human studies and, indirectly, by murine models. Herein, we summarize the experimental approaches taken in order to study the possible mechanisms of Treg involvement in atherosclerosis as well as the beneficial clinical potential of Treg in stabilizing atherosclerotic plaques.

Atherosclerosis, Inflammation and Autoimmunity

The immune system plays a pivotal role in the pathogenesis of atherosclerosis, the underlying cause of many cardiovascular diseases, including myocardial infarction, stroke and ischemic gangrene.[1,2] Atherosclerosis involves the innate immune responses with the recruitment and activation of monocytes/macrophages that respond to the accumulation of modified lipids, mainly the oxidatively modified LDL (OxLDL) within the arterial wall. These events are possibly followed by adoptive immune responses comprising differential antigen-specific T-lymphocytes. Most of the effector T-lymphocytes in atherosclerotic lesions are $CD4^+$ T-helper cells with the phenotype characteristic of a proinflammatory T-helper 1 (Th1) subset.[3-5] Most of the T-cells bear T-cell receptors (TCR)[6,7] and are often found in clusters in shoulder regions of the lesion.[8,9] These cells specifically recognize antigens that are produced in relative abundance in hypercholesterolemic individuals or in plaques, including Ox-LDL and HSP 60/65 in the form of antigen-presenting cells(APC) such as macrophages or dendritic cells.[10] The accumulation of inflammatory cells within the arterial wall leads to local production of chemokines, interleukines and proteases that enhance the influx of monocytes and lymphocytes, among which are IFN-gamma, tumor necrosis factor (TNF)-alpha and membrane CD40 ligand, thereby amplifying the immune response and promoting progression of atherosclerotic lesions.

Regulatory T-Cells, Developmental and Functional Aspects

Many mechanisms have evolved to maintain immunologic self-tolerance and to limit responses to foreign antigens.[13] One of these mechanisms involves regulatory T-cells that actively suppress responses of effector T-cells, via homing in on peripheral tissues in order to maintain self-tolerance and to prevent autoimmunity by inhibiting pathogenic lymphocytes. Several types of regulatory

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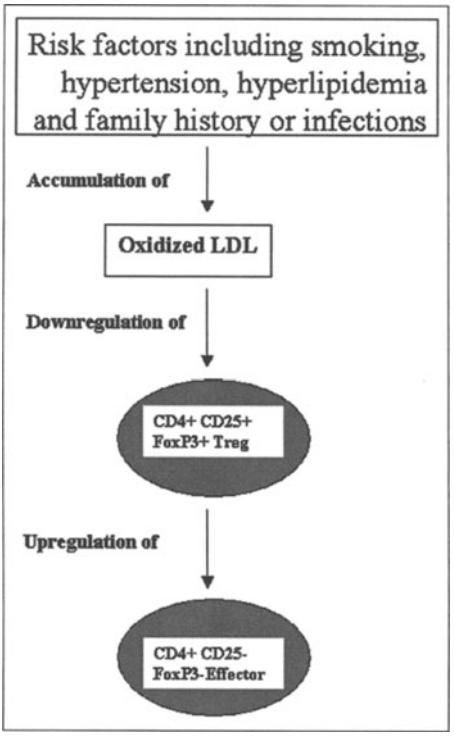

Figure 1. Potential involvement of Treg in atherosclerosis.

T-cells have been identified, including IL-10-producing Type 1 regulatory T-cells (Tr1), transforming growth factor beta (TGF beta)-producing Th3 cells[14,15] and the $CD4^+CD25^+$ (interleukin-2 receptor-α chain) $FoxP3^+$ regulatory T-cells (Treg) which are best ones characterized. Tregs are natural regulatory T-cells that mature in the thymus and comprise 5% to 10% of the peripheral $CD4^+$ T-cells.[16] FoxP3, a forkhead family transcription factor, is a lineage-specific factor for Treg, which plays a crucial role in their suppressive function as outlined in Figure 1. Whereas initial studies characterized these cells by their co-expression of CD4 and CD25 surface markers, subsequent reports identified expression of other surface markers including CTLA-4 (Cytotoxic T-Lymphocye Antigen 4 also known as CD152) and GITR (Glucocorticoid-Induced TNF Receptor)[17,18] as well as CD103, CD62L, lymphocyte activation gene 3 protein (LAG 3), C-C chemokine receptor Type 5 (CCR5) and neurophilin, and the concomitant absence of certain markers such as CD127 (the alpha chain of the IL-7 receptor).[18-21] Major progress in the understanding of the homeostasis of naturally occurring Tregs was made with the identification of FoxP3 as a requisite factor for the development of Tregs and for their suppressive functions, as will be described in detail in the section below.

Natural Treg are generated during thymic development, but are also induced in peripheral tissues during immune responses[16] and atherosclerosis(Fig. 2). Treg express antigen receptors typical of effector T-cells and are presumably activated by peptide antigens presented by APCs. They also acquire interleukin (IL)-2 receptor for development and survival. In this context, two populations of potential Treg have been described: those that originate from a committed lineage of FoxP3-expressing cells in the thymus and those that convert from mature $CD4^+$ cells in the periphery.[22] The basic characteristics of natural Treg, and adaptive Treg versus effector T-cells are summarized in Table 1. Three general models of suppression have been proposed to explain the inhibitory actions of Treg cells on activated T-cells, none of which have been completely elucidated: 1. Cell contact-dependant suppression

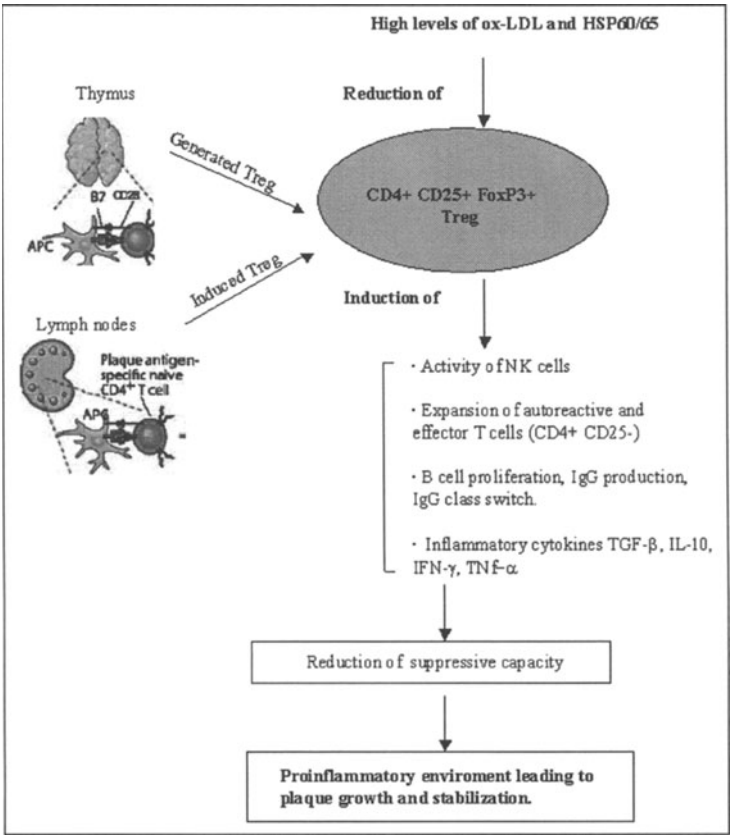

Figure 2. Treg and atherosclerosis. Natural Treg develop in the thymus and may be induced in peripheral tissues. Naïve T-cells specific for plaque antigens (such as oxidized LDL and HSP60) are activated by APCs and differentiate into Th1 effector cells, which migrate into atherosclerotic lesions, reactivated by lesional APCs, secrete IFN-gamma and promote disease. The reduced numbers and functional impairment of Tregs could promote atherosclerosis by several mechanisms as described.

mediated by engagement of CTLA-4 (expressed on Treg cells) with CD80 molecules (expressed on effector T-cells) or interaction of CTLA-4 with CD80/CD86 on APCs.[23] 2. Consumption and limitation of growth factors such as IL-2. Effector T-cells secrete IL-2 upon activation, which binds to CD25 on Treg cells, thus maintaining and activating Treg cell genes such as FoxP3, which in turn down-regulate IL-2 secretion in a feedback loop. This action results in deprivation of effector T-cells from the essential growth factor IL-2. This feedback process might therefore induce apoptosis of activated T-cells in vitro and in vivo.[24] 3. Production of inhibitory cytokines, including IL-10, TGF-β or IL-35.[25] Production of these cytokines may induce deactivation of dendritic cells, leading to a loss of ability to activate effector T-cells with distinct antigen specificity to Treg cells, a mechanism called 'bystander immune suppression'. In addition, TGF-β inhibits the proliferation, activation and differentiation of T-cells towards Th1 and Th2.[26,27]

FoxP3 (Forkhead Box Protein P3), a member of the forkhead winged helix protein family of transcription factors, was demonstrated to govern mouse $CD4^+CD25^+$ Treg function.[28,29] Loss of function mutations of FoxP3 were shown to eliminate $CD25^+$ Treg and result in lethal lymphoproliferative autoimmune syndrome in mice associated with extremely enlarged spleens

Table 1. A basic comparison between naturally-occurring Tregs, induced Tregs and effector T cells

	Natural Treg	Induced Treg	Effector T cells
Generation site	Thymus	Peripheral lymph nodes	Thymus
CD25 expression	High	Variable	No
FoxP3 expression	Yes	Yes	No
IL-2 dependency	Yes	Yes	No
Specificity	Self	Self and foreign	Foreign

and lymph nodes and lymphocytic infiltrates in multiple organs, associated with deficiency or malfunction of Treg.[30] This finding was strongly supported by the observation that patients with the rare immune system dysregulation, polyendocrinopathy, enterophathy and X-linked inheritance (IPEX), have a severe inflammatory disease accompanied by a mutation in the FOXP3 gene.[31] The requirement of FoxP3 in $CD4^+CD25^+$ regulatory T-cell development was demonstrated upon generation of a mixed bone marrow (BM) chimeric mice in which lethally- irradiated C57BL/6 (B6) $Thy1.1^+$ congenic mice were reconstituted with T-cell-depleted BM from congenic B6 $Ly5.1^+$ mice mixed at a 1:1 ratio with BM from either $FoxP3^-$ or $FoxP3^+$ mice. The $CD4^+CD25^+$ regulatory T-cell population in the ($Ly5.1^+B6^+FoxP3^-$) chimeras was solely of $Ly5.1^+B6$ origin in both the thymus and lymph nodes, whereas, both $Ly5.1^+B6$ and $FoxP3^+$ BMs contributed equally to the $CD4^+CD25^+$ regulatory T-cell compartment in the ($Ly5.1B6^+$ $FoxP3^{+/+}$) chimeras.[28] Moreover, ectopic FoxP3 expression was found to be sufficient to activate a program of suppressor function in peripheral $CD4^+CD25^-$,[28,29] pointing to FoxP3 as a unique marker of $CD4^+CD25^+$ Treg, distinguishing them from activated $CD4^+CD25^-$ T-cells and as a master transcriptional regulator of Treg homeostasis. Therefore, in contrast to other molecular markers used to identify regulatory T-cells, such as GITR, CTLA-4 and CD25, FoxP3 is not upregulated by activated $CD4^+CD25^-$ T-cells.

Recently, several monoclonal antibodies specific for human Foxp3 became available for detection of endogenous human FoxP3 by flow cytometry and immunohistochemistry.[32] Similar to murine FoxP3, the majority of human FoxP3 was also expressed by the majority of the $CD4^+CD25^{high}$ T-cells in peripheral blood, enabling investigation of human FoxP3 for clinical use. FoxP3-GFP knock-in mice[33] as well as FoxP3-GFP-hCre bacterial artificial chromosome transgenic mouse[34] were recently created. Those mouse strains may pave the way for better characterization of the different $FoxP3^+$Treg subpopulations and thus provide a better analytic tool to identify the subpopulation mostly involved in atherosclerosis progression.

FoxP3 in Experimental Models of Autoimmunity and Atherosclerosis

In recent decades, the role of the immune system in atherosclerosis development has received considerable attention.[1,2] The general belief is that risk factors such as hypertension, hyperlipidemia, family history of premature atherosclerosis as well as infectious pathogens could promote LDL oxidation within the vessel wall and in the circulation. These downregulate the numbers and functions of FoxP3-expressing Treg (Fig. 1).[35] In the last decade experimental approaches successfully used in other disease model, have been employed to test the importance of autoimmunity in the development of atherosclerosis. Initial studies have identified putative autoantigens within atherosclerotic plaques, including heat shock proteins, oxidized LDL and β2-glycoprotein.[36-38] Several studies, some of which were performed in our laboratory, demonstrated that adoptive transfer of antigen-responsive lymphocytes or alternatively passive transfer of antibodies, significantly enhance development of atherosclerosis in experimental models.[39-41] Furthermore, induction of immune tolerance to plaque-associated components, such as OxLDL, attenuated the progression of atherosclerosis in mice.[42-44] Several studies were later conducted in an attempt to elucidate the potential role of the

$CD4^+CD25^+FoxP3^+$ Treg cell repertoire in the control of atherosclerotic plaque development. Since CD25-deficient (IL2rα−/−) mice die prematurely from severe autoimmune disease with cachexia and malabsorption,[45] they are not suitable for the study of the effect of Treg cell deficiency on atherosclerosis. Therefore, two alternative transgenic atherosclerosis-prone mice strains have been studied for assessment of the development of atherosclerotic plaques: 1. The apolipoprotein E-deficient (ApoE−/−) mice. These mice develop complex atherosclerotic lesions that result from plasma accumulation of cholesterol-rich lipoproteins.[46] The number and functional properties of Treg were found to be compromised in ApoE−/− mice compared with those in wild-type C57BL/6 littermates.[47,48] 2. The low-density lipoprotein receptor-deficient (Ldlr−/−) mice, known to be susceptible to development of atherosclerosis when fed a high-fat, high- cholesterol diet.[49] The experimental design included depletion of Treg cells by either genetic or antibody-mediated means[50] and by enrichment of Treg by adoptive transfer, as reported by our research group and others.[50,51] Using these approaches, a direct effect of Treg on atherosclerosis was demonstrated. Our group has recently shown that compared with controls, ApoE−/− mice exhibit reduced Treg numbers and compromised Treg function.[51] Interestingly, proatherogenic Ox LDL triggered a more robust depletion in the splenic Treg population than in the effector T-cell population, and the ApoE−/− mice were more susceptible to this attenuation than control animals. Moreover, Treg deficiency related to genetic ablation of the B71/2-CD28 costimulatory pathway in the hematopoietic compartment was shown to enhance atherosclerotic lesion development in Ldlr−/− mice.[52] Treg depletion using an anti-CD25 antibody also enhanced atherosclerosis in ApoE−/− mice.[50] Deficiency of the T-cell costimulatory molecule ICOS resulted in enhanced atherosclerosis in Ldlr−/− mice, which can be attributed to an impaired Treg development and function.[53] Interestingly, Treg depletion did not influence lesion size or inflammatory phenotype when a host effector T-cell population was genetically engineered to be insensitive to TGFβ.[50] This finding together with a previous work showing markedly enhanced atherosclerosis in ApoE −/− mice with TGF-β resistant T-cells,[50] suggests that TGF-β is required for the atheroprotective effects of Treg. Reduction in atherosclerosis in Apo E −/− mice has also been achieved through adoptive transfer of $CD4^+$ $CD25^+$ regulatory T-cells,[50,51] possibly through expression of distinct forms of TNF-alpha in ApoE (−/−)mice[54] or via induction of oral tolerance to HSP60 in Ldlr (−/−) mice.[44] A recent study performed in our laboratory demonstrated an association between hypoxia and the homeostasis of Treg mediated by upregulation of HIF-1α (hypoxia-inducing factor alpha), pointing to the additional potential mechanisms of vasculo-protective effects of Treg.[55] In vivo expression of HIF-1α achieved by hydrodynamic injection of HIF-1α expressing vector induced an increase in FoxP3 expression and an increase in the number of functionally active $FoxP3^+CD4^+CD25^+$ Treg. We therefore assume that hypoxic sites (tumoral, ischemic, inflammatory) may downregulate local early Th1-mediated inflammatory response by inducing expression of HIF-1α within local lymphocytes with consequent upregulation of the Treg pool.

Foxp3, Regulatory T-Cells and Atherosclerosis in Humans

When comparing the data from human studies investigating the potential involvement of Treg in atherosclerosis with data from murine studies, it is important to keep in mind one crucial factor. Whereas most murine studies test plaque burden as determined by lipid accumulation, in humans it is practically impossible to quantitatively evaluate the extent of atherosclerotic vasculatur.[56] In humans, a more realistic marker for assessing atherosclerosis may be the clinical syndrome, namely the presence of plaque rupture as evidenced by the occurrence of acute coronary syndromes (ACS). It is now recognized that most plaques that cause ACS exhibit angiographic obstruction of less than 70%[57,58] and that the onset of ACS is mainly associated with changes in the inflammatory response in these lesions, including a shift in the phenotype of intraplaque T-cells.[59,60] The majority of ACS-related atheromas are caused by rupture of plaques consisting of a large, thrombogenic core of lipid and necrotic debris, including foci of macrophages, T-cells, old haemorrhage, angiogenesis and calcium. The factors that govern the transition of the plaque from a stable to a rupture-prone lesion are not entirely understood. However, accumulating evidence supports the role of immune system dysregulation, including reduction and impaired function of the pool of the naturally

occurring $CD4^+CD25^+$ $FoxP3^+$ Tregs in the alteration of the plaque phenotype. Indeed, in two independent studies, one of which was performed in our laboratory, Treg purified from peripheral blood of patients with ACS exhibited a significantly reduced expression of FoxP3 compared to blood from patients with stable angina or from normal coronary arteries.[61,62] An additional in situ study pioneered by De Boer et al[63] demonstrated significantly reduced mean numbers of intimal as well as adventitias FoxP3 and GITR in atherosclerotic lesions compared to inflammatory skin lesions,[63] as opposed to normal vessel fragments in which T-cells were virtually absent. This novel finding may account for the chronic inflammatory process that takes place throughout the long-standing course of atherosclerosis. In addition, high-risk lesions contained significantly-increased numbers of Treg compared to early lesions. Similarly, the frequency of $FoxP3^+$ cells in high-risk lesions was somewhat higher compared to the stable ones. Similar to Treg, the frequency of activated T-lymphocytes is reported to be significantly increased in unstable lesions[59,60] and the onset of ACS was shown to be associated with the antigen-driven proliferation of certain T-cell subpopulations.[64] It appears that the overall increase in T-cell-mediated inflammatory activity within the unstable plaque environment may account for the subsequent increased frequency of Treg in these unstable lesions. De Boer et al[63] speculated that the reason for this low frequency of Treg in atherosclerosis may rise from local inhibition by oxidized lipids already present in the intima or from the direct contact with plaque-derived lipoproteins transported via microvessels to the adventitia. The mechanisms involved in Treg suppression of proatherogenic immune responses, however, have yet to be resolved. The mechanisms may involve contact-dependant or cytokine-dependant suppression as some studies would suggest.[44,50,53] However, when interpreting the data, caution should be taken when extrapolating findings from animal models to humans. One should keep in mind that unlike mice, in which most $CD4^+CD25^+$ Treg express FoxP3, this master transcription factor is less abundant in humans in an equivalent population.[65] Moreover, in humans, T-cell receptor engagement is sufficient to stimulate a notable expression of FoxP3, whereas this is not evident in $CD25^-$ cells from mice.

Our laboratory has recently demonstrated that several statins (HMG-CoA reductase inhibitors), which are in widespread use due to their LDL-reducing properties and concomitant improvement of clinical outcome in patients with and without preexisting atherosclerosis, induce expansion of functionally active $CD4^+CD25^+Foxp3^+$ Treg in humans in vitro and in vivo.[66] Increased numbers of Treg cells by statins in the atherosclerotic lesion may be beneficial by reducing the pathogenic responses mediated by the effector T-cells in the atheroma and thus possibly enhance the stability of the atheromatous plaque. Altogether, those studies shed light on the encouraging beneficial clinical potential of Treg in stabilizing atherosclerotic plaques.

Treg and Atherosclerosis: Prospects

The last decade has witnessed very important progress in our understanding of the pathophysiology of atherosclerosis. The discovery of endogenous counter-regulators of the pathogenic immune response in atherosclerosis led to the identification of an important role for Treg cells in the control of lesion development and/or progression. FoxP3 was demonstrated to be a "master regulator" gene for this subset of T-cells. Data gathered from in vivo data in general, and in particular data demonstrating that increasing the numbers of Tregs in the atherosclerosis prone (ApoE−/−) mice by means of adoptive transfer leads to smaller atherosclerotic lesions, suggests that the Treg population appears to be capable of modifying plaque burden in vivo. Reduced numbers of functionally active intraplaque Treg as well as in peripheral blood in patients with ACS compared to bood from patients with stable plaques or blood from healthy individuals further supports the perception of Treg involvement in immunomodulatory reactions protecting from coronary diseases. Although the data reviewed here suggest that Treg function has a central role in the regulation of the proatherogenic T-cell response, much effort should be directed towards the delineation of the major determinants of the regulatory response and to the molecular mechanisms involved in their survival, homing and suppressive function. The potential for treating atherosclerosis by manipulating Treg responses will require a better characterization

of the antigens that proatherogenic T-cells recognize and the antigens that drive development of peripheral induction of Treg which migrate into atherosclerotic lesions. Identification of such antigens might pave the way for vaccination-like strategies using such antigens to promote a disease-specific regulatory response and reduce atherosclerosis development. In addition, greater knowledge about the long-term behavior of Treg after transfer to humans is also essential in order to establish treatment protocols. Thus preliminary human trials of adoptive Treg transfer may provide further insights into the use of Treg- modulating strategies in the treatment of patients with atherosclerosis and ACS. Lastly, although the expression of FoxP3 is now accepted as the gold standard for defining either thymic-derived Treg cells or Treg cells that might be generated in the periphery, one must consider the potential role of subpopulations of $FoxP3^+$ Treg with different functional properties, especially in humans in whom the CD25 expression levels might vary among the $CD4^+FoxP3^+$ T-cell expressors.

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