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Athero-protective immunity and cardiovascular disease: therapeutic opportunities and challenges

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Abstract

Emerging knowledge of the role of atheroprotective immune responses in modulating inflammation and tissue repair in atherosclerotic lesions has opened promising opportunities to develop novel therapies directly targeting the disease process in the artery wall. Regulatory T cells (Tregs) have a protective role through release of anti-inflammatory cytokines and suppression of auto-reactive T effector cells. Studies in experimental animals have shown that blocking the generation or action of Tregs is associated with a more aggressive development of atherosclerosis. Conversely, cell transfer and other approaches to expand the Treg populations *in vivo* have been shown to result in reduced atherosclerosis. There are still relatively few clinical studies of Tregs and cardiovascular disease but the available evidence is also in line with a protective function. These observations have raised hope that it could be possible to develop therapies that act by enforcing the suppressive activities of Tregs in atherosclerotic lesions. One approach to achieve this goal has been through development of vaccines that stimulate immunological tolerance for plaques antigens. Several pilot vaccines based on LDL-derived antigens have demonstrated promising results in pre-clinical testing. If such therapies can be shown to be effective also in clinical trials this could have import impact on cardiovascular prevention and treatment. This review article summarizes current knowledge of the mode of action of athero-protective immunity and ways to stimulate such pathways in experimental settings. The challenges in translating this knowledge into the clinical setting are also discussed and set into the perspective of the experience of the introduction of immune-based therapies for other chronic, non-infectious diseases.

Atherosclerosis is the major cause of acute cardiovascular events such as myocardial infarction and stroke. Current therapies focusing on risk factor reduction, primarily by treatment with cholesterol-lowering statins, have proved to reduce the risk of ischemic cardiovascular events by up to 40% in randomized clinical trials [1]. Although this has significantly lowered mortality in cardiovascular disease, statin therapy still leaves the majority of treated subjects without effective protection. Accordingly, it is important to identify novel targets for therapy that can act on top of current medications. Experimental, clinical and epidemiological research has identified inflammation as a major pathogenic mechanism in atherosclerosis and inflammatory markers as novel risk factors for cardiovascular disease [2]. Therefore, novel therapies targeting the inflammatory disease process in the atherosclerotic plaque represents an exciting new frontier for therapy development. Accumulating evidence suggests that autoimmune responses against plaque antigens are an important cause of vascular inflammation [3]. Modulation of these processes represents a new and promising approach to reduce cardiovascular risk. This review will discuss possible ways to modify immune responses of relevance for atherosclerosis to prevent and treat cardiovascular disease as well as the challenges in translating such novel therapies into clinical application.

Immune tolerance to atherosclerotic antigens

The contribution of adaptive immune responses to atherosclerosis has been robustly demonstrated in murine models, and adaptive immunity has been inferred to be of relevance to human atherosclerosis based on descriptive and correlative studies (recently reviewed in [3, 4]). A cardinal feature of adaptive immunity is self-tolerance, which is actively induced by various

mechanisms in the thymus during lymphocyte development and after development in the peripheral immune system. Immunologic tolerance is induced by exposure of lymphocytes to the antigens they specifically recognize under conditions that result in death or enduring inactivation of the lymphocytes. After tolerance induction, re-exposure to the same antigens under typically immunogenic conditions will not activate a response. Autoimmunity results from the failure of these mechanisms of self-tolerance [5]. The adaptive immune responses that promote atherosclerosis may be thought of as autoimmune in that they are directed against self-antigens that are generated by modification of normal self-molecules. Such modified self-molecules include oxidatively-modified components of lipoproteins, which accumulate in arterial walls, and are recognized by both innate immune pattern recognition receptors and by the adaptive immune system's T cells and antibodies. One of the key goals of research in the field of atherosclerosis immunology is to develop approaches to induce tolerance to these atherosclerosis antigens. In order to achieve this goal, we need to better understand what the relevant antigens are, and/or lean how to induce tolerance to these antigens. Indeed, there is ample evidence that major mechanisms of tolerance discovered by immunologists, including regulatory T (Treg) cells and immune inhibitory molecules do regulate pro-atherogenic immune responses in mice [6, 7] (figure 1). However, these mechanisms are overwhelmed in the typical severely hypercholesterolemic mouse models. If similar mechanisms are at play in limiting pro-atherogenic immune responses in human, they are also clearly inadequate to prevent what is a ubiquitous disease in immunologically normal individuals. The challenge is to enhance physiological tolerance mechanisms that may already target the relevant harmful lymphocytes, but which are nonetheless ineffective without intervention.

Inducing long lived antigen specific regulatory T cells

FoxP3$^+$CD4$^+$CD25$^+$ Treg cells are clearly required for the maintenance of self-tolerance, and their absence in mice and humans results in lethal autoimmunity [8]. The protective impact of Treg on atherosclerosis has been demonstrated by depletion and adoptive transfer studies in mice [9] **(Table 1).** We are likely to need answers to many unresolved questions about athero-protective Treg cells before we can take therapeutic advantage of these cells. The generation of stable long lived memory Treg cells specific for self-antigens may be a major physiological mechanism by which antigen-specific tolerance is induced to self-antigens. In order to induce true T cell tolerance to antigens that drive pro-atherogenic effector T cell responses, it would be necessary to establish a long lived or self-renewing population of atherosclerosis-relevant antigen-specific Treg cells in patients. The desired result would be to increase numbers of athero-protecive Treg cells, or to favorably change the ratio of Treg to pro-atherogenic effector T cells. One general way to achieve this result is to expand the number of pre-existing Treg, either by in vitro expansion and adoptive transfer or by in vivo expansion using drugs. The assumption underlying these approaches is that the starting population of Treg cells in the patients will include those of the relevant antigen specificity. Clinical trials of adoptive Treg therapy for the treatment of type 1 diabetes [10] and allograft rejection [11] have been conducted, the former with in vitro expanded polyclonal autologous Treg cells, and the latter with in vitro expanded alloantigen-specific autologous Treg [12, 13]. There are no data available that these transfers increase long lived memory Treg in recipients. Given the technical and logistical hurdles related to expanding enough autologous Treg cells, it is unlikely that Treg cell transfer would ever be practical for treatment of atherosclerosis. A more practical approach would rely on expanding

endogenous Treg cells in vivo. One way this can be accomplished is by treatment with IL-2/anti-IL2 conjugates which selectively expand Treg in vivo [14] (see below).

A second general approach to establish tolerance to atherosclerosis-relevant T cell antigens is to specifically induce Treg cells specific for those antigens by delivery of the antigens via tolerogenic routes or in tolerogenic forms. For example, many studies have shown that immunization with various derivatives of LDL, including oxidized-LDL and ApoB100 peptides, via putatively tolerogenic routes, reduces atherosclerosis lesion development in mouse models, reviewed in [15]. In many of these cases, increases in Treg cells in lymphoid organs, blood, or lesions has been reported. Nonetheless, there is inadequate data showing that robust antigen-specific tolerance can be reliably induced by these methods, and there is only limited data showing that the increase in Treg is the mechanism by which lesion development is controlled. Treg cell activation requires antigen recognition by the TCR on the Treg cells. The identity of the antigens that are the relevant targets of autoreactive effector T cells in tissue specific autoimmune diseases are unknown in general and it is not clear these would be the same antigens that must be recognized by Treg to control those diseases. Thus in atherosclerosis, we remain ignorant about both the precise antigens (e.g. peptides) that pro-atherogenic T cells recognize, and the peptides recognized by Treg cells that control such responses.

Many molecules that mediate suppression or inhibition of immune responses in the context of tolerance have been identified and well characterized. Some of these are cytokines elaborated by Treg cells and other cell types, including TGFβ and IL-10 (figure 1). The atheroprotective properties of these cytokines have been well documented by the accelerated lesion development and increase in lesion inflammation in mice genetically lacking these cytokines [3]. Therapeutic administration of these cytokines would only accomplish passive temporary immunosuppression;

the value of knowing their role in immune tolerance is for understanding the desired phenotype of

long lived regulatory cells that we need to induce to control disease.

Coinhibitory and costimulatory molecules as targets for tolerance induction

Another important class of regulatory molecules that appear to be essential for self-tolerance is

cell surface coinhibitory receptors expressed by T cells. At the same time the TCR binds antigen,

these coinhibitory receptors bind ligands expressed on antigen presenting cells, generating

inhibitory signals that block antigen driven activation of the T cell (figure 1). The best defined

coinhibitory receptors are the CD28 family members cytotoxic T-lymphocyte–associated protein

4 (CTLA-4, CD152), which binds B7-1 (CD80) and B7-2 (CD86), and programmed cell death 1

(PD-1, CD279), which binds PD-L1 (CD274) and PD-L2 (CD273). Both CTLA-4 and PD-1 are

targets of clinically approved blocking antibody drugs to enhance T cell immunity to tumors [16].

Other inhibitory receptors on T cells that are potential therapeutic targets include lymphocyte-

activation gene 3 (LAG-3, CD223), which binds class II MHC, and T -cell immunoglobulin and

mucin domain 3 (TIM3), which is reported to bind Galectin-9 [17] . All these molecules are

expressed on activated effector T cells, and more highly expressed on "exhausted" effector T

cells in individuals with chronic infections or tumors, where they function to block responses.

Interestingly, these same molecules are also found on Treg cells, where they function to enhance

Treg cell development and to mediate their suppressive activities [18-21]. The atheroprotective

influence of some of these T cell inhibitory molecules has been demonstrated in mouse models

by enhanced disease after antibody blockade or genetic deletion. Blocking these inhibitory

receptors has proven useful to enhance wanted T cell response to tumors and possibly chronic

viral infections, but such blockade also enhances risk for autoimmunity in patients. In mice, blocking or genetic absence of these inhibitory receptors enhances atherosclerotic lesion growth and inflammation. For example, treatment of $Ldlr^{-/-}$ mice with blocking anti-TIM3 enhances atherosclerotic lesion development and lesion inflammation [22]. Genetic absence of PD-1 or its ligands PD-L1 and PD-L2, also enhances lesion development and CD4+ and CD8+ T cell infiltration of lesions [23-25]. On the other hand, agonists of these inhibitory receptors, which have not yet been developed for clinical use, could theoretically be therapeutic for autoimmunity and atherosclerosis. Analogous to recently developed approaches in tumor immunotherapy, in which drugs target and release these physiological molecular "brakes" on tumor-specific T cells in order to enhance anti-tumor immune responses, in atherosclerosis, therapies are needed to target and tighten those brakes on atherosclerosis-antigen specific lymphocytes. An important question about these coinhibitory pathways is if they can be therapeutically engaged in a way that results in true long lasting tolerance without the need for chronic treatment.

Costimulatory pathways, especially mediated by B7-1 (CD80) and B7-2 (CD86) on APCs binding to CD28 are essential for activation of naïve T cells and their differentiation into effector cells. Furthermore B7-1 and B7-2 deficiency in atherosclerotic prone mice results in less lesion formation and inflammation and impaired T cell responses to the atherosclerosis-associated antigen HSP60 [26]. One of the best defined experimental methods to establish antigen-specific T cell anergy is antigen exposure in the absence of co-stimulation. In mice B7-costimulation in combinations with blockade of the CD40-CD40-Ligand pathway, which induces expression of costimulators on APCs, can induce long lasting alloantigen-specific tolerance and prolonged survival of allografts [27]. CD40 blockade is reported to prolong allograft graft survival in nonhuman primates [28], and there is some evidence that blockade of B7 and CD40 in primates

may also synergize in inducing alloantigen tolerance [29]. B7 blockade by CTLA-4-Ig (abatacept and belatacept) is an approved therapy for rheumatoid arthritis and for immunosuppression in renal allograft recipients. The potential of using costimulatory blockade with combined reagents, such as CTLA-4-Ig and anti-CD40 to tolerize patients to autoantigens that drive autoimmune disease or atherosclerosis has not been fully explored because of thrombotic complications of earlier trials with anti-CD40 ligand reagents, and the logistics of combining two reagents in clinical trials.

Experimental approaches for induction of atheroprotective immunity

As discussed above, our recent understanding that atherosclerosis is promoted by the alteration in the balance between the suppressive activity of Treg cells and activation of harmful effector Th1 immunity has opened new opportunities for manipulating Treg cells to stimulate atheroprotective immunity through the induction of immune tolerance (Table 2), a therapeutic approach that is being tested to prevent or treat autoimmune, inflammatory, or allergic diseases.

One way to promote early immune tolerance is to administer the antigen when the immune system is developing. Exposing mice to high dose of antigens during thymic T-cell education induces immune tolerance to the administered antigens. This strategy has been used successfully in newborn *ApoE-/-* mice that were injected with oxLDL at birth [30]. Remarkably, neonatal tolerance to oxLDL reduced atherosclerosis in adult *ApoE$^{-/-}$* mice, and normalized the T-cell repertoire that was altered by the development of the disease [30]. This was the first evidence that immune tolerance can protect against atherosclerosis. Subsequently, additional evidence was provided for the possibility to promote atheroprotection by the adoptive transfer of IL-10

producing Tr1 cells, a subtype of Treg cells that can induce a bystander immune suppression and limit the development of atherosclerosis [31]. *ApoE*$^{-/-}$ mice injected with clones of ovalbumin (OVA)-specific Tr1 cells with their cognate antigen developed less atherosclerosis compared with mice receiving only OVA without Tr1 cells or Tr1 cells without OVA.

Numerous different approaches have been developed to manipulate Treg cells as a crucial component of immunotherapy. These include strategies to enhance Treg cell generation, through cytokines or sub-immunogenic antigen administration, as well as strategies to modulate the activation, expansion, survival or suppressive function of Treg cells by interfering with intracellular signaling pathways [32]. In the context of atherosclerosis, most of these strategies have shown beneficial atheroprotective effects.

Treg cell generation using self-antigens

Using self-antigens for the specific induction of Treg cells is the most attractive approach to specifically target the deleterious effects of self-reactive immune cell functions while maintaining the ability of the immune system to fight infections. Specific atherosclerosis-related antigens have not yet been clearly identified, but candidates, including oxLDL, Hsp60 or ApoB100-peptides, have been suggested [4, 33]. Nasal or oral administration of small doses of Hsp65 in *Ldlr*$^{-/-}$ mice reduced atherosclerosis [34, 35], by shifting the immune response toward a Th2 response and production of IL-4 [34] and IL-10 [35] in tolerized animals. Others have used mucosal administration of oxLDL [36], Hsp60 [37], or ApoB-100 peptides fused to the B subunit of the cholera toxin [38] to limit atherosclerosis development. In these models, the induction of

immune tolerance was associated with an increase in Treg cells, and TGF-β and/or IL-10 production.

Extrathymically induced Foxp3+ Treg cells can be expanded by antigen delivery [39]. Remarkably, van Boehmer's group has shown that long-term subcutaneous infusion of adjuvant-free, low-dose antigen can transform mature effector Th cells into Treg cells [40]. A similar approach has been found effective in inducing atheroprotective immunity in *Apoe*-/- mice that were subcutaneous administered with low doses of ApoB-100 peptides for 2 weeks. This "tolerogenic vaccination" promoted antigen-specific Treg cells and reduced cytokine production by Th1 and Th2 cells [41]. Although no direct evidence has yet been provided that antigen-specific Treg-cell responses were responsible for the atheroprotective effect obtained with these therapeutic approaches, they have provided evidence that enhancement of Treg cells is feasible in atherosclerosis.

Vaccines activating atheroprotective immunity

The first indications that it could be possible to use a vaccine approach to treat atherosclerosis came from unexpected observations of decreased atherosclerosis in hypercholesterolemic rabbits immunized with oxidized LDL [42, 43]. Subsequent studies identified certain peptide sequences in Apo-B-100 [44] and phospholipids [45] as the key antigens responsible for this effect and suggested that immunization had shifted a pre-existing Th1 response towards Th2 and Tregs [46]. Prototype vaccines based on the Apo B-100 peptides p45 (amino acids 661-680) and p210 (amino acids 3136-3155) have demonstrated promising results in pre-clinical studies [47-49] and are expected to enter clinical testing within a few years. The mechanisms through which these

vaccines function remains to be fully elucidated but the protective effect a prototype vaccine containing p210 was blocked by co-administration of CD 25-blocking antibodies suggesting the involvement of Tregs [50]. Administration of a recombinant IgG against aldehyde-modified p45 has been shown to inhibit atherosclerosis in mice indicating that activation of Th2 responses also may be of importance [51, 52].

Approaches to induce Treg cell expansion

<u>Anti-CD3</u>. Antibodies directed against CD3 have been shown to be able to reconstitute self-tolerance in autoimmune diseases, including type 1 diabetes [53]. In atherosclerosis, intravenous or oral anti-CD3 therapy enhanced TGF-β and Foxp3 mRNA expression in lymphoid organs and reduced the development of the disease in Ldlr$^{-/-}$ mice [54, 55].

<u>IL-2</u>. IL-2 is a critical cytokine for Treg cell homeostasis. IL-2 combined with the IL-2 specific monoclonal antibody (JES6-1) rapidly and specifically expanded Foxp3+ Treg cells in mice [56]. Recently, in clinical studies low-dose IL-2 preferentially expanded Treg cells in patients with chronic graft-versus-host disease without altering effector T cell numbers, and provided clinical improvement in half of the [57]. In another study, low-dose IL-2 increased Treg/Teff cell ratios in patients with autoimmune vasculitis induced by hepatitis C virus infection and showed beneficial clinical effects in 9 out of 12 patients [58]. More recently, the same favorable effect of low dose IL-2 on Treg/Teff cell ratios was obtained in patients with type 1 diabetes [59]. In *Apoe$^{-/-}$* mice, functional delivery of IL-2 to pre-established atherosclerotic lesions [60] or IL-2/anti-IL-2 mAb (JES6-1) treatment [61] resulted in Treg expansion and reduced atherosclerotic lesion development.

Statins. Statin treatment is also reported to enhance circulating Treg numbers in atherosclerotic plaques [62] and in circulation of patients [63], and this may reflect in part direct effects of statins on T cell differentiation, distinct from indirect immune effects secondary to lipid lowering [64].

Vitamin D3. Calcitriol, an active form of vitamin D3, can promote Treg cell expansion by inducing tolerogenic dendritic cells [65], or acting directly on CD4+ CD25- T cells to generate Foxp3+ T cells expressing high levels of CTLA-4 [66]. In *ApoE-/-* mice oral administration of calcitriol promoted the induction of both Treg cells and tolerogenic dendritic cells, and reduced atherosclerosis [67]. Interestingly, there exists a relationship between low plasma levels of vitamin D3 and predisposition to adverse cardiovascular events [68].

Tolerogenic Dendritic Cells. Treg cell survival and homeostatic proliferation critically depend on continued interactions with DCs, presumably presenting the relevant autoantigen in a tolerogenic context. Several studies have highlighted the important role of dendritic cells in maintaining atheroprotective Treg cells. Blocking DC maturation through CD11c-specific knockdown of MyD88 altered the Treg cell pool and accelerated atherosclerosis [69]. Moreover, the lack of ICOS or PD-1, inhibitory pathways of the TNF superfamily, reduced Treg cell capacity and lead to increased atherosclerosis in *Ldlr*^{-/-} mice [23, 70]. However, DCs receiving strong anti-inflammatory signals, such as TGF-β or IL-10, show tolerogenic properties, which have exploited for prevention and therapy of autoimmune diseases [71]. Interestingly, DCs made tolerogenic to LDL by treating them with IL-10 and loading them with human ApoB100, and administered to LDLr-/- mice transgenic for human apoB100 (huB100[tg]×*Ldlr*^{−/−} mice) induced T-cell tolerance to ApoB100, with dampened Th 1 and Th2 immune responses to apolipoprotein B (ApoB)-100, and reduced atherosclerosis [72].

Microbiota. Bacterial metabolites from commensal microorganisms regulate the immune system by promoting peripheral Treg cell expansion [73]. Butyrate, a short-chain fatty acid produced by commensal microorganisms from dietary fibres after fermentation in the large intestine, promoted extrathymic Treg cell differentiation from CD4+ T cell precursors and enhanced the ability of DCs to stimulate Treg cell differentiation [73]. Interestingly, dietary supplementation of butyrate reduced atherosclerosis in apoE$^{-/-}$ mice [74], associated with diminished vascular inflammation, but this report made no mention of the status of Treg cells. Yet, these resent studies open new avenues for studying the communication between nutrition, microbiota, regulatory immunity and atherosclerosis.

Translating findings from mouse into clinical application

The concept that adaptive immunity plays an important role in atherosclerosis is primarily based on studies performed in mouse models of atherosclerosis. The challenge of translating these findings into new clinical therapies for cardiovascular disease should not be underestimated. Although lesion morphology is similar in mouse and man it remains to be fully understood if the disease mechanisms are the same. Accordingly, novel therapies developed solely on the basis of findings from mouse models are likely to have a large risk of failure when brought directly into clinical testing. One approach to decrease this risk is to use translational models such as "humanized" mice (with a human immune system) as well as mechanistic studies on cultured human immune cells and artificial ex vivo human immune organs. Indirect support can also be obtained from clinical studies of genetic and biomarker associations with risk for development of cardiovascular disease. It should be kept in mind that also findings from such studies need to be

interpreted with some caution because statistical association does not necessarily prove the existence of causality. However, such associations can still provide strong support of the validity of findings from experimental models.

Clinical evidence for the existence of atheroprotective immunity

In spite of the extensive support for a role of adaptive immunity in atherosclerosis from experimental studies the clinical evidence for this is still mostly circumstantial. Associations between oxLDL autoantibodies, the severity of atherosclerosis and risk for CVD events have been reported in a large number of studies [75, 76], but the results have been inconsistent possible due to technical difficulties in standardizing the assays used. Analysis of autoantibodies against certain peptide sequences in Apo B (primarily p45 and p210) have provided more consistent findings and shown that high antibody levels correlate with less severe atherosclerotic burden and a lower risk for myocardial infarction [77-79]. In a prospective study with 15 years follow-up Wigren et al reported that high levels of CD4$^+$FoxP3$^+$ Treg cells were associated with a decreased risk for development of acute coronary events [80]. In accordance with a protective role of Treg cells in cardiovascular disease several case-control studies have also identified reduced levels of Treg cells in patients with acute myocardial infarction and unstable angina [81-83]. Treg cell numbers have been shown to be low in vulnerable plaques [84], but interestingly to be enriched in thrombi adhered to ruptured plaques [85]. The association of other immune cell types with cardiovascular disease is less well studied but high levels of Th2 cells and CD19$^+$CD40$^+$ B cells have been found to be associated with lower risk for coronary events in one prospective study [86, 87]. Detailed immune cell phenotyping requires the use of multi-channel

flow cytometers and antibodies that have become available only during more recent years. Accordingly, the association between immune cell subtypes and cardiovascular risk has so far only been addressed in a few prospective studies but it is likely that much more information will become available in the next couple of years.

The risk for several types of chronic inflammatory diseases with autoimmune characteristics such as type 1 diabetes, systemic lupus erythematous (SLE), rheumatoid arthritis, and psoriasis is associated with certain HLA genotypes but such associations are weak for cardiovascular disease [88, 89]. Nonetheless these autoimmune disease are associated with significantly increased risk of accelerated atherosclerotic disease. The reason for this discrepancy is not clearly understood Pathway analysis of data from genome wide association studies have identified strong associations between coronary artery disease and networks linked to inflammation [90, 91]. However, with the exception of the IL-6 receptor, which is of importance for differentiation of Th17 cells, no genes that that can be specifically linked to adaptive immunity have so far reached genome-wide significance.

Experience from clinical translation of immune-based therapies in other diseases

Conventional approaches for treatment of autoimmune diseases have in the past largely relied on non-specific immunosuppressive drugs leading to substantial side effects. This situation has changed dramatically with the introduction of antibody-based therapy targeting specific immune pathways. The use of TNF-α and IL-1β receptor blocking antibodies has revolutionized the treatment of rheumatoid arthritis and several other autoimmune diseases. Novel therapies are also being developed to more specifically target T and B cell-dependent immunity including vaccines

and recombinant antibodies. For diseases such as type 1 diabetes mellitus (T1DM), Alzheimer's disease, rheumatoid arthritis, SLE, psoriasis, Crohn's disease, Bechet's disease and inflammatory bowel disease such therapies are already in Phase II and III clinical testing mostly targeting, B cells or Th17 cells **(Table 3).**

T1DM is an autoimmune disorder directed against the pancreatic islet β cells. The first manifestation of islet autoimmunity is the occurrence of autoantibodies against islet antigens such as insulin, GAD65, IA-2 and the ZnT8 transporter. The aim of immune therapy in type 1 DM has been to induce tolerance against islet antigens. Clinical trials have been performed to test the ability of parental, oral and intra-nasal administration of insulin to prevent T1DM in islet autoantigen positive subjects or to preserve β cell function in early stages of the disease but the results have so far been disappointing [10]. A subcutaneously administered Alum-formulated GAD65 vaccine has demonstrated some positive effects on preserving residual C-peptide levels phase II clinical trials and this effect was associated with induction of Treg cells [92, 93]. A phase I trial (NCT01210664, http://clintrials.gov) to study the effect of *ex vivo* expanded Treg cells in T1DM is also ongoing.

In Alzheimer's disease both active and passive immunization therapies have been developed to reduce the accumulation of amyloid-β peptide in brain tissue [94]. The proposed mechanisms include amyloid-β peptide clearance, binding of immune complexes to inhibitory receptors on immune effector cells and effects of amyloid-β peptide on neurons. Attempts to develop vaccines for Alzheimer's disease have suffered several setbacks including difficulties to induce a good antibody response in elderly patients. One clinical trial was halted because of the development of T-cell-mediated menigoencephalitis caused by activation of Th1 T cells [95]. Therapy based on

recombinant amyloid-β peptide antibodies has been proven safe but have so far not demonstrated convincing effects on clinical outcome.

B cells and B cell-derived autoantibodies play a central role in the pathogenesis of many autoimmune diseases [96, 97]. Depletion of B cells by treatment with anti-CD20 antibodies have demonstrated beneficial effects rheumatoid arthritis and is used clinically in patients that are inadequate responders to anti-TNF therapies. While anti-CD20 therapy failed to prevent clinical relapse in SLE more promising results have been obtained in clinical trials using antibodies against BAFF (belimumab) that selectively targets B2 cells [98]. This is of considerable interest from a cardiovascular perspective since experimental studies have demonstrated that B2 cells are pro-atherogenic while B1 cells have a protective function [99]. However, a long-term use B cell depletion therapy in cardiovascular disease is less attractive due to increased risk of adverse effects including the risk for infections.

Attempts have also been made to treat autoimmune disease by targeting generation of Th17 cells or the Th17 signature cytokine IL-17A (reviewed in [100]). Antibodies against the IL-6 and IL-12/IL23 receptors have been approved for clinical use in moderate rheumatoid arthritis and psoriasis and an antibody against IL-17A have shown promising results in phase III trials for the same diseases. Anti-Th17 therapy could represent a possible therapy also in CVD although the role of Th17 cells in atherosclerosis remains to be fully characterized. The generation of plaque antigen-specific Treg cells represents a very attractive option for treatment CVD. Antigen-specific Treg ells have been shown to prevent and suppress disease in experimental models of SLE, T1DM, multiple sclerosis and rheumatoid arthritis [101-103] but this approach remains to be clinically evaluated.

Approaches for immune therapy in CVD

There is still limited experience from clinical testing of immune-based therapy for cardiovascular disease. Several of established therapies for prevention of cardiovascular disease also have effects on the immune system. For instance statin therapy is known to favor expansion of Treg cells [63] but the clinical importance of this effect remains to be fully understood. There are no ongoing clinical trials directly targeting the adaptive immune system but the possible protective effect of anti-inflammatory therapy is being evaluated in two large phase III trials. The Cardiovascular Inflammation Reduction Trial (CIRT) randomized 7000 patients with prior myocardial infarction and T2DM or metabolic syndrome to low-dose methotrexate or placebo [104]. CIRT started in 2012 and is designed to accrue a total of 530 major cardiovascular events over an estimated average follow up of 3 to 4 years. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) randomized 17200 postmyocardial infarction patients with persistent elevation of hsCRP to placebo or 50, 150 or 300 mg of the IL-1β inhibitor Canakinumab every 3 months [105]. The trial was started in 2011 and completion is estimated to occur after the accrual of at least 1400 major cardiovascular events. The Goal of Oxidized Ldl and Activated Macrophage Inhibition by Exposure to a Recombinant Antibody (GLACIER, http://clintrials.gov) is a multicenter, placebo controlled, randomized phase II trial to study the effect of MDA-p45 Apo B antibody (see above) on carotid plaque inflammation. Plaque inflammation was determined by measuring uptake of radioactively labeled glucose by PET/CT. The results of this study have not yet been published but according to a press release from the sponsor it failed to demonstrate a significant effect on the primary endpoint. As discussed above several prototype atherosclerosis vaccines have demonstrated beneficial effects in experimental animal models. They primarily act

by inducing tolerance against LDL-associated antigens and in this respect resemble the immunization strategies developed for prevention of T1DM. These vaccines are still in preclinical development but are likely to enter into clinical testing within a few years.

Future perspectives

Today almost all patients that develop a recurrent cardiovascular event are already on state-of-the-art preventive treatment. To offer help to these patients new therapies need to be develop that directly target disease processes in the atherosclerotic plaque to dampen inflammation and to stimulate vascular repair responses. Targeting immune responses against plaque antigens represents a novel and attractive approach since these responses modulate both inflammation and repair processes. Such an approach is also attractive because it is likely to be additive to that of current therapies. The pathophysiological importance of adaptive immunity in atherosclerosis is well documented in animal models of the disease and evidence for a role also in clinical disease is becoming more and more convincing. Several different approaches to reinforce atheroprotective immunity have demonstrated good efficacy in experimental models and a first generation of immune-based therapies for cardiovascular disease are now in or about to enter clinical testing. There is considerable experience of development of immune-based therapies from other chronic inflammatory and autoimmune diseases such as T1DM, Alzheimer's disease, rheumatoid arthritis, SLE, psoriasis and Crohn's disease. These novel therapies have for some disease revolutionized daily care but have for others proven much more difficult than anticipated. For a successful clinical development of immune-based therapies for cardiovascular disease it will be important to learn from these experiences. One important lesson is that mechanistic

findings from animal models of disease not always can applied be to humans and that appropriate translational models need to be developed. An important advantaged of vaccines against infectious diseases is that they are antigen specific. This would be attractive from an efficacy and safety perspective also for cardiovascular disease but it remains to be fully established if this will be possible. Another important challenge will be how to monitor the response to immune-based therapies in cardiovascular disease. Again in depth understanding of the mechanism of action of the therapy will be of crucial importance. However, in spite of these hurdles we believe that that enforcing atheroprotective immunity represents a promising and realistic novel target for cardiovascular therapy and that such therapy has the potential to have significant clinical impact by acting directly on the disease process in the atherosclerotic lesion. **The CANTOS trial is the first large randomized, placebo-controlled study to specifically evaluate the potential of immune-based therapy for CVD. When the results of this trial become public a few years from now they will be of critical importance for determining the potential of the immune system as a target for prevention and treatment of CVD. If this turns out to be true the next and even greater challenge will be to develop immune-based therapies that selectively target the arterial wall. To reach this goal a much better understanding of the role adaptive immunity in the human atherosclerotic disease process will be required.**

Disclosures

Jan Nilsson and Alain Tedgui are signed as co-inventors on patents involving atheroprotective vaccines.

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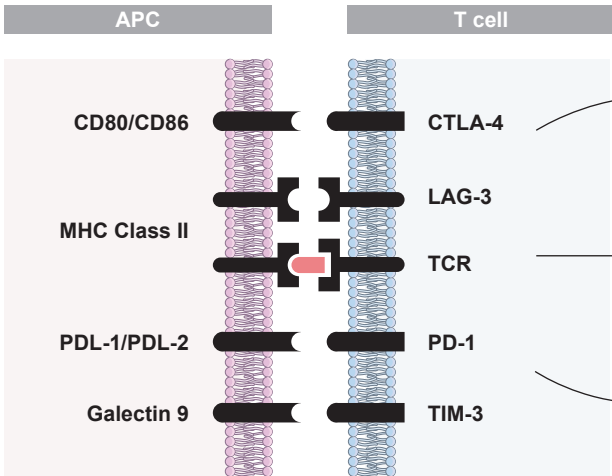
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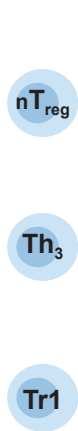
Figure legends

Figure 1: Atheroprotective immune response by Tregs. Actrivation of Tregs in lymphatic tissues is mediated by stimulation of coinhibitory receptors that bind ligands expressed on antigen presenting cells. Dependent on cellular origin and mode of stimulation different types of Tregs are generated and enter the circulation. Tregs that encounter their cognate antigen in the vascular wall suppress T effector cells with similar antigen-specificity through local depletion of IL-2 and suppress inflammation through the release of anti-inflammatory cytokines such as TGF-β and IL-10.
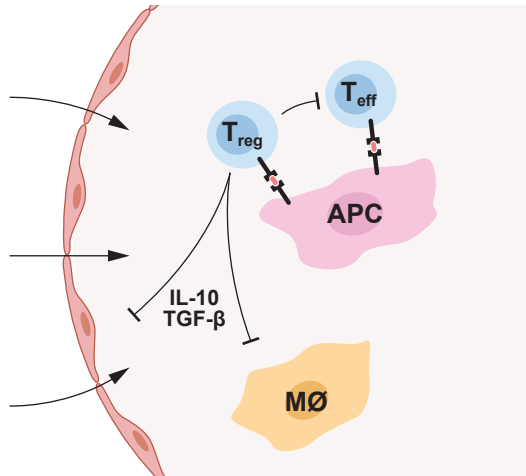
Lymphatic tissue

APC

CD80/CD86	CTLA-4
MHC Class II	LAG-3
	TCR
PDL-1/PDL-2	PD-1
Galectin 9	TIM-3

T cell

Circulation

nT_{reg}

Th_3

$Tr1$

Vascular wall

T_{eff}

T_{reg}

APC

IL-10
TGF-β

MØ

Table 1

Immune tolerance and atherosclerosis
• *Tregs are required for maintaining self-tolerance*
• *Generation of Tregs takes places in the thymus (natural Tregs) or in peripheral tissues (induced Tregs)*
• *Th1-type immune responses against plaque antigens promotes plaque development*
• *Tregs suppress activation of auto-reactive naïve and effector T cells, through blockade of costimualtion, competition for T cell growth factors, and release of anti-inflammatory cytokines Depletion of Tregs aggravates atherosclerosis*

Table 2

Experimental approaches to enforce athero-protective immunity
• *Anti-CD3 antibodies*
• *IL-2 and IL-2/anti-IL-2 immune complex*
• *Vitamin D3*
• *Tolerogenic dendritic cells*
• *Changing of the microbiota*
• *Tolerogenic vaccines*
• *Treg transfer*

Table 3

Autoimmune diseases and immune therapy
• *Non-specific immunosuppressive drugs are often effective but are also associated with serious side effects*
• *TNF-α and IL-1β antibodies are established therapies for RA and several other autoimmune diseases*
• *IL-17 antibodies in clinical trial for RA and psoriasis*
• *CD20 antibodies approved for RA therapy*
• *BAFF antibodies in clinical trial for SLE*
• *Costimulatory blockade with CTLA-4-Ig is approved for RA therapy*
• *Tolerogenic GAD65-based vaccines in clinical trial for T1DM*
• *Amyloid-β peptide vaccine and antibodies in clinical trials for Alzheimer's disease*