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THE PRESENT AND FUTURE

STATE-OF-THE-ART REVIEW

Vaccine for Atherosclerosis

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ABSTRACT

Atherosclerosis is an immune-mediated inflammatory disease of the arterial wall, with both the innate and adaptive immune systems responding to many endogenous and exogenous antigens. Both proatherogenic as well as atheroprotective roles have been identified for the immune system in atherosclerosis. Hence, it is conceivable that an immunomodulatory strategy via active immunization against many of these antigens could potentially alter the natural history of atherosclerosis. This review discusses: 1) the complex role of important components of the innate and adaptive immune systems in atherogenesis; 2) the nature of many antigens that have been tested successfully in vaccine formulations to reduce atherosclerosis in pre-clinical experimental models; and 3) the potential opportunities and challenges for clinical application of vaccination for atherosclerosis in the future. (J Am Coll Cardiol 2014;64:2779–91) © 2014 by the American College of Cardiology Foundation.

Substantial data from experimental and clinical investigation support the role of immune-mediated inflammatory mechanisms in atherogenesis. Cells of both the innate and adaptive immune systems, such as macrophages, dendritic cells (DCs), B and T lymphocytes, and mast cells, are ubiquitous in atherosclerotic plaques (1,2). Immunoinflammatory mediators such as pathogen- and danger-associated molecular pattern molecules, immunoglobulins, cytokines, chemokines, and complement proteins are all present to varying degrees in the atherosclerotic plaque (3,4). In atherosclerosis-prone areas of the murine aorta, especially in the adventitia, antigen-presenting cells are already present in greater numbers than in atheroresistant segments, even before hyperlipidemia or atherosclerotic lesions are introduced, suggesting that there is immunoinflammatory priming in these segments (5).

INNATE IMMUNITY AND ATHEROSCLEROSIS. Innate immunity reflects a nonspecific immediate response to pathogens and other danger signals. Cells of the innate immune system, such as DCs and macrophages, act as sentinels and first responders, sampling the host environment to detect molecular signatures of damage or danger, such as oxidatively-modified low-density lipoprotein (oxLDL). These molecular signatures of damage or danger, which are perceived by the host as molecular insults to the vascular wall, interact with toll-like receptors. Toll-like receptors act as pattern-recognition receptors, leading to the activation of genes involved in acute inflammation and the eventual release of inflammatory cytokines that characterize the acute innate immune response. Disruption of genes involved in this innate immune signaling pathway reduces atherosclerosis, plaque inflammation, and circulating inflammatory proteins in mice, independent of changes in circulating cholesterol levels (6).

Proatherogenic roles for innate immune cells are demonstrated by the deletion of genes involved in their differentiation and proliferation. Hypercholesterolemic mice deficient in the macrophage colony-stimulating factor gene, which is essential

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ABBREVIATIONS AND ACRONYMS

DC = dendritic cell

HSP = heat shock protein

Ig = immunoglobulin

LDL = low-density lipoprotein

LDLR = low-density lipoprotein receptor

MHC = major histocompatibility class

NK = natural killer

oxLDL = oxidatively-modified low-density lipoprotein

T_{reg} = regulatory T

for macrophage survival and proliferation, have markedly reduced atherosclerosis despite severe hypercholesterolemia (7). Accumulating evidence also suggests diversity in the subtypes of mononuclear cells involved in innate immune responses. Studies in mice identified 2 subtypes of monocytes, 1 of which is considered proinflammatory, bearing high Ly6C expression as a marker (8). This inflammatory subset of high Ly6C monocytes was proposed to be precursors of proinflammatory M1 macrophages found in the plaques. Another phenotypic subset of macrophages is M2 macrophages, which, in a simplified phenotypic classification, are involved in resolution of plaque (9,10). Although this simplified view of monocyte-macrophages helps investigators to better understand the phenotypic heterogeneity of monocyte-macrophages in vitro, real, in vivo phenotypic changes are likely much more complex and not quite so discrete (9–11).

Natural killer (NK) cells are another innate immune cell type that contributes to atherosclerotic lesion formation. Low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice repopulated with bone marrow cells from Ly49A transgenic mice deficient in functional NK cells developed smaller atherosclerotic lesions in the aortic root and arch compared with $LDLR^{-/-}$ mice receiving bone marrow cells from nontransgenic donors (12). Depletion of NK cells in apolipoprotein (apo) $E^{-/-}$ mice using anti-asialo-GM1 antibodies reduced atherosclerosis. Adoptive transfer of wild-type NK cells into $apoE^{-/-}Rag\text{-}2^{-/-}$ interleukin $2rg^{-/-}$ mice augmented lesion size, confirming the proatherogenic role of NK cells. The proatherogenic function of NK cells appears to be dependent on mediators such as perforin and granzyme B (13).

Mast cells also accumulate in atherosclerotic lesions, and hypercholesterolemic mice lacking mast cells have reduced inflammation and lesion formation (14–16). Interestingly, 2 of these studies reported lower circulating cholesterol levels in mast cell-deficient mice, suggesting a link between innate mast cell signaling and cholesterol homeostasis (15,16).

DCs bridge the innate immune system with the adaptive immune response and are the upstream component in the chain of immune cell activation. This unique position makes DCs an important target when considering potential strategies to modulate atherosclerosis. Resident intimal DCs rapidly ingest lipid in hypercholesterolemic conditions, whereas depleting DCs using CD11c-specific diphtheria toxin receptor (DTR) transgenic mice resulted in reduced early lesion formation in $LDLR^{-/-}$ mice (17), supporting a proatherogenic role for conventional DCs. This study confirmed a previous report using genetic deletion of CD11c in $apoE^{-/-}$ mice (18). Murine and human atherosclerotic lesions contain plasmacytoid dendritic cells (pDCs). Exposure to oxLDL enables pDCs to elicit antigen-specific T cell responses. Depleting pDCs using antimouse plasmacytoid dendritic cell antigen-1 antibody in $apoE^{-/-}$ mice reduced atherosclerosis in the aortic sinus and was associated with reduced plaque inflammation and global suppression of T cell activation (19). The atherosclerosis-enhancing role of pDCs was mediated by activation of immune responses by autoantigens through protein-deoxyribonucleic acid (DNA) complexes (20).

ADAPTIVE IMMUNITY AND ATHEROSCLEROSIS. Compared with the blunt, nonspecific nature of the innate immune system, the adaptive immune response is more specific and develops over time through stochastic rearrangement during immunoblast development, generating a wide variety of T and B cell receptors that recognize specific antigens. Innate immune cells, such as DCs and macrophages, present antigens in the context of the major histocompatibility complex for recognition by T cells. T cell activation occurs upon presentation of the antigen in the setting of an inflammatory state, resulting in clonal proliferation. $CD8^+$ T cell clonal proliferation involves increased cytokine production and cytotoxic function targeted against cells presenting the specific antigen. $CD4^+$ T cell activation also results in cytokine production, which, in turn, skews subsequent B cell activation to produce specific immunoglobulins.

Prior work with B cells suggested a protective role against atherosclerosis in hypercholesterolemic mice (21). A series of elegant studies by the Witztum group showed that natural antibodies of the immunoglobulin (Ig) M isotype are reactive with the phosphorylcholine head group present in oxidized low-density lipoprotein (LDL), apoptotic cells, and the cell wall of Pneumococcus. These natural antibodies of the IgM isotype attenuated atherosclerosis (22–24), supporting the role of molecular mimicry in atherogenesis. These IgM antibodies are produced by self-renewing B1 cells, lending support to the protective role of B cells in atherogenesis (25). In contrast to these studies, B cell depletion using anti-CD20 antibody reduced atherosclerosis (26), suggesting that a more intricate balance of B cell subtypes is likely involved. Studies targeting the B cell activating factor pathway in murine atherosclerosis support this concept (27,28). B cell activating factor deletion resulted in reduced B2 cells with a preserved B1 cell population, and was associated with reduced atherosclerosis. Thus, cumulative evidence suggests that there is cell

subtype specificity in the role of B cells in atherosclerosis, with B1 cells having an atheroprotective effect, whereas B2 cells have a proatherogenic effect. Recently, the effect of passive immunomodulation using single- or multiple-dose infusion of a monoclonal anti-oxLDL antibody (MLDL1278A, aka BI-204) was tested in a small phase 2 multicenter, randomized, double-blind, placebo-controlled trial involving stable atherosclerotic cardiovascular disease patients (NCT01258907). The 12-week study was designed to measure the effect of the antibody on carotid and aortic vascular inflammation, as determined by ^{18}F 2-deoxyglucose positron emission tomography imaging. The study failed to meet its primary endpoint of reduction in vascular inflammation, as measured by target to background ratio for vascular ^{18}F 2-deoxyglucose uptake with the antibody treatment (29). The full and final results of this trial have not yet been published. The precise reasons for this trial's negative results remain unclear, but may include the failure to enrich the population with subjects more likely to have lipid-rich and highly-inflamed plaques, who would have been the most likely to respond to treatment. Uncertainty about the accuracy of the imaging technique used in the trial may also have contributed to the negative results. Nevertheless, these negative findings do not have any direct implications for active vaccination strategy, especially when the strategy works by altering cell-mediated immune responses.

Human atherosclerotic plaques contain macrophages and DCs, both of which can function as antigen-presenting cells (APCs), as well as T cells that express markers of activation (30). Evidence of APC-T cell interaction suggests antigen-specific immune activation through immunologic synapses in the plaque (31). $CD4^+$ T cells were initially reported to have a generalized proatherogenic role. This was supported by HLA-DR-restricted oxLDL activation of $CD4^+$ T cells cloned from atherosclerotic plaques (32). Adoptive transfer of $CD4^+$ T cells into immunodeficient hypercholesterolemic mice aggravated atherosclerosis (33). The proatherogenic role of $CD4^+$ T cells is, in part, due to the exaggerated proinflammatory effect of the Th1 cytokine response, particularly interferon-γ (34,35). Increased atherosclerosis after adoptive transfer of $CD4^+$ T cells reactive to LDL supported the postulated role of LDL as an antigen source for $CD4^+$ T cell responses in atherosclerosis (36). Other reports suggested that certain Th2 cytokine responses, such as IL-10, are protective against atherosclerosis (37,38). However, not all Th2 cytokine responses are atheroprotective. Deficiency of IL-4, another prototypical Th2 cytokine, in bone marrow-derived cells achieved by a bone marrow transplant strategy in $LDLR^{-/-}$ mice resulted in reduced atherosclerosis in specific sites of the vascular tree, suggesting a proatherogenic role of IL-4 (39). $ApoE^{-/-}$ mice genetically deficient in IL-4 also developed reduced atherosclerosis formation when compared with $apoE^{-/-}$ mice, further supporting a proatherogenic role of IL-4 (40). In addition to $CD4^+$ Th2 cells, other types of immune cells, such as mast cells, macrophages, and NK cells, secrete Th2 cytokines. The heterogeneous involvement of these many different types of cells, and their possible interaction with Th1 response, makes a unified interpretation of the role of Th2 response in atherogenesis difficult, as outlined in a recent excellent review (41).

Another subtype of $CD4^+$ T cells that has gained attention in the field of atherosclerosis research is $CD4^+$ regulatory T (T_{reg}) cells (42,43). The conventional immunology paradigm divides $CD4^+$ T_{reg} cells into natural or adaptive T_{reg} cells. Natural T_{reg} cells develop in the thymus with high CD25 expression and specificity against self-antigens, whereas adaptive T_{reg} cells develop from mature T-cell populations under antigenic stimulation with variable levels of CD25 expression and specificity against tissue and foreign antigens (44). However, CD25 is not exclusively a marker for T_{reg} cells. A more reliable T_{reg} cell marker is expression of the transcription factor, FoxP3, which distinguishes $CD4^+CD25^+$ T_{reg} cells from their $CD4^+CD25^+$ T effector cell counterparts. Depletion of $CD4^+CD25^+$ T_{reg} cells by deletion of CD80/CD86, CD28 or with CD25 neutralizing antibody resulted in increased atherosclerosis (42,45). Selective deletion of $FoxP3^+$ cells using either a diphtheria toxin receptor transgenic approach (46) or by vaccination against FoxP3 (47) resulted in increased atherosclerosis in hypercholesterolemic mice. Thus, experimental evidence from pre-clinical studies supports the notion that $CD4^+$ Treg cells have atheroprotective properties. Clinical studies also showed that the number of $CD4^+CD25^+$ T_{reg} cells in peripheral blood from patients with acute coronary syndrome was reduced and their ability to suppress responder $CD4^+$ T cell proliferation was compromised compared with cells from patients with stable angina and subjects with normal coronary arteries (48,49). Similar reductions of FoxP3 expression in peripheral $CD4^+CD25^+$ T_{reg} cells and decreased frequency of $CD4^+CD25^+FoxP3^+$ T cells in patients with aortic aneurysm have also been reported (50). Another prospective study revealed that low levels of baseline $CD4^+FoxP3^+$ T cells were associated with an increased risk of developing acute coronary syndrome 11 to 14 years later, suggesting that patients

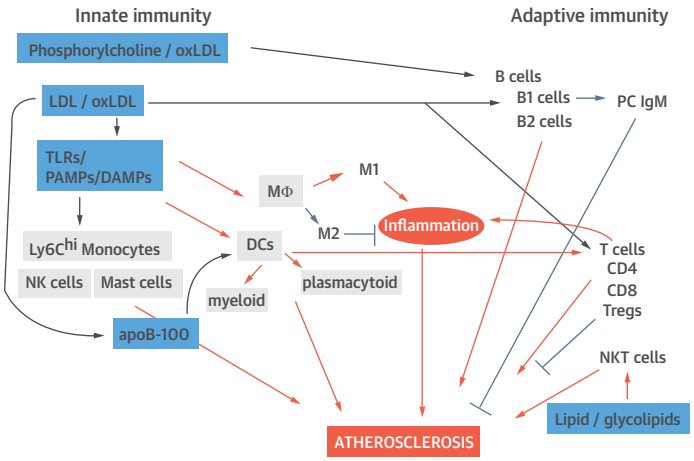
with low baseline levels of $CD4^+FoxP3^+$ T cells have the propensity to develop a higher burden of atherosclerotic coronary disease (51).

Investigators are keenly interested in the natural killer subtype of T cells (NKT), due to their ability to recognize lipid antigens presented in a CD1-restricted manner. CD1 molecules are present in atherosclerotic plaques and colocalize in areas of the arterial walls containing T cells, indicating potential interaction between $CD1^+$ cells and T cells (52). Genetic deletion of CD1d in hypercholesterolemic mice reduced lesion size (53,54), and activation of NKT cells using a synthetic glycolipid, α-galactosylceramide, increased atherosclerosis (53–55). However, not all NKT cells are atherogenic. $ApoE^{-/-}$ mice rendered NKT cell-deficient by day-3 neonatal thymectomy developed smaller atherosclerotic lesions. Adoptive transfer of $CD4^+$, not double negative NKT cells, into thymectomized $apoE^{-/-}$ mice promoted atherosclerotic lesion formation. These data suggest differential roles of NKT cell subtypes in atherogenesis (56).

$CD8^+$ T cells were initially sidelined, partly due to the lack of observed effects on atherogenesis in mice with gene deletions that severely reduced or eliminated $CD8^+$ T cells (57,58). However, subsequent studies reported that $CD8^+$ T cell activation occurred earlier than $CD4^+$ T cell response in the setting of hypercholesterolemia (59). A hypercholesterolemic milieu was reported to enhance T cell activation and function in both $CD4^+$ and $CD8^+$ cell subtypes (60). A study that used depleting antibodies in hypercholesterolemic mice showed a pathogenic role for $CD8^+$ T cells in atherosclerosis, supporting the role of $CD8^+$ T cells in atherogenesis (61). In a recent prospective clinical study, a higher fraction of $CD8^+$ T cells correlated with characteristics of insulin resistance such as a high waist-hip ratio and high fasting plasma glucose, insulin, and triglyceride levels. Patients with the 2 highest tertiles of $CD8^+$ T cells displayed a trend toward increased incidence of coronary events during follow-up for 11 to 14 years (62). Additional work with $CD8^+$ T cells in atherogenesis showed complexity in the subtypes involved. $CD8^+CD25^+$ T cells adoptively transferred into hypercholesterolemic mice reduced atherosclerosis associated with immunomodulatory functions (63). Thus, similar to $CD4^+$ T cells, regulatory subtypes of $CD8^+$ T cells may also function to down-regulate immune responses in atherosclerotic diseases. This function of $CD8^+$ T cells is in line with the historic reference to this cell type as suppressor T cells. Further investigations are still needed for a more clear understanding of the role of $CD8^+$ T cell subtypes in atherogenesis.

As summarized in the preceding text, numerous components of the innate and adaptive immune systems participate in modulating atherogenesis, with intricate interactions among these components that can lead to either worsening or amelioration of atherosclerosis (**Figure 1**). Given a major role for the adaptive immune system in atherosclerosis, its modulation with vaccination provides an opportunity for a potential atherosclerosis treatment strategy. Several candidate antigens for vaccination are under investigation, with LDL and its protein component, apoB-100, at the forefront.

FIGURE 1 Intricate and Complex Interactions Among Components of Innate and Adaptive Immunity in Atherogenesis



Salmon arrows depict proatherogenic factors, **slate lines with capped ends** depict antiatherogenic functions, and **periwinkle boxes** indicate antigen sources. apo = apolipoprotein; DAMP = danger-associated molecular pattern; DC = dendritic cell; Ig = immunoglobulin; LDL= low-density lipoprotein; NK = natural killer; NKT = natural killer T cell; oxLDL = oxidatively-modified LDL; PAMP = pathogen-associated molecular pattern; PC = phosphorylcholine; TLR = Toll-like receptor; T_{reg} = regulatory T cells.

CONCEPT OF A VACCINE FOR ATHEROSCLEROSIS

RATIONALE FOR A VACCINATION STRATEGY. Given that immune-mediated inflammation is a cardinal feature of atherosclerosis, it is tempting to consider specific strategies to target immune or inflammatory components as a novel approach against inflammation and atherosclerosis. The pathophysiologic role of inflammation in atherosclerosis is currently being tested in human trials using anti-inflammatory drugs (64,65). These approaches are predicated on the notion that dampening the inflammatory response may favorably affect the clinical outcome by reducing atherothrombotic cardiovascular events.

Modulation of the athero-promoting adaptive immune response is another potentially attractive strategy against atherosclerosis. Immune activation during atherogenesis is an inevitable host response; thus, fine-tuning this response may prove beneficial in atherosclerosis. The challenge of this approach is to identify specific antigens relevant to atherogenesis so that they can be used to activate an antigen-specific atheroprotective immune response. Most studies in search of potential antigens are in pre-clinical experimental stages; hence, unless otherwise stated, data discussed in the subsequent sections of this review come from animal models of atherosclerosis.

EXOGENOUS SOURCES OF REACTIVE ANTIGENS. A reasonable approach to searching for antigens would be to focus on the disease itself: the atherosclerotic plaque. Many foreign antigens, including bacteria such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Porphyromonas gingivalis* and viruses such as hepatitis C virus, enteroviruses, human immunodeficiency virus, and cytomegalovirus, have been identified in atherosclerotic plaques (66). It is unclear if these pathogens are causative agents, directly participating in the pathogenesis of atherosclerosis, or if they provide conditions that skew the immune response to more inflammatory activity through molecular mimicry, rendering arterial walls more prone to atherosclerosis formation. Many clinical trials using antibiotics to treat *Chlamydia* infection failed to prevent cardiovascular events (67-74). Because the results can be viewed either as evidence not in support of such a role or as suggesting that failure resulted from initiating antibiotic treatment too late to have significant clinical impact, they do not resolve the issue of whether pathogens play a causative role in atherogenesis.

Periodontal pathogens have recently gained attention in the research community, as many are found in human atherosclerotic lesions (66) and have been linked to atherosclerosis (75-78). Exposure of experimental animals to *P. gingivalis* accelerates atherosclerosis formation (79-81), whereas immunization against *P. gingivalis* attenuates such pathogen-induced atherosclerosis (82). At this time, a direct role for these pathogens in human atherosclerosis remains uncertain, thus it is unclear whether vaccines targeting periodontal pathogens would be a useful strategy in human subjects.

ENDOGENOUS ANTIGENS. Search for LDL and apoB-100–derived antigens. Because LDL and other apoB-100–containing lipoproteins are the primary culprits with the strongest causative link with atherothrombosis, antigens derived from them remain prime candidates for vaccine development. Immunization of experimental animals with homologous whole native or oxidatively-modified LDL incorporated into a vaccine formulation with different adjuvants has clearly and consistently demonstrated atheroprotective effects; however, the precise mechanisms and antigenic epitopes are not fully defined (**Table 1**).

Because LDL is a large, heterogeneous molecule containing a diverse cargo of apolipoproteins, cholesteryl esters, triglycerides, and phospholipids, it would be impractical to use whole homologous LDL as an antigen in a clinically-usable vaccine formulation. Its complexity makes it difficult to determine the exact immunogenic epitopes within the LDL molecule. Safety issues during isolation of large quantities of LDL for vaccine formulation are another concern. Given the promising experimental data from immunization using LDL as antigen, it

TABLE 1 Summary of the Studies of Active Immunization Using LDL or its Modified Form as Antigens to Modulate Atherosclerosis

Animal (Ref. #)	Antigens	Immunization Route	Effect on Atherosclerosis
LDLR$^{-/-}$ rabbits (132)	MDA-LDL	Subcutaneous	Reduced (aorta)
NZW rabbits on high cholesterol diet (133)	Native LDL or Cuox-LDL	Subcutaneous	Reduced (aorta)
LDLR$^{-/-}$ mice (134)	Native LDL or MDA-LDL	Subcutaneous	Reduced (aortic sinus)
ApoE$^{-/-}$ mice (135)	MDA-LDL	Subcutaneous	Reduced (aortic sinus)
ApoE$^{-/-}$ mice (136)	Plaque homogenate or MDA-LDL	Foot pad injection	Reduced (aortic sinus)
ApoE$^{-/-}$ mice (137)	Native LDL	Subcutaneous	Reduced (aortic sinus)
ApoE$^{-/-}$ or apoE/CD4 double knockout mice (138)	MDA-LDL	Subcutaneous	Reduced (aortic sinus)
LDLR$^{-/-}$ mice (120)	Cuox-LDL	Intravenous delivery of oxLDL-pulsed dendritic cells	Reduced (accelerated carotid atherosclerosis induced by pericarotid collar)
ApoE$^{-/-}$ mice (139)	Cuox-LDL	Nasal delivery	Reduced (aortic sinus and aorta)

Apo = apolipoprotein; Cuox = copper-oxidized; LDL = low-density lipoprotein; LDLR = low-density lipoprotein receptor; MDA = malondialdehyde; NZW = New Zealand white; oxLDL = oxidatively-modified low-density lipoprotein.

would be of great interest to identify the atheroprotective antigenic epitopes in LDL. To do this, we initiated an extensive effort to identify potential antigenic epitopes within apoB-100, the major protein component of LDL and other atherogenic lipoproteins, which could then be used as antigens to achieve atheroprotective effects via immunization. We initially screened the entire 4,536-amino acid human apoB-100 protein and designed a library of 302 peptides (each 20 amino acids long with a 5-amino acid overlap with the preceding sequence) spanning the entire apoB-100 sequence. Out of these 20-mer peptide sequences, 102 peptides to which an immune response with antibodies was detected in pooled human plasma were identified (83). Subsequent testing revealed that several immunoreactive peptides, including p2, p143, and p210, resulted in a 40% to 70% decrease in atherosclerosis and reduction in plaque inflammation when used in a vaccine formulation in hypercholesterolemic mice (84,85). Our research teams in the United States and Sweden have been using p210 as a prototype antigen in vaccine formulations because the p210-based vaccine has delivered the most consistent atheroprotective effects (86–88). Because knowledge of the defined epitope makes more definitive immunologic studies possible, this move away from using the whole LDL particle as an antigen and toward using more defined apoB-100 peptides as antigens is a crucial step in development of pre-clinical prototypes of an atherosclerosis vaccine.

Mechanisms of action of p210 vaccine. Immunization with the p210 vaccine resulted in a significant reduction of aortic atherosclerosis compared with control subjects (88). Given that immunization activates both B and T cells (88), it is important to delineate the subset(s) of lymphocytes that mediate the p210 vaccine's atheroprotective effect. At this time, no strong experimental evidence supports a role for the humoral response in the protective effect of active immunization using the p210 vaccine. The atheroprotective effects of apoB-100 peptide immunization occurred without an increase in peptide-specific IgG, as reported with $LDLR^{-/-}$ human apoB-100 transgenic mice (86), and the induced antibody titers did not correlate with the lesion size (87). Adoptive transfer of B cells from p210-immunized mice to nonimmunized recipient mice did not confer the atheroprotective effect (88). However, in the course of the immunization study, we observed changes in p210 antibody levels in $apoE^{-/-}$ mice. Both p210 IgM and IgG titers were low before immunization, with p210 IgM titers increasing over time, regardless of whether or not mice were immunized with p210 vaccine. IgG titers against p210 followed a different trend: in control mice, there was no significant change of IgG titer between baseline and 25 weeks, whereas mice receiving adjuvant only or p210 vaccine developed high p210 IgG titer at 25 weeks. Interestingly, the IgG titer at 25 weeks from p210-immunized mice was significantly lower than that of mice receiving adjuvant only. This observation suggests that: 1) there is an endogenous IgM immune response against p210, with adjuvant recipients or p210 vaccine recipients inducing antibody class switching to IgG; and 2) induction of p210 IgG could potentially serve as a marker of vaccination effect, even though it does not correlate with the atheroprotective efficacy of the p210 vaccine.

In addition to reducing aortic atherosclerosis, p210 vaccine also activated $CD8^+$ T cells, along with a reduction of dendritic cells at the site of immunization and within the atherosclerotic plaques. These observations led us to postulate that $CD8^+$ T cells could be the immune cells that mediate the vaccine's atheroprotective effect. Subsequent experiments involving adoptive transfer of $CD8^+$ T cells from p210 immunized mice into naïve, nonimmunized mice indeed recapitulated the atheroprotective effect of active immunization and, thus, confirmed our postulate. Modulation of dendritic cells by p210 vaccine appeared to be antigen-specific, because effector $CD8^+$ T cells from p210-immunized mice developed a preferentially higher cytolytic response against p210-loaded dendritic cells in vitro. This may explain the reduction in dendritic cells seen in the immunization sites and atherosclerotic plaques. The observed modulation of DCs and cellular immune responses did not alter the efficacy of subsequent T cell-dependent or independent immune response to other irrelevant antigens (88).

In addition to the $CD8^+$ T cell response, p210 immunization also elicited a $CD4^+$ T cell response. The reduction of atherosclerosis by p210 immunization was associated with a $CD4^+CD25^+$ T cell response. Administration of antibodies against CD25 reduced $CD4^+CD25^+$ T cells and abrogated the atheroprotective effect of p210 immunization (89). In addition, Dr. Klingenberg's group (87) immunized female $apoE^{-/-}$ mice intranasally twice weekly for 12 weeks with a recombinant protein consisting of p210 fused with the cholera toxin B subunit (CTB). Control mice received ovalbumin peptide fused with CTB or PBS as control. Mucosal immunization using p210-CTB reduced atherosclerosis in aortic sinuses of mice. The investigators also observed that splenic $CD4^+$ T cells from p210-CTB-immunized mice contained a higher percentage of the $IL\text{-}10^+$ subset, which

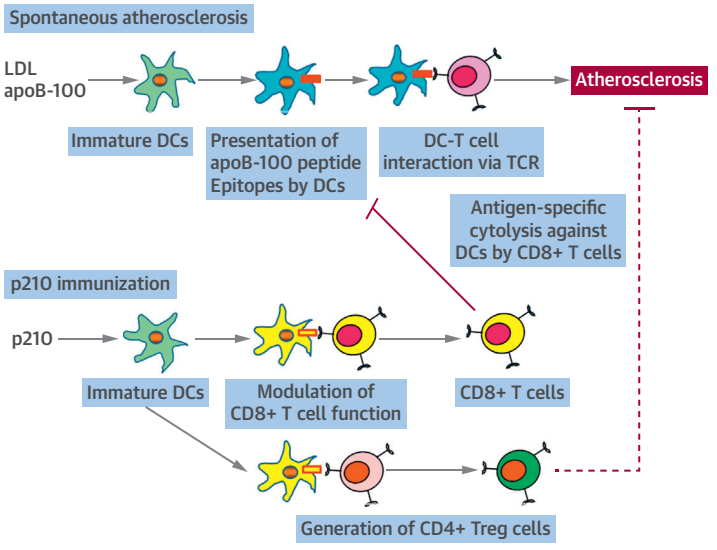
were functional in their ability to suppress effector $CD4^+$ T cells, without any differences between p210-CTB and control subjects in FoxP3, IL-10, or TGF-β messenger ribonucleic acid expression in the aorta (87). Furthermore, there was no difference in the numbers of $FoxP3^+$ cells in aortic lesions or $CD4^+FoxP3^+$ T cells in lung mucosa (87). Further evidence of the atheroprotective effects of p210 was provided by Herbin et al. (90), who implanted a mini-osmotic pump to subcutaneously deliver a mixture of apoB-100 peptides (p210, malondialdehyde-modified [MDA]-p210 and p240) or p210 alone for 2 weeks. Such treatment reduced atherosclerotic lesions in aortic sinuses 10 weeks later compared with control subjects and also retarded the progression of established atherosclerotic lesions in old female mice (90). Subcutaneous peptide delivery was associated with reduced activation of $CD4^+$ T cells and an increased $CD4^+CD25^+FoxP3^+$ subset of T cells in lymph nodes. Ablation of $CD25^+$ T cells by CD25-depleting antibody abrogated the atheroprotective effects of subcutaneous infusion of apoB-100 peptides, similar to the study by Wigren et al. (89). On the basis of these reports, there is clearly a CD4+ T cell response (be it induction of $CD4^+CD25^+$ or $CD4^+IL\text{-}10^+$ T cells) with p210 immunization. However, there is no direct evidence suggesting that these $CD4^+$ T cell responses mediate the atheroprotective effect of p210 immunization, nor what the target cell types of these $CD4^+$ T cells are.

These different cellular immune responses elicited by p210 in various reports could be due to the difference in: 1) the form of p210 delivered—carrier conjugated, recombinant with CTB, or free form; 2) route of delivery—subcutaneous versus mucosal; 3) doses of p210; or 4) duration of p210 delivery—long-term depot effect of carrier-conjugated p210 in subcutaneous tissue versus infusion for 2 weeks. Nevertheless, the consistent reduction of atherosclerosis after p210 immunization, regardless of how and which form was delivered, strongly suggests that p210 is a promising candidate antigen for potential vaccine formulation for possible future human application. Our experimental studies suggest that there is an endogenous immune response to p210 in hypercholesterolemic challenge and that p210 vaccination modulates the functions of certain subsets of $CD8^+$ T cells that regulate the endogenous p210 immune response in a feedback-dependent fashion (**Figure 2**).

Other apoB-100–related antigens. A peptide with apoB-100 amino-acid residues 688 to 707 has been incorporated into a multiantigenic construct with peptidic epitopes from *Chlamydophila pneumoniae* and heat shock protein (HSP) 60. Immunization with such multiple antigenic epitopes reduced atherosclerosis accompanied by a reduction of macrophage infiltration and an increase of $CD4^+FoxP3$ T cells in the plaques (91).

Using a different approach to identify possible apoB-100 epitopes in a major histocompatibility class (MHC)-II restricted context, Dr. Klaus Ley's group (92) recently surveyed the murine apoB-100 protein for peptide fragments that were predicted by modeling algorithms to bind to the mouse MHC-II molecule I-Ab. The survey predicted 2 peptide fragments, $ApoB_{3501\text{-}3516}$ and $ApoB_{978\text{-}993}$, which also reduced atherosclerosis when used to immunize $apoE^{-/-}$ mice, possibly via an IL-10-dependent mechanism (92). Recently, Dr. Hansson's group (93) generated T cell hybridomas from human apoB-100 transgenic mice immunized with human oxLDL and identified ApoB-100-responding $CD4^+$ T cell hybridomas. These hybridomas were MHC class II-restricted and expressed a single T cell receptor V beta chain, TRBV31. Use of a TRBV31-derived peptide in a vaccine formulation induced anti-TRBV31 antibodies that blocked T cell recognition of apoB-100 and significantly reduced atherosclerosis (93). Thus, the

FIGURE 2 $CD8^+$ T Cells Elicited by p210 Immunization Modulate Spontaneous Atherosclerosis



Processing and presentation of endogenous LDL and its ApoB-100 peptide epitopes by DCs induce atherogenic antigen-specific T cell responses leading to atherosclerosis. Immunization with p210 generates cytolytic $CD8^+$ T cells that lyse antigen-presenting DCs, and hence down-regulate atherogenic immune response to LDL and its apoB-100 peptides. **Dotted line** depicts lack of direct evidence to show how $CD4^+$ T_{reg} cells induced by p210 immunization reduce atherosclerosis. Abbreviations as in **Figure 1**.

investigators used an innovative approach by identifying a potentially pathogenic $CD4^+$ T cell population and used antigen-specific humoral immunity, determined by the specific signature of these $CD4^+$ T cells to block a proatherogenic cellular immune response, and hence confirming the pathogenic role of $CD4^+$ T cells in atherosclerosis.

OTHER ANTIGENS IN VACCINE DEVELOPMENT FOR ATHEROSCLEROSIS. The approach of using vaccines to modulate immune responses in atherosclerosis has not been limited to LDL and apoB-100-related antigens. The complexity of atherosclerotic vascular disease presents the opportunity to target other potential sources of antigens.

Other lipid-related antigens. A natural IgM antibody recognizing the epitopes in oxLDL (94,95) and phosphorylcholine (PC) headgroups on the surface of apoptotic cells, and which inhibits uptake of oxLDL and apoptotic cells by macrophages, has been extensively studied (22,94,96). Protection against infection from *Streptococcus pneumoniae* is attributed to anti-PC antibodies (97,98). Active immunization with *S. pneumoniae* in $LDLR^{-/-}$ mice to induce anti-PC antibodies resulted in increased oxLDL antibodies, primarily of the IgM isotype, and reduced atherosclerosis (23). The increase in oxLDL-specific IgM is attributed to the cross-reactivity of the phosphorylcholine moiety on oxLDL with *S. pneumoniae*-induced antibodies, suggesting molecular mimicry between *S. pneumoniae* and oxLDL. This molecular mimicry was investigated and expanded further in the context of a vaccine by Caligiuri et al. (99) using phosphorylcholine (PC), the reported mimotope. The group used the PC-keyhole limpet hemocyanin-conjugate coupled to CpG oligonucleotides as adjuvant and immunized $apoE^{-/-}$ mice with multiple interperitoneal injections. The PC immune serum significantly increased IgG and IgM antibodies against PC and oxLDL, with reduced macrophage oxLDL uptake, and reduced atherosclerosis (99). However, observational cohort studies in humans, using myocardial infarction or stroke as endpoints, did not show consistent protective effects of pneumococcal vaccines (100–103). Whether active immunization against PC will result in reduced atherosclerosis still awaits validation.

In rabbits, active immunization with cholesteryl ester transfer protein (CETP), a key enzyme involved in high-density lipoprotein (HDL) metabolism, induced neutralizing antibodies and markedly increased HDL-C levels concomitant with reduced atherosclerosis (104–106). However, a phase 1 human trial did not show consistent induction of CETP antibody or significant changes in CETP function or HDL levels with CETP immunization (107).

Vaccines targeting HSP antigens. HSPs are stress proteins that are highly conserved in all organisms. They are present in cells under normal conditions and can be expressed at high levels when cells are exposed to stresses, such as altered pH or oxygen deprivation. HSPs have also been implicated in atherogenesis (108–111). However, the effect of immunization with HSPs on atherosclerosis has been inconsistent. Several groups reported that immunization with HSP65 induces atherosclerotic lesions (112–114), whereas others reported reduced atherosclerotic lesions (115,116). The difference in outcomes highlights the likely effect of the adjuvant used, as well as the mode of antigen delivery. Prior studies reporting increased atherosclerosis with HSP65 used Freund's adjuvant, which induces a strong inflammatory response. However, Klingenberg et al. (115) used alum as the adjuvant, with the authors proposing that, given its nature as a microbial constituent, HSP65 itself may act as both antigen and adjuvant, with alum acting as a modulating factor. The immune response constituted an increase in B cell activity, most noticeably in HSP65 IgG1, with a similar trend in oxLDL IgG and reduced atherosclerosis (115).

In addition to the subcutaneous route, vaccines may also be delivered through nasal mucosa, and mucosal immunity appears to elicit a down-modulation of immune responses to specific antigens. In a recent report, the vaccines used either DNA, whole protein HSP65, or both in PBS with multiple intranasal vaccinations in rabbits. All vaccine formulations induced HSP65 IgG responses, increased serum interleukin (IL)-10, and reduced interferon-γ, and reduced atherosclerosis accompanied by decreased cholesterol levels (116).

Vaccines against host cell surface and extracellular matrix proteins. The involvement of certain inflammatory cells in atherosclerotic plaque formation suggested that specific cell surface markers could be potential antigens for immunization. Strategies targeting cell surface proteins thought to contribute to atherosclerosis have been tested using DNA vaccines. DNA vaccines formulated for oral administration have been used experimentally with success. Also called transgenic vaccination, the antigen is delivered via an expression plasmid that encodes the antigen. Oral immunization is thought to work through transfer of the genetic material from the carrier to host phagocytes in the gastrointestinal tract. The phagocytes then express the antigen de novo in the cytosol, and present it on MHC molecules (117). In studies using this approach in

atherosclerosis, constructs were designed to encode CD99 (118) or vascular endothelial growth factor receptor-2 (119) carried by live attenuated *Salmonella typhimurium* and delivered orally. In these studies, the expressed antigens were presented in the context of MHC-I, which elicited a $CD8^+$ cytolytic T cell response targeting cells that expressed vascular endothelial growth factor receptor 2 or CD99, resulting in reduced atherosclerosis.

LDL retention in the arterial wall by extracellular matrix (ECM) is an early step in the development of atherosclerotic lesions. Fibronectin is an ECM protein found in plaques. Immunization with fibronectin formulated with alum as the adjuvant significantly reduced atherosclerosis in $apoE^{-/-}$ mice, and was associated with increased Th2-type antibody production and increased regulatory T cells (120). Interestingly, plasma cholesterol was significantly reduced in the immunized mice, suggesting that there is an interaction between immune responses to ECM proteins and cholesterol metabolism.

Dendritic cell vaccines. Because DCs are the most efficient antigen-presenting cells, antigen delivery by DCs into the host should provoke an efficient response. This is achieved by transferring autologous or syngeneic DCs loaded with the specific antigen to naïve recipients. Several groups tested this in preclinical studies using different antigen formulations. Habets et al. (121) used oxLDL as the antigen with lipopolysaccharide (LPS) as the maturation factor, and transferred the cells into naïve recipient mice intravenously 3 times. Transfer of oxLDL-loaded DCs induced a Th1 response, increased oxLDL IgG titers, and reduced atherosclerosis (121).

Using apoB-100 as the antigen, Hermansson et al. (122) again treated DCs with LPS but added IL-10 to induce a tolerogenic phenotype. Using only a single intravenous transfer strategy, atherosclerosis in mice was significantly reduced. This was accompanied by increased $CD4^+IL\text{-}10^+$ T cells, increased FoxP3 messenger ribonucleic acid expression in the spleen, and reduced lymphocyte proliferation after apoB-100 stimulation, suggesting an immunoregulatory function. In another mouse study, the same investigators loaded DCs with MDA-modified LDL, also with LPS as a maturation factor, but without cytokine treatment, and injected the loaded DCs subcutaneously multiple times over several weeks (123). This resulted in increased atherosclerosis with increased MDA-LDL IgG response and MDA-LDL–stimulated T cell proliferation. Because the group had previously shown reduced atherosclerosis with MDA-LDL immunization formulated with CFA, they performed a short study to determine immune responses with the 2 vaccination approaches. They found that MDA-LDL emulsified in CFA-induced $CD4^+CD25^+FoxP3^+$ T regulatory cells, whereas MDA-LDL loaded DCs did not, suggesting that vaccine formulation and delivery significantly affects the outcome.

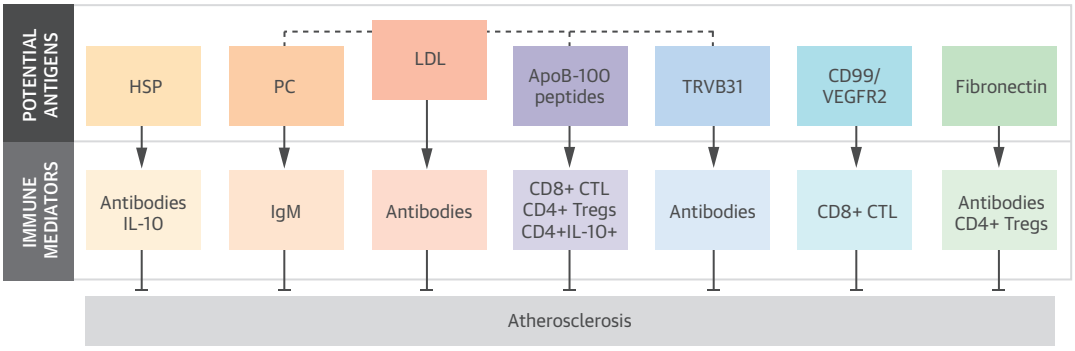
Influenza vaccination and cardiovascular events. Documents from professional societies advocate influenza vaccination to patients with cardiovascular disease for secondary prevention of further cardiovascular events (124). Studies have shown increased rates of acute myocardial infarction and death during the influenza season, suggesting that there is an association between cardiovascular events and influenza infection (125). Although its causal role in triggering cardiovascular events remains to be elucidated, experimental studies have shown that the influenza virus promotes arterial and plaque inflammation (126,127). Three small, randomized clinical trials conducted outside of the United States demonstrated consistent reductions in cardiovascular events (such as rehospitalization for ischemia), but less consistency in reduction of cardiovascular death (128–130). A recent meta-analysis further suggested a benefit of influenza vaccine for secondary protection of cardiovascular events (131). These reports provide evidence to support further studies on the use of the influenza vaccine to reduce rates of acute myocardial infarctions and to understand its mechanism of action, which is not yet known. It is possible that vaccination results in reduced inflammatory signaling in atherosclerotic plaques, leading to reduced acute cardiovascular events, a mechanism different from those for the antigens described in the preceding sections.

IMPLICATIONS AND CLINICAL PERSPECTIVES

Vaccines and clean water are the 2 major public health advances of the past 100 years. Vaccines have had a major impact in virtually eradicating many infectious diseases; therefore, the idea of developing vaccination strategies for the chronic, highly prevalent disease of atherosclerosis, which takes a considerable toll on human lives across the globe, is both exciting and daunting. In this review, we discussed the experimental evidence for induction of antigen-specific immune responses relevant in atherosclerosis by vaccination (**Central Illustration**). The adaptive immune system is a powerful tool, used by the host to attempt homeostasis when challenged. Successful harnessing of this powerful tool will be an important contribution to the host arsenal for combatting atherosclerotic disease. However, there are clear challenges for clinical translation.

Translation of the aforementioned promising preclinical observations into the clinical arena is in its

CENTRAL ILLUSTRATION **Potential Antigens and Their Respective Immune Mediators**



Shah, P.K. et al. J Am Coll Cardiol. 2014; 64(25):2779–91.

Selected representative antigens that have shown positive results in published experimental models are depicted. **Dashed arrows** originating from low-density lipoprotein (LDL) indicate LDL as a shared antigenic source for 3 different strategies. Apo = apolipoprotein; CTL = cytotoxic T cell; HSP = heat shock protein; Ig = immunoglobulin; IL = interleukin; PC = phosphorylcholine; T_{reg} = regulatory T cell; VEGFR = vascular endothelial growth factor.

infancy. Many questions remain to be answered while designing proper clinical studies. These include (but are not limited to) questions regarding: vaccine safety and stability; schedule and durability of immunization; proper selection of patient populations for testing; and determination and monitoring of efficacy endpoints in clinical studies. In addition, how an approach would potentially impact current patient management with strategies such as lipid lowering and lifestyle changes must be considered. With all of these challenges in mind, we are cautiously optimistic about the potential for future clinical application. Currently, as discussed in this review, we have many choices of antigens that can be tested for human application. Firm evidence from numerous clinical trials has established the causative role of LDL in atherogenesis, which makes LDL and apoB-100 reasonable and logical initial targets for immunomodulatory therapy. Pre-clinical studies using LDL or apoB-100 peptides as candidate antigens in vaccine formulation further support such a claim. It is time to muster together the academic investigators, professional societies, government agencies, funding organizations, and pharmaceutical industries needed to initiate this long-term journey into clinical application.

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