Review

Anti-inflammatory and immune-modulatory therapies for preventing atherosclerotic cardiovascular disease

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Atherosclerosis is believed to be a chronic inflammation of the arterial wall and various immune cells of innate and adaptive immunity involves in the pathogenesis of atherosclerosis. Based on this notion, several anti-inflammatory strategies for prevention of atherosclerosis have been examined mainly using animal models. Vaccination or mucosal immunization with athero-antigens comes under candidate therapeutic methods for antigen-specific prevention of atherosclerosis. Immune suppression mediated by regulatory T cells (Tregs) could be another method to regulate pathogenic chronic inflammation in atherogenesis. Inducible Tregs are reported to differentiate peripherally in the intestine and we have been interested in the oral tolerance, in which not only Tregs but also tolerogenic dendritic cells play crucial roles. We demonstrated that modulation of the intestinal immunity including oral tolerance could be a novel therapy against atherosclerosis. Further, downregulation of effector T cell response and/or Treg predominant condition was shown to induce atherosclerosis regression and inhibit the progression of aneurysm.

In clinical situations, none of the approaches to specifically and directly treat inflammation to prevent cardiovascular events or reduce atherosclerosis in human individuals were successful, although high-sensitive C-reactive protein is shown to have a strong relationship with recurrent events of cardiovascular diseases in several randomized clinical trials. Now two randomized placebo-controlled clinical trials evaluating anti-inflammatory agents are being conducted in the USA and Canada to clarify whether targeting the inflammation itself will reduce cardiovascular events and risks.

In this review, we present the current understanding of anti-inflammatory and immune-modulation therapies against atherosclerosis and discuss the future perspectives.

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Introduction

Cardiovascular disease (CVD), a leading cause of mortality in many developed and developing countries, is caused mainly by atherosclerosis. Clinical studies and animal experiments have established that high plasma concentrations of cholesterol, mainly transported by low-density lipoprotein (LDL), promote atherosclerotic CVD and statin-based lipid lowering therapy reduces CV events. However, some clinical trials revealed residual cardiovascular risks cannot be ignored even after the aggressive reduction of LDL cholesterol to target levels. Despite great advances in treating acute coronary syndrome with catheter-based therapies and controlling risk factors of CVD, there is still an enormous need for additional therapies as the recurrent rate of CV events remains at about 20% within 3 years even with optical medical treatment [1].

Atherosclerosis is considered not only a disorder of lipid accumulation in the arterial wall but also a chronic inflammatory disease that contains components of both innate and acquired immune systems [2–5]. Several immune responses are critical factors in the initiation and progression of atherosclerosis (Fig. 1). The first step preceding the atherosclerotic lesion formation is endothelial activation or dysfunction and LDL-cholesterol deposition in the arterial wall, which are mediated by coronary risk factors such as dyslipidemia, hypertension, diabetes mellitus, and smoking. Secondly, the accumulated LDL is oxidized and the resultant formation of oxidized LDL (OxLDL) has been suggested to be the critical event in deteriorating inflammation in vascular wall. Thirdly, not only monocytes but also various types of leukocytes adhere to the activated endothelium, migrate into the arterial wall via upregulated adhesion molecules, and produce pro-inflammatory cytokines or chemokines. Subsequently, monocyte-derived macrophages take up OxLDL via scavenger receptor leading to the formation of lipid-laden foam cells. Following such steps, the initial fatty streaks contain lipids and numerous immune cells such as macrophages, dendritic cells (DCs), and T lymphocytes. Progressed atherosclerotic lesions involve the migrated smooth muscle cells (SMCs), debris, apoptotic cells, and extracellular matrix such as collagen and proteoglycans [5]. B lymphocytes and their producing immunoglobulins including IgG and IgM are thought to be associated with atherogenesis. Finally, such indolent progressed atherosclerotic plaques may suddenly rupture and induce life-threatening coronary thrombosis presenting as an acute coronary syndrome. The notable features of unstable rupture-prone plaque are infiltration of many inflammatory cells, large lipid core, and thin fibrous cap [6,7].

Fig. 1. Role of inflammatory cells and immune responses in atherogenesis. Low-density lipoprotein (LDL) is deposited in the subendothelial space, and the accumulated LDL is oxidized to OxLDL (oxidized LDL) that activates the endothelium. Coronary risk factors also activate the endothelium and induce the adhesion molecules. Monocytes migrate into the subendothelial space using the adhesion molecules, differentiate into macrophages, take up OxLDL, and change to foam cells. The protein components of the OxLDL particle are processed and presented as antigens to T cells by macrophages and dendritic cells (DCs). Other self and foreign antigens may also trigger similar immune reactions. T cells differentiate into effector T cells (Th1, Th2, and Th17) and release cytokines and chemokines, and stimulate the migration of smooth muscle cell (SMC) and other inflammatory reactions. Migrated SMCs change their phenotype from contractile SMCs to synthesized ones that produce cytokines. Synthesized SMCs and foam cells contribute to form the atherosclerotic plaques including the lipid core and fibrous cap formation. Proatherogenic cytokines including IFN-γ secreted by Th1, and IL-12 secreted by DCs and macrophages deteriorate the lesion formation, might be associated with destabilizing the plaque, and induce the plaque rupture. Regulatory T cells (Tregs) suppress effector T cell activation, the differentiation of naive T cell into effector T cells, and downregulate antigen presentation of DCs via secretion of anti-inflammatory cytokines including interleukin (IL)-10 and transforming growth factor (TGF)-β. Tolerogenic DCs, characterized by downregulated expressions of CD80/CD86, maintain the tolerance to self-antigens by inducing Tregs or by inhibiting effector T cells. Immunoglobulins produced by B cells are also thought to play a role in atherogenesis. apo B, apolipoprotein B; CRP, C-reactive protein; HSP, heat shock protein; IFN, interferon; Ig, immunoglobulin.
Taken together, we might prevent atherosclerotic CVD by intervening at each step or factor that is associated with the inflammatory processes in atherogenesis, although none of the approaches treating specific inflammation to prevent the CV events in humans were successful to date. Several anti-inflammatory drugs are being examined for their potential to reduce the residual CV risks in CVD patients. In this review, we focus on the anti-inflammatory therapies of clinical trials and animal experiments and consider the future perspectives of fighting against atherosclerotic CVD.

Clinical trials of direct anti-inflammatory therapies for cardiovascular disease

To date, no clinical trial has addressed whether targeting the inflammation itself will reduce CV events and risks. Now two randomized placebo-controlled clinical trials evaluating anti-inflammatory agents are being conducted in the USA and Canada (Fig. 2) [8,9]. High-sensitive (hs) C-reactive protein (CRP) has been shown to have a strong relationship with recurrent events of CVD in several randomized clinical trials [10]. hsCRP is produced in the liver stimulated by interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6). Based on the basic animal studies, inhibition of such cytokines and chemokines could reduce atherosclerosis.

The Canakinumab Anti-inflammatory Thrombosis Outcomes Study [CANTOS] is the first large randomized controlled clinical study testing whether the use of canakinumab, human anti-IL-1β monoclonal antibody, can prevent secondary CV events in subjects with stable coronary artery disease considered at high inflammatory risk despite usual therapy [8]. Results of this CANTOS trial are expected in 2016. If successful, this trial would provide a new cytokine-based therapy for secondary prevention of CVD.

Another one is the National Heart Lung and Blood Institute initiated Cardiovascular Inflammation Reduction Trial [CIRT], in which patients with chronic atherosclerosis and either diabetes mellitus or metabolic syndrome will be randomized to usual care or usual care plus low-dose methotrexate (15–20 mg/week) [9]. This low-dose methotrexate is routinely and safely used as an anti-inflammatory regimen for the treatment of rheumatoid arthritis and the primary endpoint is major cardiovascular events. Results of this clinical trial can be expected in 2018.

If both or either of the two clinical trials are successful, they would support the inflammatory hypothesis of atherosclerosis and provide a novel IL-1β-, IL-6-, and CRP-associated cytokine-based therapy for inhibiting CV events and we could have new anti-inflammatory therapies for secondary prevention of CVD.

Why does chronic inflammation exist in atherosclerotic lesions and how to regulate it?

Numerous studies of human atherosclerotic lesions and genetically altered mouse strains including the apolipoprotein E knockout (apoE−/−) and LDL receptor knockout (LDLr−/−) mice have provided many important clues on the link between immune cells and the pathogenesis of atherosclerosis [5]. Following antigen presentation by antigen-presenting cells such as macrophages or DCs to naive T cells, adaptive T cell-mediated immune responses are initiated, and many activated CD4+ T cells are observed in atherosclerotic lesions in both humans and mice [11,12]. CD4+ T cell clones which respond to some specific antigens derived from atherosclerotic plaques have been shown to recognize suspected self-antigens important for atherosclerosis. To keep the chronic inflammation in the atherosclerotic lesions, several continuous antigenic stimuli might be needed, although sterile inflammation (inflammatory) was also reported to play critical roles in atherogenesis [13]. The candidate antigens related with atherosclerosis are OxLDL, heat shock proteins (HSP), and several foreign antigens including bacteria such as Chlamydia pneumonia and Porphyromonas gingivalis [14]. However, the true antigens responsible for driving atherosclerosis remain unidentified.

The elimination of foreign antigens, immunization of autoantigens, or tolerance induction to antigens might alleviate inflammatory disease. To eliminate the bacterial antigens, several human clinical trials using antibiotics for prevention of atherosclerotic CVD were performed. Unfortunately, no antibiotic clinical trials were effective for preventing CV events in humans and antibiotics cannot be considered as anti-atherogenic agents [15]. The question, whether this is the proof that infections and their related foreign antigens have no role in atherogenesis or whether the treatment was too late to start for preventing CV events, is still unanswered.

The immunization study with OxLDL or modified LDL demonstrated a remarkable protective effect on atherosclerosis, reducing lesion area in hypercholesterolemic rabbits and mice by 30–50% [16–18] (Fig. 3). Since LDL particle contains apoproteins, mainly apolipoprotein B (apoB), and many lipid species, the key antigens associated with atherosclerosis could not be identified to date. In any case, this series of experiments were the first trial of immunization (vaccine) therapies against atherosclerosis and they proposed a new notion that we might prevent CVD via vaccination with some specific antigens. Immunization with some specific apoB peptides also reduced atherosclerotic lesion area in mice [19,20]. Further, whole atherosclerotic plaque homogenates were used as antigens for immunization in mice [21]. Their immunization could induce antibodies against modified LDL and inhibited atherosclerotic lesion formation. Several reports indicated the atheroprotective role of anti-oxLDL IgM, but the functional role of anti-oxLDL IgG remains elusive [18]. We had already reported that xenogenic macrophage homogenates immunization could reduce atherosclerosis in mice via inducing anti-macrophage antibodies that inhibited macrophage functions [22].

The cytotoxic chaperon Hsp60 has a high degree of homology to mycobacterial Hsp65. Different from the case of OxLDL, the immunization against Hsp60 or Hsp65 aggravate atherosclerosis in rabbits and mice [23] (Fig. 3). On the other hand, oral administration or mucosal immunization with Hsp60 could reduce the atherosclerotic lesions [24,25]. Taken together, immunization with
all antigens including atherosclerotic lesions is not always atheroprotective. The ultimate purpose of this research including ours is development of anti-atherosclerogenic vaccine to inhibit atherosclerosis or reduce the incidence of CV events [26]. ApoB peptides and other antigens as atherosclerosis vaccines are now clinically tested to develop new immune-therapies against atherosclerotic CVD [20]. We hope some of the antigens can be used for anti-atherogenic vaccines, although we should be careful to apply the experimental results in mice to humans.

**Anti-atherogenic strategy by enhancing regulatory immune response**

Recent compelling data suggest that several subsets of Tregs, responsible for maintaining immunological tolerance and suppressing excessive immune responses [27], inhibit atherosclerosis development or progression by dampening effector T cell responses (Fig. 1). Natural Tregs are defined as a population of T cells expressing CD4, CD25 (IL-2 receptor), and the major transcription factor forkhead box P3 (Foxp3). These T cells are differentiated mainly in the thymus and have unique immunosuppressive activities without antigen exposure in the periphery. Deficiency of Treg function or development has been shown to deteriorate atherosclerotic lesion formation [28–30]. Other Tregs are inducible Tregs (iTregs) including type 1 regulatory T cells (Tr1) and transforming growth factor-β (TGF-β) producing Tregs (Th3).

iTregs are derived from naive or effector T cells and differentiated in the periphery such as intestine. They are also CD4 CD25-positive T cells, but do not require Foxp3 to be functional. Tr1 secrete IL-10 and Th3 produce TGF-β. iTregs maintain immunologic homeostasis mainly through secreting immunosuppressive cytokines and inhibit atherosclerotic lesion formation in mice [31–33]. Both natural and iTregs can prevent autoimmunity and redundant immune responses by suppressing proliferation of naive and effector T cells, their differentiation into Th1, Th2, and Th17 lineage, and the function of other types of lymphoid cells including B cells, macrophages, and DCs. Since Tregs exert suppressive functions regardless of their antigen specificity after activation by antigen presentation [27], we speculate that induction of polyclonal activated Tregs as well as antigen-specific Tregs could be a possible therapeutic approach to atherosclerosis.

Recently, our clinical study demonstrated that the number of circulating natural Tregs and the Treg/effector T cell ratio was decreased in coronary artery disease patients compared with controls [34]. Other previous papers also indicated that low levels of circulating Foxp3-positive cells were associated with an increased risk of coronary events. In consideration of previous studies, we believe that increasing the Treg/effector T cell ratio, by suppressing effector T cell responses and increasing the number of Tregs or promoting Treg responses, could be a promising therapeutic approach for atherosclerotic CVD [35].

IL-2 has long been thought to be a critical cytokine for T cell proliferation and differentiation. In particular, it is crucial for the maintenance of Tregs, because the Treg marker CD25 is a component of the high-affinity IL-2 receptor and is functionally essential for the development of Treg by binding IL-2. Low doses of a recombinant mouse IL-2/anti-IL-2 monoclonal antibody complex (IL-2 complex) administration induced selective expansion of CD4+CD25+Foxp3+ Tregs [36]. IL-2 complex therapy was shown to attenuate atherosclerosis and to inhibit aneurysm formation via selective Tregs expansion (Fig. 4) [37,38].

**Intervention to the Treg/effector T cells ratio**

Parental administration of FcR-non-binding anti-CD3 monoclonal antibody has been shown to suppress effector T cell immune responses and be effective for treating autoimmune diabetes in mice and humans [39], and acute transplant rejection in humans [40]. Notably, this therapy also inhibits atherosclerosis development and progression in atherosclerosis prone mice (Fig. 4) [41]. Previous studies demonstrated induction of CD4+CD25+ Tregs, along with reduced number of effector T cells, from CD3-Ab
The intestine, explaining the long-term protective effects observed in mouse models of autoimmune diseases [42], although this increase in CD4⁺CD25⁺ Treg number was not observed in atherosclerosis-prone mice [41].

To test the hypothesis that under inflammatory conditions such as hypercholesterolemia, suppression of effector T cell immune responses before Treg expansion is important in augmenting regulatory immune responses for efficient reduction in atherosclerosis, we employed the combination of anti-CD3 antibody and IL-2/anti-IL-2 monoclonal antibody complex in apoE⁻/⁻ mice. We demonstrated that this novel combination therapy could effectively reduce atherosclerotic lesion formation and plaque inflammation in mice by strikingly enhancing a regulatory immune response (Fig. 4) [43]. We believe that our findings are highly relevant for shaping future clinical strategies for preventing atherosclerotic diseases.

Recently, we examined the impact of T cell modulation by anti-CD3 antibody on atherosclerosis regression in a mouse model previously reported [44], in which LDLR⁻/⁻ mice were fed a high-cholesterol diet for 8 weeks to form atherosclerotic lesions and were then changed to a standard diet to lower plasma cholesterol. We demonstrated for the first time that intravenous administration of anti-CD3 antibody, in addition to lowering plasma cholesterol, could induce rapid regression of atherosclerosis by reducing CD4⁺ T cells and increasing the proportion of Foxp3⁺ Tregs in both lymphoid organs and atherosclerotic plaques [45]. Depletion of Tregs by anti-CD25 antibody injection abolished the anti-CD3 antibody-mediated atherosclerosis regression, indicating the essential role for Tregs in this process.

**Intestine as a therapeutic target**

Based on the experimental results that oral administration or mucosal immunization of Hsp60 or Hsp65 inhibited atherosclerotic lesion formation in animal models [24,25], we have been interested in the intestinal mucosal immunity and we thought that the intestine could be a therapeutic target for preventing atherosclerosis. Further, we focused on the mechanism of "oral tolerance" to apply its mechanism for prevention of atherosclerosis [46,47]. Accumulating evidence indicated that DCs and Tregs play critical roles in maintaining the tolerance (Fig. 5).

Tregs differentiate from naïve T cells in the periphery under certain conditions, as well as in the thymus [48]. Gut-associated lymphoid tissue (GALT) is known to be the main site for generation of peripheral iTregs, where there are multiple preferential stimuli such as commensal microbiota and food antigens for the induction of Tregs [46]. It was demonstrated that iTreg might play a crucial role in the maintenance of mucosal immune tolerance and in the suppression of allergic inflammation in the intestine. However, the differences between iTregs and natural Tregs or functional stability of iTregs in vivo remain to be determined, and further studies are needed.

The intestinal immune system must protect our body from invading pathogens, but never attacks food antigens and commensal bacteria. Recent studies have revealed that DCs in the small intestine have a crucial role in determining inflammatory or tolerogenic immune responses, but distinct subtypes have not been clearly defined. In the small intestine, DCs are identified by their CD11c expression and can be divided into two subsets depending on their CD80 and CD86, or CD103 expressions [46,47]. CD80⁻CD86⁺ DCs are thought to be active and mature DCs and work as immune activated APCs. On the other hand, CD80⁻CD86⁻ DCs are immature or tolerogenic DCs and induce tolerance in the intestine together with Tregs [46]. CD103⁺ DCs can be divided into two subsets that do or do not express CD11b. Lamina propria CD103⁺CD11b⁺ DCs capture antigens and migrate into mesenteric lymph nodes. Although the precise function of CD103⁺CD11b⁺ DCs in GALT for regulating Tregs remains to be determined, lamina propria CD103⁺CD11b⁺ DCs promote the generation of Tregs through the production of TGF-β and vitamin A.
metabolite retinoic acid. CD103⁺ DCs regulate the balance between immunity and tolerance in the intestine. Elucidation of the mechanisms of how different subtypes of intestinal DCs respond to various antigens and determination of the response toward either immunity or tolerance will contribute to effective tolerance induction therapy.

Studies have shown that oral or nasal anti-CD3 antibody is biologically active and induces TGF-β producing CD4⁺LAP⁺ (latency-associated peptide) Tregs = Th3 that suppress experimental autoimmune encephalitis (EAE) [49] and autoimmune diabetes [50] in a TGF-β dependent fashion. Autoimmune diseases are shown to be suppressed by only low doses of oral anti-CD3 antibody in association with an increase in LAP⁺ Tregs, but not by high doses, although there is no evidence of antigen specificity with oral anti-CD3 antibody [49]. As observed in mucosal tolerance induction, only low doses of oral anti-CD3 antibody administration may result in the induction of Th3 by delivering a weak signal to T cells, although further elucidation of the cellular and molecular mechanisms underlying induction of various Tregs will be needed. We applied this method for the treatment of atherosclerosis in apoe⁻/⁻ mice and demonstrated that oral anti-CD3 antibody treatment induced Th3 and CD4⁺Foxp3⁺ Tregs, which suppressed pathogenic immune processes pivotal for atherogenesis through a TGF-β-dependent mechanism and consequently inhibited atherosclerotic plaque formation [33]. Further, we examined the effect of oral anti-CD3 antibody treatment on the phenotypes of DCs in the mesenteric lymph nodes in mice and confirmed the CD80 and CD86 expressions were reduced in anti-CD3 antibody-treated mice compared to controls (Fig. 5).

Some immature DCs acquire tolerogenic properties by inducing Tregs and inhibiting naïve T cell activation. Epidemiological studies have highlighted the increasing prevalence of vitamin D3 deficiency and its association with increased risks of CVDs and mortality [51]. However, the effect of vitamin D3 supplementation on prevention of CVDs remains controversial in clinical trials and no reports have been published about the direct effects of orally administered calcitriol on atherosclerosis in animal models. We for the first time demonstrated that oral administration of active form of vitamin D3 (calcitriol) decreases atherosclerosis by promoting induction of tolerogenic DCs and Foxp3⁺ Tregs [52]. A cell-therapy strategy, using tolerogenic DCs, revealed that apoB100-pulsed tolerogenic DCs inhibit the proliferative and proinflammatory T cell response to apoB100, promoted Treg induction, and reduced atherosclerotic lesion formation [53]. Oral administration of Hsp60 might also induce Tregs in gastrointestinal tract and affect atherogenesis [25,54].

Although further studies are needed to clarify the precise role of several types of vascular DCs in atherogenesis, effective methods to induce atheroprotective DCs could be novel therapies for prevention of atherosclerosis.

**Concluding remarks**

Since the first evidence of involvement of immunity in atherogenesis was discovered more than 30 years ago, a large number of basic and clinical studies have clearly established that immune responses play crucial roles in the disease process. However, none of the approaches to specifically and directly treat inflammation to prevent cardiovascular events or reduce atherosclerosis in human individuals were successful to date, although hsCRP has been shown to have a strong relationship with recurrent events of CVD, and statin treatment could reduce hsCRP and
prevent CV events independent of lowering LDL cholesterol in several randomized clinical trials such as the JUPITER study [10]. Two large clinical trials of direct anti-inflammatory therapies for CVD [8,9] are now being conducted and we might use the results in our patients for preventing CV events in the near future. Vaccination against atherosclerosis is a hopeful method for prevention of CVD and resultingly reducing medical expenses. Vaccines targeting autoimmunity including atherosclerosis are tolerance induction against self-antigens and amplification of the regulatory responses such as Tregs and tolerogenic DCs described in this review. Since the tolerance induction can yield an immunosuppressed individual with impaired tumor surveillance and increased susceptibility to infections, we should be careful to apply these results of animal research to human clinical situations. A hopeful method to resolve this problem may be antigen-specific Treg induction that many researchers including us wish to achieve.

The intestinal immune system has been attracting much attention as a novel therapeutic target to treat atherosclerosis. Gut-associated immune tolerance induction by oral administration of drugs or therapeutic agents possessing immunoregulatory activities is simple, easy, and a hopeful way to regulate inflammatory diseases, although the detail of mechanisms of how intestinal immunity affects the systemic immune system remains to be clarified. In association with intestinal immunity in atherogenesis, we have been interested in the gut bacteria that might involve the pathogenesis of atherosclerotic CV. Now the gut flora types susceptible to CVD are under investigation.

The immune system represents a novel and promising target for prevention or treatment of CVD. The time when this concept is ready for clinical testing is coming, but it will be critical not to underestimate the difficulties that will be encountered in transferring the promising results from experimental animals to humans.

Conflict of interest

The authors report no conflict of interest.

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