

# Immunization for Atherosclerosis

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This review summarizes experimental findings that highlight the complex roles of the immune system in atherogenesis. Immune activation can have either proatherogenic or atheroprotective effects. Immune-modulation therapy via an active or passive immunization strategy aims to exploit the atheroprotective aspects of the immune system to modulate atherosclerosis. Several experimental studies have demonstrated that such an approach is feasible and effective, raising the tantalizing possibility that an atheroprotective vaccine can be developed for clinical testing. Several potential immunogens have been identified and tested for their atheroprotective efficacy with variable results. Although several questions such as choice of optimal antigens, choice of most effective adjuvants, the optimal route of administration, durability of effects, and safety remain to be answered, we believe that a vaccine-based approach to manage atherosclerotic cardiovascular disease is a potentially viable paradigm.

## Introduction

Atherosclerotic cardiovascular disease continues to be the leading cause of morbidity and mortality in much of the Western world despite many advances in management. These observations highlight the need for new therapies targeting this highly prevalent disease. This review summarizes what we currently know about the complex and conflicting role of the immune system in atherogenesis and how such knowledge may be used to formulate novel immunomodulation therapies for atherosclerotic vascular disease.

## Immunity and Atherosclerosis

Atherosclerosis is a complex, chronic inflammatory disease of the arterial walls as evidenced by the presence of inflammatory cells, activated immune cells, various

molecules, and cytokines to recruit immune cells, complements, and immunoglobulins in the lesions, all of which highlight the involvement of the immune system [1,2]. The immune system can be largely divided into distinct yet overlapping innate and adaptive immunity, but both immunities modulate atherosclerosis. Innate immunity reacts quickly and recognizes common pathogen-associated microbial patterns, such as lipopolysaccharides in gram-negative bacteria and unmethylated CpG DNA motif [3]. It includes macrophages, natural killer (NK) cells, and mast cells and uses complements, various cytokines or chemokines, and cell-mediated cytotoxicity as its effector mechanism. Adaptive immunity reacts more slowly, recognizes more specific antigenic epitopes, its cellular components include T and B cells, and it uses antibodies, cytotoxic T cells, antibody-dependent cell-mediated cytotoxicity, cytokines, or chemokines as its effector mechanisms [3].

Different components of innate or adaptive immunity have been studied using gene knockout or bone marrow transplant strategies to determine their involvement in atherogenesis. Table 1 summarizes several such important examples and a few specific examples are worthy of further discussion. Toll-like receptors (TLR) are a group of pattern recognition receptors of innate immunity. Exogenous infectious triggers interact with TLRs, leading to transcription of several acute inflammatory genes and the eventual release of inflammatory cytokines that characterize the acute innate immune response [4]. Macrophages and endothelial cells in murine and human atherosclerotic lesions express TLR4 and other members of the TLR family [5,6]. Interference in innate immune signaling through genetic disruption of TLR4 or its downstream signaling adaptor molecule (myeloid differentiation factor 88) reduces atherosclerosis, plaque inflammation, and circulating inflammatory proteins in mice [7,8]. On the other hand, a naturally occurring IgM antibody with the T15 idiotype, produced by self-renewing B1 cells without T cell or thymic involvement, might have an atheroprotective role [9,10]. These natural antibodies recognize the phosphorylcholine head group present in the phospholipid moiety of oxidized low-density lipoprotein (LDL), apoptotic cells, and the cell wall of pneumococcus, and they reduce the uptake of oxidized LDL by macrophages and attenuate murine atherosclerosis [9,11]. Our laboratory

**Table 1. Effect of different immune components on atherogenesis**

Model	Immune component	Effect on atherosclerosis	Study
ApoE/RAG-1	T and B cell deficiency	Decrease; no effect if mice on high-fat diet	Dansky et al. [13]
ApoE/RAG-2	T and B cell deficiency	No effect	Daugherty et al. [15]
LDLR/RAG-1	T and B cell deficiency	Delayed	Song et al. [14]
ApoE/SCID	T and B cell deficiency	Decrease	Zhou et al. [68]
ApoE/CD1d	Natural killer T cell deficiency	Decrease	Tupin et al. [23]
ApoE/CD1d	Natural killer T cell deficiency	Decrease	Major et al. [69]
LDLR/complement 3	Defect in classical and alternative pathways	Increase	Edfeldt et al. [6]
ApoE/complement 5	Defect in terminal complement complex	No effect	Patel et al. [70]
ApoE/Myd-88 or ApoE/TLR4	Defect in innate immunity	Decrease	Michelsen et al. [7•]
ApoE/Myd-88	Defect in innate immunity	Decrease	Bjorkbacka et al. [8•]
Splenectomy in ApoE <sup>-/-</sup> mice	Defect in adaptive immunity	Increase	Caligiuri et al. [17]
B cell deficiency in LDLR <sup>-/-</sup> mice	Defect in adaptive immunity	Increase	Major et al. [16]

Apo—apolipoprotein; LDLR—low-density lipoprotein receptor.

has recently demonstrated that passive immunization of hypercholesterolemic mice with this antibody, developed by hybridoma technology, reduces accelerated vein-graft atherosclerosis [12], which further supports an atheroprotective role for innate humoral immune response to the phosphorylcholine head group.

Although the models of total T cell and B cell deficiency in the setting of hypercholesterolemia provided conflicting reports on their roles in atherogenesis [13–15], other experimental approaches have provided a somewhat clearer view regarding the role of B or T cells in atherosclerosis. Using a bone marrow transplantation strategy, Major et al. [16] demonstrated that B cell deficiency in LDL receptor (LDLR) <sup>-/-</sup> mice was associated with a reduced level of anti-oxidized LDL antibody; these mice developed a 30% to 40% increase in the lesion area in the proximal and distal aortas. Caligiuri et al. [17] reported that splenectomy aggravated atherosclerotic lesions in apolipoprotein (apo) E<sup>-/-</sup> mice and such an increase could be ameliorated by adoptive transfer of B cells from donor mice. However, adoptive transfer of naive CD4<sup>+</sup> T cells or CD4<sup>+</sup> T cells from malondialdehyde (MDA)-LDL immunized donors into hypercholesterolemic immune-deficient mice resulted in aggravation of atherosclerosis, supporting the notion that CD4<sup>+</sup> T-cell-mediated immunity is proatherogenic [18,19•]. A subset of CD4<sup>+</sup> T cells that constitutively express CD25 produces immune inhibitory cytokines, such as IL-10 (regulatory T1 cells) or transforming growth factor- $\beta$ , and inhibits the activation of other T cells and favorably influences inflammation and atherosclerosis in murine models [20–22]. Activation of yet another subset of CD4<sup>+</sup> T cells, which express NK 1.1 receptors found on NK cells and recognize lipid antigens presented by the class I-like molecule CD1, worsens early atherosclerosis

in apoE<sup>-/-</sup> mice [23]. Unlike CD4<sup>+</sup> T cells, the role of CD8<sup>+</sup> T cells in atherogenesis is currently unclear.

Through these studies, it is apparent that immune system activation could be either atherogenic or atheroprotective. Given these observations, many investigators have been interested in exploiting the concept of immune modulation as a potential therapy for atherosclerosis.

### Immune Modulation Against Atherosclerosis

There are two major ways to modulate immune responses—via immune suppressive therapy or through immunization (active or passive). In experimental animals, immune suppressive agents have variable effects on atherosclerosis. For example, cyclosporin A was reported to have the opposite effect on atherosclerosis in hypercholesterolemic rabbits in two different studies [24,25]. Although mycophenolate mofetil reduces experimental atherosclerosis [26], FK506 has an opposite effect [27]. Additionally, immune suppressive therapy results in many undesirable side effects and is not likely to have major value for routine clinical use as a therapy for atherosclerotic vascular disease.

Vaccines have successfully reduced death from infectious disease and resulted in the global eradication of diseases such as smallpox and poliomyelitis. Vaccines are effective, specific, relatively inexpensive, and generally well tolerated. In recent years, the concept of vaccination has also been extended to treat or prevent noninfectious chronic inflammatory or autoimmune diseases such as Alzheimer's disease and cancer with variable results. The observation that immunization with lipoprotein could reduce atherosclerosis can be traced back to 1950s [28]; however, it

was not until the past two decades that scientists began to understand how immunization affects atherogenesis. Several molecules have been identified as potential candidate immunogens in atherosclerosis; some are proatherogenic whereas others are atheroprotective (Table 2).

Historically, native LDL or modified LDL was used as an immunogen in immunization studies to reduce atherosclerosis [29–32]. LDL is not an ideal immunogen because it is a large, heterogeneous molecule containing apolipoprotein, cholesteryl ester, triglyceride, and phospholipids. Which components are responsible for immunization-mediated atheroprotection is not known. Although oxidation of LDL leads to degradation of apoB-100 into numerous peptide fragments that could serve as targets for recognition by the immune system [33,34], the exact nature of these peptides is not known. Use of whole LDL as an immunogen for clinical testing is also not practical because of the requirement of its isolation in large quantities and the accompanied safety issues. Therefore, to exploit the potential atheroprotective effects of LDL immunization, it would be useful to be able to identify the protective antigenic epitopes in LDL.

Our laboratories have been studying the structure of the apoB-100 component of LDL to identify potential antigenic epitopes that could mediate the atheroprotective effects of immunization with oxidized LDL [35]. We have generated a library of 302 peptide sequences spanning the entire structure of the human apoB-100 molecule, each one being 20 amino acids in length with an overlap of 5 amino acids [36]. Among these peptides, we have identified 102 peptides that are associated with an antibody response in pooled human serum [35]. Some of the peptide sequences, but not others, when incorporated into a vaccine formulation with alum as an adjuvant, provoke an atheroprotective response in hypercholesterolemic mice, resulting in a 40% to 70% reduction in aortic atherosclerosis along with a reduction in plaque inflammation [36,37]. Such atheroprotection could be passively transferred to nonimmunized mice through adoptive transfer of splenocytes from immunized mice [37]. Passive immunization using a monoclonal antibody to one of the atheroprotective peptide epitopes was also shown to reduce atherosclerosis in hyperlipidemic mice [38]. These studies clearly identified apoB-100-related specific peptide epitopes that are able to trigger atheroprotective immunity and provided evidence that such vaccination strategy is feasible.

A different way to identify possible immunogens is through isolation of relevant antibodies against LDL. Horkko et al. [39] and Friedman et al. [40] successfully generated a panel of B cell hybridomas from naive (nonimmunized) apoE<sup>-/-</sup> mice. A number of clones were selected that generated antibodies with specific binding to epitopes in oxidized LDL. All clones were found to secrete IgM binding either to MDA-LDL (presumably aldehyde-modified apoB-100 peptide sequences) or to copper-oxidized LDL. Subsequent studies showed that all

**Table 2. List of immunogens that have been used in immunization studies**

**Atheropromotion via active immunization**

Heat shock protein 65 [60–62]
β2-glycoprotein I [63]
Atheroprotection via active immunization
Native low-density lipoprotein [31,64]
Malondialdehyde–low-density lipoprotein [65,66]
Apolipoprotein B-100 peptides [36,37]
Phosphorylcholine head group on oxidized phospholipid [11]
Cholesteryl ester transfer protein [49,67]

antibodies binding to copper-oxidized LDL recognized oxidized phospholipids [39,40]. The antibodies recognized epitopes both in the lipid moiety of oxidized LDL and in the delipidated apoB-100, suggesting that the antigen can exist as a free lipid as well as an adduct to apoB-100. One of the IgM antibodies is of the T15 idio type, recognizes phosphorylcholine headgroups on the surface of apoptotic cells, and inhibits uptake of oxidized LDL and apoptotic cells in macrophages [9,39,41]. Antiphosphorylcholine antibodies of the T15 idio type also protect against infection from *Streptococcus pneumoniae* [42]. Binder et al. [11] studied the functional role of antiphospholipid antibodies in atherosclerosis by immunizing LDLR knockout mice with *Streptococcus pneumoniae*. This treatment resulted in induction of high levels of oxidized LDL-specific IgM and a modest reduction of atherosclerosis. We subsequently used another clone of the antiphosphorylcholine IgM antibody of T15 idio type and demonstrated its atherosclerosis-reducing effect in accelerated atherosclerosis in a murine vein-graft model via passive immunization [12]. Currently, we are testing its efficacy in reducing spontaneous atherosclerosis.

Immunization using immunogens unrelated to LDL may also have an atheroprotective effect (Table 2). The beneficial effects of high high-density lipoprotein (HDL) levels against atherosclerosis are well established [43–46]. Cholesteryl ester transfer protein (CETP) is a key enzyme involved in HDL metabolism. Inducing neutralizing antibodies against CETP via immunization results in marked elevation of HDL with concomitant reduction in atherosclerosis in hyperlipidemic rabbits [47–49]. Clinical testing of such a vaccine strategy is ongoing [50]. Interestingly, two CETP inhibitors have been developed and are now undergoing clinical trials to assess their clinical benefits.

Recently, influenza virus infection has been shown to induce vascular inflammation and a prothrombotic state in animal models; it has also been linked to increased cardiovascular events in human subjects [51,52]. Several studies have observed that influenza vaccination is associated with a reduced incidence of various cardiovascular events such as myocardial infarct-

tion, out-of-hospital cardiac arrest, and stroke [53]. A randomized controlled trial of influenza vaccination, the FLU Vaccination Acute Coronary Syndromes (FLU-VACS) study [54,55], provided the strongest evidence to support the use of influenza vaccination to reduce cardiovascular mortality in patients with atherosclerotic coronary disease. Based on these data, both the American Heart Association and the American College of Cardiology recently released a Science Advisory, widely endorsed by several professional associations, to include influenza vaccination as a part of secondary prevention measures for patients with cardiovascular disease [56]. However, only the inactivated form of vaccine should be administered to patients [56].

### Unresolved Questions in Immunization Strategy for Atherosclerosis Management

In addition to the choice of immunogens, for clinical testing of a vaccination strategy based on apo-B100-related peptides a number of critical issues will need to be addressed: 1) the timing of immunization (childhood vs adulthood or both); 2) the most suitable immune adjuvant and the optimum route of administration; 3) vaccine safety and efficacy including durability of effect; and 4) appropriate methods to document an atheroprotective benefit.

The timing of the vaccination is an important issue to consider if a widespread vaccine strategy is to be implemented. Most of the experimental studies of immunization with oxidized LDL or peptide antigens specific for oxidized LDL have demonstrated the prevention of early atherosclerotic lesions when used before a significant lesion develops; whether immunization used after lesions have already formed remains to be established. Although prevention of atherosclerosis by immunization at an early age remains a potential, long-term goal, whether immunization slows plaque progression and/or induces stabilization of existing plaques is also worthy of investigation as a secondary prevention strategy. Our experimental studies have shown that immunization favorably changes the composition of established plaques, indicated by decreased plaque inflammation and increased collagen content [31]. Studies to further address this question in detail are required.

The immune response to an antigen is determined not only by the type of antigen (protein, lipid, or carbohydrate antigen) but also by its route of administration (subcutaneous vs intramuscular vs mucosal) and the choice of the adjuvant, which can influence antigen delivery, presentation, and induction of immunomodulatory cytokines. Commonly known adjuvants include aluminum salt and Freund's complete and incomplete adjuvant. Gels containing aluminum salt are the only adjuvant approved for use in vaccines in the United States. Several new types of adjuvants are

now being tested in preclinical and clinical trials, including liposomes, immunostimulatory complexes, DNA sequences containing unmethylated CpG motifs, and biodegradable polymer microspheres. Understanding the mechanisms through which each antigen contributes to the disease may be necessary to find the optimum combination of antigen, adjuvant, and vehicle of administration.

For a vaccine to be adopted universally, it needs to be effective, safe, and conceptually acceptable to the general public. Several recent examples highlighted the importance of this issue. The concern of an increase in the incidence of autism with the measles-mumps-rubella (MMR) vaccine did raise issues around the MMR vaccination program, although no evidence suggests there is a link between autism and the MMR vaccine [57,58]. Immunization against amyloid  $\beta$ -peptide reduced amyloid peptide-induced neurotoxicity in mouse models of Alzheimer's disease; however, such a clinical trial was deemed to be unsafe and had to be terminated prematurely due to excessive occurrence of meningoencephalitis [59].

How to assess the clinical efficacy of immunization is another difficult question to answer. Atherosclerosis is a chronic disease and it is likely to take a long-term effort for any treatment to affect its hard endpoints such as death or myocardial infarction. Hence, the initial clinical trials to evaluate immunization efficacy would need to rely on the assessment of surrogate endpoints such as atherosclerotic plaque burden or composition using noninvasive and/or invasive imaging modalities. If this initial "proof-of-concept" human trial is successful, long-term trials involving hard endpoints will be required. The optimal duration of follow-up also needs to be defined.

### Conclusions

Taken together, it is apparent that modulation of immune responses related to atherogenesis by active or passive immunization is a novel and promising approach to the prevention and treatment of atherosclerotic cardiovascular disease. Even though our understanding of the complex role of the immune system in atherosclerosis is incomplete and many questions remain unanswered, experimental studies have provided encouraging data. Continued investigation of this promising approach is warranted to ensure safety and durability of benefits before clinical testing is considered. We are cautiously optimistic that there is a future for vaccination for atherosclerosis as a complementary approach to the existing antiatherogenic strategies. It is our sincere hope and dream that someday an atherosclerosis vaccination strategy will become part of the routine childhood (and/or adulthood) vaccination program so that atherosclerosis and its complications can be markedly reduced.

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