Immunotherapy for Atherosclerosis: An Emerging Paradigm

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Experimental and clinical studies have implicated the immune system in atherogenesis and its complications. Recent studies have identified a complex dual role for the immune system: some aspects of the system play a proatherogenic role, whereas other aspects are atheroprotective. Suppression of proatherogenic immune responses and activation of antiatherogenic immune responses promise to emerge as novel strategies for the prevention and treatment of atherosclerotic vascular disease.
[Rev Cardiovasc Med. *2004;5(4):194-203*]

Key words: Atherosclerosis • Immunosuppression • Vaccination • Tolerization • Low-density lipoprotein, oxidized • Malondialdehyde-apolipoprotein B-100 peptide antigens • Phospholipid antigens, oxidized • Cholesteryl ester transfer protein

A large body of data accumulated over the past several years has identified a key role for inflammation in the initiation, progression, and destabilization of atherosclerosis.[1–4] Proatherogenic risk factors and atherogenic lipoproteins that have entered and been retained in the subendothelial space of the vessel wall induce inflammatory genes that promote adhesion, chemotaxis, subendothelial retention, and activation of mononuclear cells.[1–4] The inflammatory cells contribute to the initiation and subsequent progression of atherosclerosis and its complications, including plaque rupture and thrombosis.[1–4]

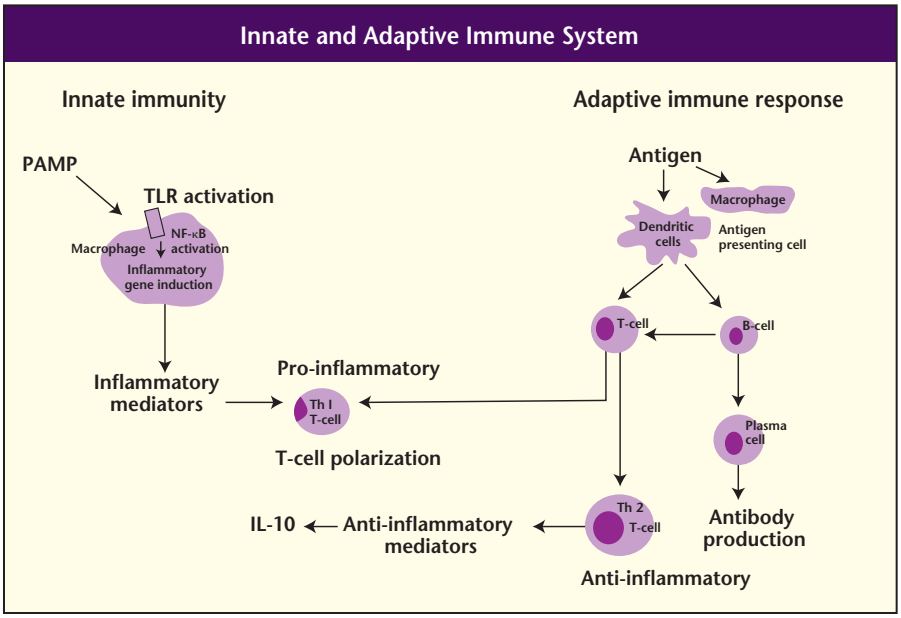

Figure 1. *Diagram of the innate and adaptive immune systems. PAMP, pathogen-associated molecular pattern; TLR, toll-like receptor.* www.medreviews.com

Immune Mechanisms in Atherosclerosis

Both innate and adaptive immune responses (Figure 1) seem to play a role in atherosclerosis.[5–8] Innate immunity is the nonspecific first line of defense against foreign pathogens and involves a number of receptors, cytokines, complements, antibodies, and other proteins encoded by germline genes.[9] The innate immune response is based on detection of pathogen-associated molecular patterns, which are part of proteins, carbohydrates, lipids, and nucleic acids, by pattern-recognition receptors on macrophages and dendritic cells, such as the scavenger receptors and the Toll-like receptors (TLR).[9–12] Scavenger receptors mediate removal of modified lipoproteins, apoptotic cells, and some microorganisms through the endocytic pathway, which not only results in degradation but might lead to antigen presentation and activation of adaptive immunity.[10] Activation of TLRs by endotoxin, heat shock proteins, and other microbial antigens induces intracellular signaling through the AP-1 and nuclear factor (NF)-κB pathways, ultimately leading to activation of proinflammatory genes and an inflammatory response.[11–13] These receptors are also expressed by endothelial cells, and their activation likely represents an important initial step in atherogenesis.[14] Several laboratories, including our own, have recently shown that TLRs are expressed in human and murine atherosclerotic lesions and might be induced by modified lipoproteins, thereby providing a potential link between lipoproteins and inflammation.[14–17] Recently, our laboratory reported marked reduction in murine atherosclerosis with genetic ablation of MyD88, a key adaptor molecule involved in TLR-mediated NF-κB activation, which further suggests a role for the innate immune system in atherogenesis.[18]

Similarly, a family of scavenger receptors, including SR-AI, SR-AII, CD36, MARCO, SR-PSOX/CXCL16, and CD68, expressed by macrophages, bind oxidized phospholipid from modified low-density lipoprotein (LDL) in an attempt to clear potentially toxic moieties. However, these receptors might also play a role in progression of atherosclerosis: mice lacking SR-A and CD36 develop less atherosclerosis.[19–24]

Unlike the innate immune response, an adaptive immune response is delayed and directed against a specific antigen. It involves a stochastic rearrangement process in immunoblasts, leading to generation of a large number of T and B cell receptors and immunoglobulins, which can recognize specific foreign antigens. In atherosclerosis, several antigens might be responsible for activation of the adaptive immune system; these antigens include heat shock proteins and oxidized LDL that has been taken up and processed by macrophages. Both the mycobacterial heat shock protein Hsp65 and the chlamydial Hsp60 show considerable mimicry of human Hsp60, which suggests that immune responses against microbial heat shock proteins could cross-react with heat shock proteins expressed by stressed arterial cells.[25] In line with this, immunization of LDLr-/- mice and hypercholesterolemic rabbits with Hsp65 was found to promote atherosclerosis.[26,27] Moreover, Lamb and colleagues[28] found an association between the magnitude of the immune response to Hsp65 after bacille Calmette-Guérin immunization and develop-

Mice lacking the scavenger receptors SR-A and CD36 develop less atherosclerosis.

ment of atherosclerosis in rabbits. It has also been reported that antibody levels against Hsp65 correlate with progress of carotid disease in humans.[29] It is possible that immune responses against viral and bacterial antigens in plaques might aggravate disease and destabilize plaques, but this remains to be shown.

Immune response against oxidized LDL has been demonstrated in experimental as well as human atherosclerosis, and both proatherogenic and antiatherogenic effects of this response have been postulated. Specific antigenic epitopes derived from oxidized LDL and other antigens are presented by major histocompatibility complex (MHC) class II proteins for recognition by specific CD4+ T cells. When T cells encounter their specific antigens on an MHC class II molecule, an adaptive immune response is activated, which includes clonal proliferation of the T cell and production of cytokines and immunoglobulins. There are two major subsets of CD4+ cells: T-helper $(Th)_1$ cells, which secrete interferon (IFN)-γ and tumor necrosis factor (TNF)-α; and Th2 cells, which produce interleukin (IL)-4, IL-5, and IL-10.[30] Analysis of the cytokine expression in atherosclerotic plaques suggests a dominance of Th1 cells.[31] The notion that Th1 cells are involved in the progression of atherosclerosis is also supported by animal experiments demonstrating that mice lacking IFN-γ develop less atherosclerosis, whereas disease is aggravated in response to the Th1 stimulatory cytokines, IL-12 and IL-18.[32–34] In contrast, there is less atherosclerosis in mice transgenic for the Th2-stimulatory cytokine IL-10.[35] Conversely, disease is exacerbated in IL-10 knockouts and reduced in IL-18-deficient apolipoprotein (apo)E-knockout mice.[36–38] Th1 cells might also increase the risk for plaque rupture: IFN-γ inhibits smooth muscle differentiation and collagen production, and TNF-α stimulates the release of matrix-degrading metalloproteinases from macrophages.[39–42] Together, these Th1 cytokines might also induce smooth muscle cell apoptosis.[43] In addition to the classic Th1 and Th2 cells, additional subsets of T cells might also modulate atherosclerosis. These subsets include the regulatory T cells, Th3 cells, and natural killer (NK) T cells. The regulatory T cells suppress antigen-induced activation of other CD4+ T cells by secreting IL-10.[44] Bystander activation of regulatory T cell type 1 was recently shown to reduce atherosclerosis in a mouse model.[45] The Th3 cell produces transforming growth factor ß, a potent immunosuppressive/anti-inflammatory cytokine that inhibits atherosclerosis and whose absence is associated with enhanced atherosclerosis and vulnerable plaque phenotype.[46,47] Another subset of T cells that could be involved in atherosclerosis is the NK1.1+ CD4+ cells that express the NK1.1 receptor usually found on NK cells. These cells recognize lipid antigens presented by the class I-like molecule CD1, which is expressed by macrophages of atherosclerotic lesions.[48] Activation of NK T cells results in strong Th1 and Th2 responses.

The notion that Th1 cells are involved in the progression of atherosclerosis is supported by experiments demonstrating that mice lacking interferon γ develop less atherosclerosis.

Studies in apoE mice lacking functional T and B cells have shown a reduction of atherosclerosis of up to 70%, which suggests that the net effect of adaptive immunity is proatherogenic.[49] Reconstitution of such mice with functional CD4+ T cells accelerates disease almost to the level of the fully immunocompetent apoE-deficient mouse.

Although most of the evidence suggests a proatherogenic role for the immune system, other studies have indicated that the immune system might also play a protective role. Evidence suggesting that this atheroprotective immunity is carried by B cells has come from experiments demonstrating that the more aggressive atherosclerosis encountered in splenectomized apoE-/- mice is reversed by injection of spleen B cells from atherosclerotic animals.[50] These observations suggest that an atheroprotective immunity can also develop during the course of disease.

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Immunomodulation for Atherosclerosis

Understanding the role of the immune system in atherosclerosis provides an impetus for development and testing of strategies that modulate the immune system to reduce atherosclerosis. Several immunomodulating strategies are being evaluated to influence atherosclerotic vascular disease (Table 1).

Table 1
Immunomodulation Strategies for Atherosclerosis

Immunosuppressive therapy	
	Corticosteroids
	Cyclosporine
	Sirolimus
Immunization (vaccination)	
	Active
	1) native LDL, oxidized LDL, apoB-related peptides, phosphorylcholine as antigens
	2) influenza and pneumococcal vaccines
	3) CETP vaccine to raise HDL levels
	Passive
	Immunoglobulin (IgG), antibody to oxidized LDL/apoB-related antigens
Tolerization	
	Mucosal exposure to heat-shock protein

CETP, cholesterol ester transfer protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Immunosuppressive Drugs

Immunosuppressive drugs include corticosteroids; cytotoxic drugs, such as azathioprine and cyclophosphamide; and inhibitors of T cell activation, such as cyclosporine A and rapamycin. Immunosuppressive drugs are primarily used to prevent acute rejection after organ transplantation. Although several animal studies have suggested that cyclosporine A and rapamycin might inhibit accelerated atherosclerosis involving arteries of the transplanted organ, the cytotoxicity of immunosuppressive drugs poses serious challenges.[51–53] Most immunosuppressive drugs also have adverse affects on cardiovascular risk factors, such as dyslipidemia, hypertension, and diabetes. The immunosuppressive compounds, cyclosporine A and rapamycin, not only act on T cells but can also inhibit smooth muscle proliferation and the response to vascular injury.[54,55] Coating of stents with rapamycin has been shown to reduce in-stent restenosis.[52] Corticosteroids have also been shown to reduce atherosclerosis in experimental animals.[56] However, the cytotoxicity and other adverse effects associated with the immunosuppressive drugs available at present make them unsuitable for wider use in the prevention and treatment of nontransplant atherosclerosis.

Immunization (Vaccination) for Atherosclerosis

Vaccines are likely to be the most important contributions to public health in the last 100 years. They have dramatically reduced death from infectious disease and have enabled the global eradication of smallpox. Modern vaccines are cheap, highly specific, and have generally few adverse effects. In recent years, attempts have been made to fight noninfectious, chronic diseases with immunization approaches. Different types of cancer vaccines based on tumor cells taken from patients and made immunogenic by addition of adjuvant or by transfection of genes encoding costimulatory molecules are being clinically tested.[57] Similarly, vaccines against Alzheimer's disease and diabetes are also under evaluation. In addition to active immunization with an antigen, passive immunization with antibodies to predefined antigens has also been tested for various diseases. The remarkable clinical benefits of anti-TNF antibodies in rheumatoid arthritis exemplify how passive immunization can be used to treat a chronic inflammatory disease in humans.

Immunization With Intact Oxidized LDL as Antigen

Oxidized LDL is believed to play a key role in atherosclerosis by causing intimal inflammation and foam-cell formation. Polyunsaturated fatty acids in phospholipids and cholesteryl esters are peroxidized, resulting in formation of reactive breakdown products, such as malondialdehyde (MDA) and 4-hydroxynonenal, which form covalent adducts with amino acids containing free amino groups in apoB-100.[58–60] Oxidation of LDL also leads to degradation of apoB-100 into numerous peptide fragments. These modifications target oxidized LDL for recognition by the immune system.[5,6] Several clinical studies have revealed that autoantibodies against oxidized LDL are common both in healthy subjects and in patients with cardiovascular disease.[61,62] Immunoglobulin (Ig)M titers are usually higher than those of IgG, which suggests a strong T cell-independent B-cell response. Several studies have reported the presence of increased antibody levels in subjects with cardiovascular risk factors, clinically manifest cardiovascular disease, and a more rapid progression of disease.[63–71]

To study the functional role of these immune responses, experimental models have been used to evaluate the effects of immunization with oxidized LDL. Unexpectedly, a 40%

to 70% reduction of atherosclerotic plaque development has been demonstrated in both rabbits and mice, thus establishing the existence of atheroprotective immune responses.[72–76] The levels of autoantibodies against oxidized LDL are low in mice on a chow diet but increase dramatically in response to a cholesterol-rich diet.[77] As in humans, these antibodies are primarily of the IgM type, whereas immunization induces a shift toward IgG.[76] After immunization, there is also an association between the increase in specific IgG levels and the extent of inhibition of plaque formation.[76] Taken together, these observations demonstrate the existence of oxidized LDL autoimmune responses in man and suggest that such responses might have atheroprotective effects. They also suggest the possibility of developing new therapeutic approaches based on selective activation of immune responses against oxidized LDL antigens.

Because oxidized LDL is a complex and poorly characterized particle containing many different epitopes that potentially could induce both atheroprotective and atherogenic immune responses, it has become important to obtain a more detailed molecular characterization of the antigens present in oxidized LDL. This information is now starting to become available, making further progress toward development of an immunization-based therapy for atherosclerosis possible. Two major subclasses of oxidized LDL antigens have been identified: specific MDA-modified peptide sequences in apoB-100 and oxidized phospholipids containing a phosphorylcholine head group, either present as an isolated lipid or covalently bound to an apoB-100 peptide sequence. Although immunization with both types of antigens inhibits atherosclerosis, the immune responses demonstrate clearly separate characteristics.

Immunization with MDA-apoB-100 Peptide Antigens

We have used a library of 302 20-amino-acid-long polypeptides covering the complete apoB-100 sequence to construct a corresponding number of MDA-apoB-100 peptide antibody enzyme-linked immunosorbent assays (ELISAs).[78] After screening of these ELISAs with pooled human plasma, more than 100 specific antibodies binding to different MDA-apoB-100 peptide sequences were identified. Antibodies recognized both hydrophobic and hydrophilic sequences in the apoB-100 molecule, and IgM was more common than IgG. Antibody binding to these synthetic apoB-100 peptides was completed by oxidized LDL but not by native LDL. Clinical studies performed on a number of the most abundant antibodies demonstrated that IgM levels decreased with age. There was also an association between high levels of IgM against these MDA-apoB-100 sequences and a low plasma concentration of oxidized LDL, which suggests that these antibodies might function by clearing oxidized LDL from the circulation and that this capacity decreases with age. Moreover, there was a significant association between high IgM levels and atherosclerosis as assessed by measurement of the carotid artery intima-media thickness. Finally, prospective studies demonstrated higher IgM levels against certain apoB-100 sequences in subjects who within 5 years developed acute myocardial infarction or sudden cardiac death. Similar but much weaker associations were observed for anti-apoB-100 peptide IgG levels. These studies identified a number of defined molecular targets for autoimmune responses against oxidized LDL in humans and demonstrated significant associations between antibody levels and disease. However, they did not clarify whether this association was due to an atherogenic effect of the immune response or reflected an association between severity of disease and activation of protective immune responses.

Immunization with several of these apoB-100 peptides was found to reduce atherosclerosis up to 70%.

The functional role of these immune responses was studied in our laboratories in apoE-/- mice immunized with the same apoB-100 peptide sequences that were found to induce autoimmune responses in humans. Immunization with several of these apoB-100 peptides was found to reduce atherosclerosis up to 70%, as well as to decrease macrophage and increase collagen contents of remaining plaques.[79,80] Interestingly, immunization with apoB-100 peptide sequences that were not homologous between human and mouse did not inhibit atherosclerosis. Immunizations resulted in a marked increase in specific IgG but had only marginal effects on IgM levels. Immunoglobulin G expression also changed from IgG2a to IgG1, which suggests activation of a Th2 response. However, it was not associated with a decreased expression of Th1 cytokines in atherosclerotic plaques or in the spleen.

Taken together, these data suggest an important but complex role for immune responses against MDA-modified apoB-100 peptide sequences in atherosclerosis. The predominat-

ing immune response to oxidation of LDL in tissues seems to be production of IgM that might help to remove circulating oxidized LDL. The level of this innate immune response correlates with disease severity[78–81] but does not seem to provide sufficient protection against development of atherosclerosis. Atheroprotective immunization with apoB-100 peptides in mice is associated with expression of IgG, which suggests that these antibodies might be more effective in inhibiting atherosclerosis. In humans, IgG levels against MDA-apoB-100 peptide sequences show only a weak or no association with disease, and it remains to be clarified whether immunization-induced activation of anti-apoB-100 IgG can provide effective atheroprotection in humans.

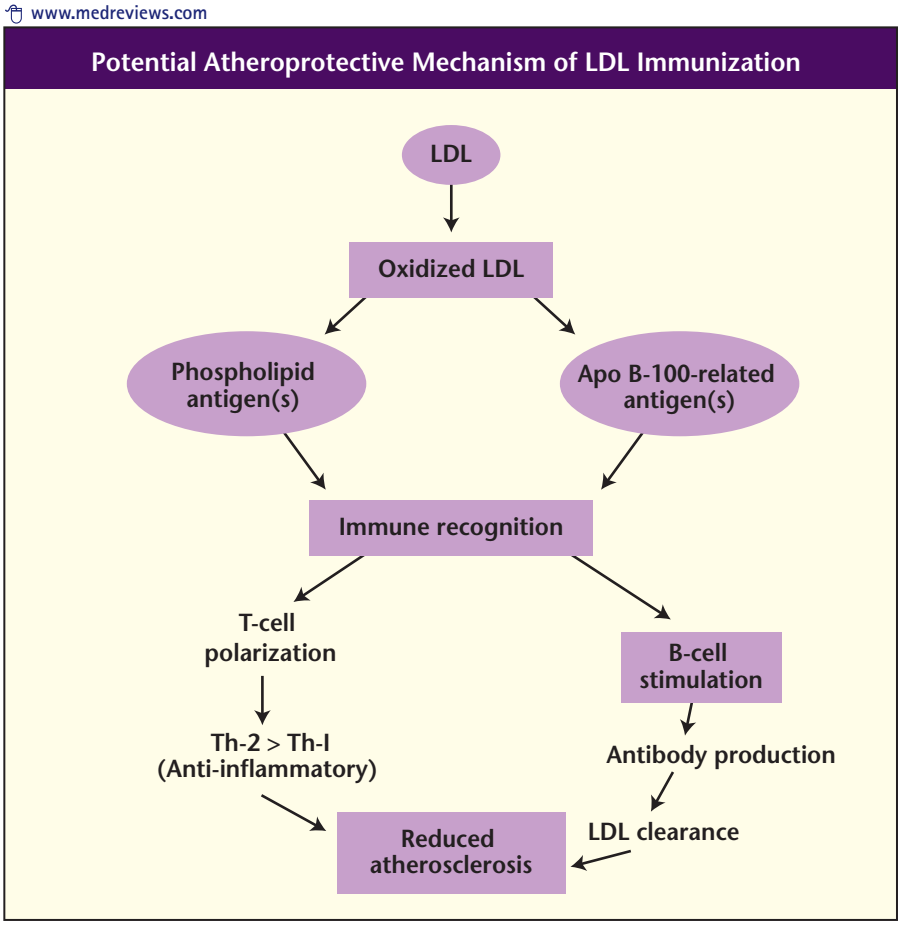

Figure 2. *Potential atheroprotective mechanism of low-density lipoprotein (LDL) immunization.*

Oxidized Phospholipid Antigens

The presence of oxidized phospholipid antigens in oxidized LDL was first demonstrated by Palinski and colleagues,[82] who established a panel of B cell hybridomas from naive (nonimmunized) apoE-deficient mice. A number of clones were selected that produced antibodies specifically 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 antibodies binding to copper-oxidized LDL recognized the same oxidized phospholipid antigen, 1-palmitoyl-2 (5-oxovaleroyl)-sn-glycero-3 phosphorylcholine.[83,84] The antibodies recognized epitopes both in the lipid moiety of oxidized LDL and in dilapidated, modified apoB-100, which suggests that the antigen can exist as a free lipid as well as an adduct to apoB-100. Epitopes recognized by these antiphospholipid IgMs were also identified on the surface of apoptotic cells, and antibody binding was shown to inhibit scavenger receptor-mediated uptake of oxidized LDL and apoptotic cells in macrophages.[83,85] Later studies revealed that the genes encoding the antigen-binding site of these antibodies were identical to those encoding the T15 antiphosphorylcholine antibodies produced by the B1 subset of B cells.[86] T15 antibodies also provide protection against several common infectious agents, including *Streptococcus pneumoniae*.[87] Binder and coworkers[88] used this information to study the functional role of antiphospholipid antibodies in atherosclerosis by immunizing LDL receptor knockout mice with *S. pneumoniae*.[88] This treatment was found to result in induction of high levels of oxidized LDL-specific IgM and a modest reduction of atherosclerosis. Antibodies in serum from immunized mice specifically recognized epitopes in atherosclerotic plaques and blocked uptake of oxidized LDL in cultured macrophages. Interestingly, immunization did not induce an increase in specific IgG. Accordingly, the responses to immunization with MDA-modified apoB-100 peptide sequences and oxidized phospholipids differ in the respect that the former induces an adaptive, T cell-dependent synthesis of IgG, whereas the latter induces on innate, B cell-dependent synthesis of IgM. Both types of immune responses seem to have atheroprotective

effects, but it remains to be determined whether they are additive.

The possible mechanisms of action for both phospholipid and Apo-B-100 antigen-triggered atherosclerosis reduction are shown in Figure 2.

Immunization with Non–LDL-Related Antigens

A few experimental studies have evaluated the possibility of inhibiting atherosclerosis by active immunization or by directly providing blocking antibodies against key proteins in the disease process. Rittershaus and colleagues[89] immunized hypercholesterolemic rabbits with a peptide containing a region of the cholesteryl ester transfer protein (CETP), an enzyme known to be responsible for transferring cholesteryl esters from high-density lipoprotein to very-low-density lipoprotein and LDL. This was found to result in an inhibition of CETP activity, increased high-density lipoprotein cholesterol, and a reduced development of atherosclerosis. A first clinical trial of a CETP vaccine is ongoing.[90] Attempts to inhibit the effect of TNF-α by immunization with a recombinant TNF-α molecule has failed to reduce atherosclerosis in ApoE-deficient mice, whereas treatment with antibodies against CD40 ligands has been shown to inhibit atherosclerosis in LDLr- mice.[91] However, early clinical trials of CD40 blockade were terminated because of unwanted side effects.

Oxidized low-density lipoprotein is believed to play a key role in atherosclerosis.

Influenza and Pneumococcal Vaccination

Observational studies, case–control studies, and a small pilot randomized trial have suggested that influenza vaccination might significantly reduce acute cardiovascular events (including coronary events) in humans.[92–95] In experimental models, influenza infection has been shown to induce an arterial inflammatory response, which could contribute to plaque destabilization; this might account for the potential benefits of influenza vaccination.[95] However, proof of definitive cardioprotective effects of influenza vaccination needs further validation in a large, prospective, randomized trial.

Similarly, pneumococcal vaccination was recently shown to reduce atherosclerosis in a murine model, presumably because of molecular mimicry between pneumococcal antigen and oxidized LDL antigen.[88] Further studies are needed to establish cardioprotective effects of pneumococcal vaccination in humans.

Tolerization with Mucosal Antigen Exposure

Many infections are associated with activation of immune responses

Main Points

- A large body of data accumulated over the past several years has identified a key role for inflammation in the initiation, progression, and destabilization of atherosclerosis.
- Modulation of immune responses involved in atherosclerosis with vaccines, passive immunization by antibody treatment, and induction of tolerance represents a new paradigm for the prevention and treatment of cardiovascular disease.
- Studies have suggested that cyclosporine A and rapamycin might inhibit accelerated atherosclerosis in arteries of transplanted organs, but the cytotoxicity and other adverse effects associated with the immunosuppressive drugs make them unsuitable for wider use in the prevention and treatment of nontransplant atherosclerosis.
- The existence of oxidized low-density lipoprotein (LDL) autoimmune responses in man has been demonstrated, and these responses might have atheroprotective effects; this suggests the possibility of developing new therapeutic approaches based on selective activation of immune responses against oxidized LDL antigens.
- Two major subclasses of oxidized LDL antigens have been identified: specific malondialdehyde-modified peptide sequences in apolipoprotein B-100 and oxidized phospholipids containing a phosphorylcholine head group; immunization with both types of antigens inhibits atherosclerosis in mice.
- Studies have suggested that influenza and pneumococcal vaccination might have cardioprotective effects; however, validation of this in larger studies is needed.
- In the case of heat shock protein (Hsp), the goal of immunomodulation has been to induce tolerance to the antigen. Mouse studies using oral and nasal administration of Hsp65 reported a significant decrease in atherosclerosis.

against heat shock protein of the invading microorganisms. In contrast to the findings with oxidized LDL-associated antigens, parenteral immunization with mycobacterial Hsp65 is strongly atherogenic.[26,27] This is believed to be owing to induction of autoimmunity against native Hsp60 in stressed vascular cells. There is also clinical evidence for an association between antibodies against Hsp65/60 and the severity and progression of carotid atherosclerosis.[29] Accordingly, in the case of heat shock protein, the goal of immunomodulation has been to induce tolerance to the antigen. The immune response to an antigen might vary significantly, depending on the route of administration. Mucosal administration of the antigen frequently generates tolerance, suppresses proinflammatory Th1 cells, and decreases organ-specific inflammation in several animal models of autoimmune disease.[96,97] This possibility has recently been studied by Harats and coworkers,[98] who exposed LDLr-/- mice to Hsp65 through oral administration, and Maron and colleagues,[99] who used both oral and nasal administration of Hsp65 in the same strain of mice. Both studies reported a significant decrease in atherosclerosis associated with decreased levels of heat shock protein antibodies and suppressed T-cell reactivity against heat shock protein. There was also a suppression of plaque inflammation and an inhibition of the expression of Th1 cytokines. The possibility of inhibiting atherosclerosis by suppressing autoimmune responses against heat shock protein is of considerable interest and needs further evaluation.

Summary

Modulation of immune responses involved in atherosclerosis with vaccines, passive immunization by antibody treatment, and induction of tolerance represent a new paradigm for the prevention and treatment of cardiovascular disease. It is hoped that over the foreseeable future one or more of these novel approaches will be tested in humans, because the implications for global cardiovascular disease prevention could be substantial. ■

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