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Nilsson, Jan; Wigren, Maria; Shah, Prediman K

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**Regulatory T cells and the control of modified lipoprotein
autoimmunity-driven atherosclerosis**

Jan Nilsson¹, Maria Wigren¹, Prediman K Shah²

¹Department of Clinical Sciences, Malmö University Hospital, Lund
University, Sweden and ²Atherosclerosis Research Center and Division of
Cardiology at Cedars-Sinai Medical Center and David Geffen School of
Medicine at UCLA School of Medicine, Los Angeles, USA

Address for correspondence: Jan Nilsson, CRC Entrance 72; 91:12,
Malmö University Hospital, SE-205 02 Malmö, Sweden, Phone: 46
40 391230, Fax: 46 40 391203, Email: jan.nilsson@med.lu.se

Abstract

It has long been recognized that arterial inflammation plays a key role in the development of atherosclerosis. More recent evidence has suggested that this inflammation is modulated by autoimmune responses against modified self antigens, such as oxidized low density lipoprotein, in the vascular wall.

However, the role of the immune system in atherosclerosis appears to be more complex than in classical autoimmune diseases, and a number of protective immune responses has also been identified. One of the most important of these is carried out by the regulatory T cells. Regulatory T cells inhibit the development of autoimmunity by controlling the activity of autoreactive T cells. If the function of regulatory T cells is compromised in hypercholesterolemic mouse models of atherosclerosis, the development of disease becomes much more aggressive. In this review we will discuss the possibility that the inflammatory activity in atherosclerotic lesions depends on the balance between plaque antigen-specific pro-inflammatory Th1-type T cells and anti-inflammatory regulatory T cells specific for the same antigen. We will also discuss the role of hypercholesterolemia in generation of these modified self antigens as well as ongoing research aiming to develop novel immune-modulating therapy for prevention of cardiovascular disease by targeting these processes.

Inflammation plays a key role at all stages of the atherosclerotic disease process. In early lesions chemokines and adhesion molecules expressed by endothelial cells recruit inflammatory cells to the arterial intima. If the acute arterial inflammatory response does not resolve and macrophages and T cells continue to accumulate in the intima this will eventually lead to activation of a fibrotic repair process and development of raised fibromuscular lesions (Hansson 2005). This process is driven by intimal leukocytes that secrete cytokines and growth factors which stimulate medial smooth muscle cells to differentiate into a fibroblast-like phenotype and migrate to the intima, where they proliferate and produce extracellular matrix proteins. However, from a more clinical perspective the most important feature of inflammation in atherosclerosis is in the advanced lesion where matrix metalloproteinases secreted from activated macrophages may degrade the connective tissue in the fibrous cap causing plaque rupture. It was initially believed that inflammation in atherosclerosis was caused solely by lipids (primarily oxidized LDL) directly interacting with pro-inflammatory signal pathways in vascular cells and monocytes/macrophages. Subsequent studies have also confirmed the importance of these mechanisms by demonstrating that mice lacking key components of innate immune signaling, such as Toll-like receptors 2 and 4 as well as their signaling

protein Myd 88, develop less vascular inflammation and atherosclerosis in response to hypercholesterolemia (Bjorkbacka et al. 2004 Michelsen et al. 2004).

However, it has also become clear that the regulation of inflammation in atherosclerosis is much more complex, and recent research has demonstrated that adaptive immune responses are of equal importance (Hansson and Libby 2006). One concept that has emerged from these studies is that atherosclerosis to some extent can be viewed as an autoimmune disease in which the adaptive immune system is targeted against vascular self-antigens modified by hypercholesterolemia (Nilsson and Hansson 2008). In this respect, atherosclerosis shares some similarities with other diseases considered to be of partial autoimmune etiology, such type I diabetes and rheumatoid arthritis. However, the role of the immune system appears to be even more complex in atherosclerosis, because there is evidence that the autoimmune responses evoked by hypercholesterolemia also may have protective effects. Moreover, acute myocardial infarction does not show the same association with a restricted number of HLA types as type I diabetes and rheumatoid arthritis, suggesting the involvement of multiple rather than

single autoantigens (Björkbacka et al 2010). The major immune pathways of importance in the development of atherosclerosis are outlined in Figure 1.

The evolving understanding of the importance of the balance between protective and disease-promoting autoimmunity in atherosclerosis has also stimulated the development of novel therapies, such as vaccines, that may be used to selectively modulate these immune processes. A particularly interesting target for such therapies is the regulatory T cell (Treg), which has been attributed a critical role in controlling the development of immune responses against self antigens. In this review we will discuss the role of Tregs in controlling autoimmune responses induced by hypercholesterolemia and the possibility to selectively activate Tregs in an antigen-specific manner by vaccines.

Autoimmunity against antigens induced by hypercholesterolemia promotes atherosclerosis

The first evidence for involvement of autoimmune responses in atherosclerosis was discovered more than 20 years ago when Jonasson and coworkers demonstrated expression of MHC class II antigen as well as the presence of activated T cells in human atherosclerotic plaques (Jonasson et

al. 1985). Findings from initial studies using hypercholesterolemic mice deficient in all lymphocytes (resulting from *Rag* or *SCID* gene mutations) were not entirely conclusive and suggested a role for immunity primarily in animals with a low cholesterol challenge (Zhou et al. 2000 Reardon et al. 2001). Detailed phenotyping of T cells in human plaques revealed that these primarily were of the pro-inflammatory Th1 type (Frostegard et al. 1999). Subsequent animal studies in which LDL receptor-deficient (*Ldlr*^{-/-}) or apolipoprotein E-deficient (*ApoE*^{-/-}) mice were cross-bred with mice lacking or over-expressing different factors involved in Th1 T cell differentiation, including CD4, the co-stimulatory molecules CD40, CD40L (CD252), OX40 (CD134), the Th1 differentiation transcription factor T-bet, IL-12, interferon (IFN) γ and the INF γ receptor (for a recent review see (Gotsman et al. 2008)) are consistent with the notion that activation of Th1 T cells contributes to a more aggressive progression of atherosclerosis. Studies evaluating the possible involvement of Th2 cells in atherosclerosis were less conclusive. While *ApoE*^{-/-} mice deficient in the Th2 cytokine IL-4 were partially protected against atherosclerosis (Davenport and Tipping 2003), several lines of evidence suggest that B cells have a protective role. Major and colleagues (Major et al., 2002) demonstrated that B cell deficiency in *Ldlr*^{-/-} mice was associated with a 30-40% increase in

atherosclerosis (Major et al. 2002). It has also been shown that the more aggressive atherosclerosis that characterizes splenectomized mice can be reversed by B cell reconstitution (Caligiuri et al. 2002). The latter observations were of particular importance because they represented some of the first evidence of the existence of protective immunity in atherosclerosis.

Oxidized LDL is a major autoantigen in atherosclerosis

The circumstance that the experimental studies describing the pathogenic role of Th1 immunity in atherosclerosis were based on induction of disease by hypercholesterolemia suggests that the relevant autoantigen is a lipoprotein or possibly a protein modified by lipids. Most attention has focused on the role of oxidized LDL in these processes. Antibodies against oxidized LDL are common in humans (Hulthe 2004). Oxidized LDL, as well as oxidized LDL autoantibodies, have also been isolated from atherosclerotic plaques (Yla-Herttuala et al. 1994). T cells specifically recognizing epitopes in oxidized LDL are present in the circulation and are enriched in human atherosclerotic lesions (Stemme et al. 1995). Collectively these studies provide strong evidence for the existence of autoimmune responses against oxidized LDL.

Paradoxically, it was studies evaluating the functional role of autoimmunity against oxidized LDL that first revealed the existence of athero-protective immune responses. We and others found that hypercholesterolemic rabbits immunized with oxidized LDL unexpectedly developed a partial protection against atherosclerosis (Palinski et al. 1995; Ameli et al. 1996). Similar observations were subsequently made in mouse models of atherosclerosis (George et al. 1998 Zhou et al. 2001).

Immune-modulation as a novel therapeutic approach for prevention of atherosclerosis

The finding that athero-protective immune responses could be activated by immunization with oxidized LDL pointed to the fascinating possibility of developing an atherosclerosis vaccine. Several laboratories initiated studies to reveal the active antigens in oxidized LDL and to characterize mechanisms involved in the protective effect. The result of these studies demonstrated the presence of multiple antigens related either to peptide fragments of B-100 (Fredrikson et al. 2003a) or to oxidized phospholipids (Binder et al., 2003) that, when incorporated into pilot vaccines significantly inhibited the development of atherosclerosis (Fredrikson et al. 2003b Chyu et al. 2005). Unexpectedly, it was also found that part of the protective effect

appeared to be unrelated to the oxidized LDL antigens and instead was due to a direct effect of the adjuvant (Khallou-Laschet et al. 2006). Zhou and coworkers (Zhou et al. 2005) reported that the protective effect of the adjuvant was lost in CD4-deficient mice, suggesting that presentation of peptide antigens still was of importance even though no antigen had been co-administered with the adjuvant. A possible explanation of this apparent paradox was provided by Wigren et al (Wigren et al. 2009), who showed that adjuvants in a hypercholesterolemic environment may capture oxidized antigens locally at the injection site, thus mimicking the mode of action of oxidized LDL-antigen based vaccines. If adjuvants also have athero-protective properties in humans with hypercholesterolemia remains to be determined.

Many vaccines function through the generation of neutralizing antibodies. Initial studies suggested that this was also the case for oxidized LDL antigen-based vaccines. The protective effect of immunization can be transferred into non-immunized mice by transplantation of spleen cells (Chyu et al. 2005), and it has been shown that the more aggressive development of atherosclerosis that occurs in splenectomized mice can be reversed by B cell substitution (Caligiuri et al. 2002). Immunization with

apo B peptides results in generation of apo B peptide-specific antibodies, and high levels of antibodies against apo B peptides have been associated with less severe atherosclerosis and a lower risk for development of acute cardiovascular events in humans (Fredrikson et al. 2007 Sjogren et al. 2008). Finally, treatment with recombinant antibodies specific for the aldehyde-modified 661-680 amino acid sequence of apo B has been shown to inhibit the development of atherosclerosis (Schiopu et al. 2004) and to induce regression of existing lesions (Schiopu et al. 2007) in hypercholesterolemic mice. The mechanisms through which recombinant antibodies against apo B peptides protect against atherosclerosis remain to be fully characterized, but experimental studies have implicated a removal of oxidized LDL from plasma, inhibition of the proinflammatory effect of oxidized LDL, as well as stimulation of reverse cholesterol transport by facilitating the uptake of extracellular oxidized LDL by plaque macrophages. Taken together, these observations have provided strong support for the notion that the protective effects of apo B peptide-based vaccines are mediated through generation of peptide-specific antibodies. However, subsequent studies have revealed that immunization with apo B peptides may inhibit atherosclerosis also in the absence of a specific antibody response (Fredrikson et al. 2008) demonstrating that alternative mechanisms must exist. Ongoing studies in

several laboratories point to the regulatory T cells (Tregs) as the most likely candidate for this alternative protective immune pathway.

Tregs control autoimmunity

The most important function of the immune system is to recognize and eliminate infectious microorganisms. In doing so it is vital that the immune system can differentiate these pathogens from self antigens. However, a complete deletion of all autoreactive T cells would impair the effectiveness of the infectious defense, because some self antigens share structural similarities with foreign pathogens. As a result a fraction of moderately self-reactive T cell escape deletion in the thymus and activation of such cells can potentially lead to development of autoimmune disease. Several mechanisms exist to control autoreactive T cells, but the most important is the Tregs, and loss of Treg function results in development of autoimmune diseases.

Several subtypes of Tregs have been identified. The natural Tregs (nTregs), characterized by expression of the α -subunit of the IL-2 receptor CD25 and the transcription factor FoxP3, are developed in the thymus in response to weak stimulation by self-antigens. These cells migrate to the periphery where, if they encounter their self-antigen, they secrete inhibitory cytokines such as IL-10 and TGF- β or simply deprive effector T cells of the IL-2 these

cells require to proliferate. A second class of inducible Tregs (iTregs) is generated in the periphery and is characterized by expression of IL-10 (Tr1 cells) or TGF- β (Th3 cells; Figure 1). It is likely that these Tregs control autoimmunity against modified self antigens since modified self-antigens are unlikely to be presented in the thymus. Accordingly, it can be logical to assume that autoimmunity against preserved native components present in oxidized LDL is controlled by nTregs, while that against modified structures is controlled by iTregs.

Tregs and atherosclerosis

Several lines of evidence have implicated dysregulation of Treg function in atherosclerosis (Mallat et al. 2007). Atherosclerotic plaques contains relatively few FoxP3 positive Tregs (1-5% of all T cells) as compared to normal arterial tissue or inflammatory skin lesions, where Tregs constitute about 25% of all T cells, suggesting that local tolerance protection is impaired in atherosclerotic plaques (de Boer et al. 2007). Treg numbers are lower in old, atherosclerotic *ApoE*^{-/-} mice than in young, non-atherosclerotic *ApoE*^{-/-} mice (Mor et al. 2007) and they are also lower in *ApoE*^{-/-} than in age-matched wild-type mice (Wigren et al. 2009). Depletion of Tregs through deletion of CD80/86, CD 28 or ICOS, as well as anti-CD25 antibody

treatment, significantly increases plaque formation (Ait-Oufella et al. 2006 Gotsman et al. 2006). Similarly, inhibition of Th3 cells through deletion of the T cell receptor for TGF- β markedly enhance the progression of the disease (Robertson et al. 2003), while administration of a clone of ovalbumin-specific Tr1 cells together with its cognate antigen inhibited plaque development in *ApoE*^{-/-} mice (Mallat et al. 2003). Inhibition of atherosclerosis in *ApoE*^{-/-} mice has also been observed in response to transfer of CD4⁺CD25⁺ Tregs (Mor et al. 2007). An interesting approach to study the role of FoxP3 expressing Tregs in atherosclerosis was recently published by van Es and coworkers, who immunized *Ldlr*^{-/-} mice with FoxP3-transfected dendritic cells, thereby inducing autoimmunity against the cells supposed to control autoimmunity (van Es et al. 2009). This procedure was found to result in depletion of Tregs and increased atherosclerosis. A similar approach was used by Xiong et al (Xiong et al. 2009), who used antisense technology to induce apoptosis selectively in Tregs and showed that this was associated with aggravated vascular inflammation. Collectively, these studies provide convincing evidence for a protective role of Tregs in experimental atherosclerosis. The role of Tregs in development of atherosclerosis in humans remains to be explored. Treg numbers are lower and their function appears to be compromised in patients with acute

coronary syndromes (Mor et al. 2006). However, it has not been elucidated whether the decrease in Tregs is the cause or the consequence of the acute event.

Therapeutic targeting of Tregs

The identification of Tregs as an important protective factor in atherosclerosis has focused the attention on these cells as possible targets for intervention. As discussed above, there is accumulating evidence that the rate of progression of the disease is determined by the balance between pro-inflammatory Th1 and anti-inflammatory Treg responses against modified vascular antigens. The possibility to modulate this balance towards a predominance of Tregs represents an attractive therapeutic possibility. At the same time it is important that this is done in a strictly antigen-specific manner to avoid compromising infectious immunity. Ideally, such therapy would induce Tregs that remain inactive until they encounter their specific antigen (such as, for example, oxidized LDL) in an atherosclerotic lesion. They would subsequently release factors that inhibit plaque inflammation and stimulate repair responses to stabilize the plaque. One example of a Treg-targeting therapy for atherosclerosis was recently proposed by Sasaki et al (Sasaki et al. 2009), who showed that oral anti-CD3 antibody treatment

was associated with increased Tregs and inhibition of atherosclerosis. However, it is unclear if this approach will be sufficiently selective in respect to the antigen-specificity of the induced Tregs. Vaccines based on oxidized LDL antigens represent one possible approach for activation of plaque antigen-specific Tregs. An association between Treg activation and inhibition of atherosclerosis has been observed following oral immunization with oxidized LDL (van Puijvelde et al. 2006) as well as in response to a subcutaneous capture of oxidized LDL antigens by Alum (Wigren et al. 2009). Ongoing work in our laboratories suggests that apo B peptide based atherosclerosis vaccines also activate Tregs and that this effect is of critical importance for the athero-protective effect of immunization.

Future perspectives

The emerging recognition of the role of plaque-antigen autoimmunity in atherosclerosis has focused attention on the possibility of developing immune-modulating therapy for prevention of cardiovascular disease. A first generation of such therapies is now approaching clinical testing and phase I safety studies of an apo B peptide-based atherosclerosis vaccine are expected to be initiated during 2010. However, the relatively incomplete understanding of the mode of action of these novel therapies, in combination

with a rudimentary characterization of clinical associations between dysregulation of autoimmunity and cardiovascular disease, constitute important obstacles to the translation of the promising experimental findings to the clinic. Increased focus on these issues should be a matter of high priority. However, in spite of these limitations we believe that it is realistic to assume that one or more immune-based therapies for prevention and treatment of cardiovascular disease will have become clinically available within the coming 5-10 year period.

Figure 1.

Immune activation in atherosclerosis

Antigens are presented by MHC class II (MHCII) molecules on antigen presenting cells (APCs). T cells with a T cell receptor (TCR) recognizing the antigen will become activated. Beside the TCR-MHC class II interaction, activation of a naïve T cell requires co-stimulation. The co-stimulatory molecules CD80 and CD86 on APCs interact with CD28 on T cells. CD80/86-CD28 interaction is required for any T cell activation. CTLA-4 is another co-stimulatory molecule that interacts with CD80 and CD86. In contrast to CD28, CTLA-4 provides an inhibitory signal leading to an inhibition of T cell activation. APCs also express ICOS ligand (ICOSL), which is a co-stimulatory molecule of the same family as CD80 and CD86. ICOS expressed on T cells interacts with ICOSL. APCs also express CD40 that interacts with CD40L on T cells. The antigen taken up by the APC determines the pattern of co-stimulatory molecules and cytokines secreted from the APC. This pattern will determine the further fate of the naïve T cell. Differentiation into Th1 cells results in an inflammatory, pro-atherogenic immune response. IFN- γ is an important cytokine in Th1 cell differentiation and function. CD40-CD40L interaction, ICOSL-ICOS interaction and IL-4 secretion are characteristic of a Th2 cell response. Differentiation into Th2

cells leads to activation of B cells. B cells will secrete antigen-specific IgG that help to clear the antigen. In atherosclerosis, Th2 cells and B cells are considered anti-atherogenic because of their ability to remove oxidized LDL. Antigens presented on APCs can also induce T cells with a regulatory phenotype. Regulatory T cells are immune inhibitory cells that dampen immune responses. Tr1 and Th3 cells are regulatory T cells that secrete the anti-inflammatory cytokines IL-10 and TGF- β , respectively.

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