

Immunomodulation of Atherosclerosis Implications for Vaccine Development

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Abstract—A number of studies have shown activation of the immune system throughout various stages of atherosclerosis. Recent observations have suggested that activation of immune responses may promote atherosclerosis on one hand by inducing and perpetuating arterial inflammation, whereas on the other hand, selective activation of certain immune functions may inhibit atherosclerosis and arterial inflammation. These observations suggest the possibility that selective suppression of proatherogenic immune responses or selective activation of antiatherogenic immune responses may provide new approaches for atherosclerosis prevention and treatment. Several antigens activating immune responses affecting development of atherosclerosis have been identified. These immune responses may be modulated by presenting the antigens together with different types of adjuvants as well as through the route of administration. In this review, we summarize recent experimental studies using immunomodulatory approaches for treatment of atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2005;25:18-28.)

Key Words: atherosclerosis ■ vaccine ■ immunity ■ lipoproteins ■ heat shock proteins

Over the last few decades, our understanding of the basic mechanisms involved in development of atherosclerotic plaques and acute cardiovascular events has progressed significantly. The important role of inflammation at all stages of the disease process is now recognized^{1,2} and has focused attention on the immune system as a possible novel target in prevention and treatment of cardiovascular disease. Development of atherosclerosis is influenced by innate and adaptive immune responses.^{3,4} Innate immunity represents a fast but relatively blunt inflammatory and toxic response to invading microorganisms but also interacts with several modified endogenous antigens. It is based on detection of pathogen-associated molecular patterns by pattern recognition receptors on macrophages and dendritic cells.⁵ There is a repertoire of pattern recognition receptors binding a wide range of proteins, carbohydrates, lipids, and nucleic acids, but those considered most important in atherosclerosis are the scavenger receptors (SRs) and the Toll-like receptors (TLRs). Adaptive immunity is much more specific than innate immunity but may take several days or even weeks to be fully

mobilized. 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 foreign antigens. Immunomodulatory therapies, such as immunization and immunosuppressive drugs, usually target adaptive immune responses. However, the effect of immunization on adaptive immune responses can in turn be modulated by targeting elements of innate immunity, such as the TLRs.

Manipulating the Immune System to Treat Disease

There are 2 major ways of manipulating the immune system: immunosuppressive drugs and immunization. Immunization can either be active, in which an immune response is induced through exposure to an antigen, or passive, in which preformed antibodies are administered directly.

The immunosuppressive drugs available today include (1) anti-inflammatory corticosteroids, (2) cytotoxic drugs such as azathioprine and cyclophosphamide, and (3) fungal and

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bacterial derivatives inhibiting T-cell activation such as cyclosporine A and rapamycin. Immunosuppressive drugs are used to prevent acute rejection after organ transplantation and to treat certain autoimmune diseases. Because aggressive arteriosclerosis (transplant vascular sclerosis [TVS]) in arteries of the transplanted organ is the major cause of late rejection, it would be beneficial if immunosuppressive drugs also inhibited TVS. Although several animal studies have suggested that cyclosporine A⁶ and rapamycin⁷ may have an inhibitory effect, data suggest that the cytotoxicity of immunosuppressive drugs causes vascular injury, thus contributing to TVS progression.⁸ Most immunosuppressive drugs also have adverse effects on cardiovascular risk factors such as dyslipidemia, hypertension, and diabetes.

The immunosuppressive compounds cyclosporin A and rapamycin not only act on T cells but can also inhibit smooth muscle proliferation and the response to vascular injury.^{9,10} Coating of stents with rapamycin may therefore help prevent restenosis.⁷ Corticosteroids have also been shown to reduce atherosclerosis in experimental animals.¹¹ However, cytotoxicity and other adverse side effects associated with the immunosuppressive drugs available at present make them less suitable for wider use in prevention and treatment of nontransplant atherosclerosis.

Passive immunization denotes transfer of preformed antibodies and includes treatment of certain infections with hyperimmune sera. Recombinant antibodies could also be considered examples of passive immunization. The remarkable effects of anti-tumor necrosis factor (TNF) antibodies in rheumatoid arthritis and of antibodies to platelet glycoprotein IIb/IIIa in unstable angina and restenosis prevention may be viewed as examples of how passive immunization can be used to treat noninfectious disease in humans.

Active immunization uses antigen preparations, vaccines, to induce a protective immune response. Vaccines are likely to be the most important medical contribution to public health during the last 100 years. They have dramatically reduced death from infectious disease and resulted in the global eradication of smallpox. Modern vaccines are cheap, highly specific, and have generally few adverse side effects. In recent years, attempts have been made to fight noninfectious chronic diseases by using 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, as are vaccines against Alzheimer's disease and diabetes.

When considering the possibility of developing vaccines against atherosclerosis, it will be important to learn from the experience of developing immunotherapeutic approaches to other chronic inflammatory diseases, such as rheumatoid arthritis, type I diabetes, and Alzheimer's disease. Alzheimer's disease is believed to develop in response to accumulation of toxic levels of amyloid β peptide in cognitive brain regions caused by a failure of endogenous clearance processes (ie, a mechanism with some similarities to that proposed for oxidized low-density lipoprotein [LDL] in atherosclerosis). Immunization with synthetic amyloid β peptides in mice models of Alzheimer's disease has resulted

in decreased amyloid deposits with concomitant beneficial neuropathological and behavioral changes.¹² Subsequent human trials have shown beneficial effects of amyloid β peptide immunization on cognitive functions, but a phase II trial was discontinued shortly after its initiation when $\approx 5\%$ of the treated patients developed what appeared to be a central nervous system inflammatory reaction. Similar disappointing results have been encountered in vaccination trials for other inflammatory diseases.¹³ The mechanism responsible for these adverse events remains to be fully understood but may have involved the use of a Th1-inducing adjuvant in the clinical trials as opposed to the Th2-favoring administrations used in the animal studies.

Packaging and Administration Determines Immune Effector Responses to Vaccination

The outcome of an immunization is determined not only by the antigen but also on the adjuvant in which it is administered and the route used for administration. Although immunologists have focused their efforts almost exclusively on identifying and designing antigens, it has long been known that adjuvants and route of administration play a decisive role for the response. The tendency to ignore the role of these factors prompted Charles Janeway to label the adjuvant effects "the dirty little secret of immunology."¹⁴ Immunologic adjuvants are agents that act to enhance, accelerate, modify, or prolong specific immune responses to vaccine antigens. They function through three basic mechanisms: (1) effects on antigen delivery and presentation, (2) induction of immunomodulatory cytokines, and (3) effects on antigen-presenting cells. The original adjuvants were based on aluminum salt-containing gels (alum) primarily functioning by complexing and retaining the antigens at the site of injection. These still remain the only adjuvants in US-licensed vaccine formulation. Subsequent development in adjuvant technology has shown that adjuvants work more effectively if they also interact directly with immune cells. Most of these adjuvants are mixtures of oils, salts, killed bacteria, etc, that are empirically known to promote adaptive immune responses. For instance, parenteral administration using Freund's complete adjuvant usually induces strong Th1/delayed-type hypersensitivity (DTH) and antibody responses.¹⁵ This adjuvant contains mineral oil and heat-killed mycobacteria (with substantial amounts of heat shock proteins [Hsps]). Incomplete Freund's adjuvant, which lacks mycobacteria, induces antibody production but does not promote DTH reactions to the same extent as complete Freund's adjuvant. Dendritic cells can be activated by coadministration of DNA sequences containing unmethylated cytosine guanine dinucleotides (CpG) motifs, which ligate TLRs; this increases antigen presentation, leading to enhanced immune responses to the antigen.¹⁶ Several new types of adjuvants have been tested in preclinical and clinical trials including liposomes, immunostimulatory complexes, and biodegradable polymer microspheres. Some of these may become relevant for testing in immunomodulation of atherosclerosis because they mimic the pattern through which, for example, oxidized LDL antigens are presented. Other adjuvant approaches introduced more recently include coadministration of cytokines, such as

Types of Immunological Adjuvants

| | Example | References |
|---|--|-------------|
| Gel-type | Aluminium hydroxide calcium phosphate | 118,119 |
| Microbial | Muramyl dipeptide bacterial exotoxins endotoxin-based adjuvants bacterial DNA | 120–123 |
| Particulate | Biodegradable polymer microspheres Immunostimulatory complexes liposomes | 124–126 |
| Oil-emulsion and surfactant-based adjuvants | Freund's incomplete adjuvant microfluidized emulsions saponins | 127–130 |
| Synthetic | Muramyl peptide derivatives nonionic block copolymers polyphosphazane synthetic polynucleotides | 129,131–133 |
| Cytokines | IL-12 granulocyte-macrophage colony-stimulating factor INF- γ | 134,135 |
| Genetic | Cytokine genes or genes encoding costimulatory molecules delivered as plasmid DNA | 136,137 |

Adopted from R. Vogel and C.R. Alving. Progress in Adjuvant Development, 1982–2002. The Jordan Report.

interleukin-12 (IL-12) and interferon- γ , and genetic synthesis of fusion proteins containing peptide sequences of antigen and costimulatory molecules. A summary of the different types of immunologic adjuvants available is presented in the Table.

Mucosal Immunization and Induction of Tolerance

Several different strategies have been used to target immunogens to selectively induce protective responses. Activation of the mucosa-associated lymphoid tissue can induce protective immune responses or tolerize against the immunogen, depending on the precise protocols.¹⁷ This strategy uses oral or nasal administration of antigen to activate immune cells in mucosal membranes. The mechanisms remain incompletely understood but appear to involve recruitment of local dendritic cells and possibly activation of immune regulatory cytokines (eg, IL-10 or transforming growth factor- β [TGF- β]). Depending on the dose and precise protocol for administration of antigen, mucosal vaccination can result in tolerance or production of high-titer antibodies. This route can be stimulated further if the antigen is conjugated with a molecule that binds to epithelial glycolipids, such as the cholera toxin B subunit (CTB).¹⁸ Oral immunization with autoantigens, with or without previous conjugation to CTB, can ameliorate certain experimental autoimmune diseases.^{17,19} In animal models of experimental autoimmune encephalitis (EAE), type 1 diabetes, and collagen-induced arthritis, disease development was markedly inhibited by oral or nasal administration of a few low-dose treatments with chemical conjugates or gene fusion proteins between CTB and, respectively, myelin basic protein, insulin, and collagen II. Accordingly, recent studies show effects of mucosal immunization also on experimental atherosclerosis (see below). The mechanisms in-

involved are proposed to include activation of regulatory T cells (Tregs), immunoregulatory cytokines, or protective antibodies.

There are other approaches for induction of immune tolerance that may have relevance for immunomodulation of atherosclerosis. Antigen presentation by dendritic and other antigen-presenting cells (activated protein C) in the absence of costimulation inactivates naïve T cells, inducing a state known as anergy. This helps ensure tolerance of T cells to self-antigens but has also been used experimentally to induce tolerance. Dendritic cells exposed to antigens in the absence of costimulatory cytokines in vitro develop into a tolerogenic state.²⁰ Injection of such tolerogenic dendritic cells has been found to halt the progression of EAE by a mechanism involving activation of regulatory T cells.²¹ Blockade of the costimulatory receptors required for T-cell activation is yet another approach that has shows promise in induction of tolerance after tissue transplantation.²² Finally, exposure of T cells to ligands with minor alterations, such as a single amino acid substitution, may also result in induction of tolerance.²³

What Adjuvant and Route of Administration Should be Used When Immunizing Against Atherosclerosis?

Identifying the key antigens responsible for activation of immune responses involved in atherosclerosis is a prerequisite for development of an immunization therapy. However, finding the most suitable adjuvant and route of administration represents an equally important challenge. Understanding the mechanisms through which each antigen contributes to the disease is also necessary for making the best combination of antigen and vehicle of administration. There is accumulating evidence that activation of immunity in atherosclerosis pri-

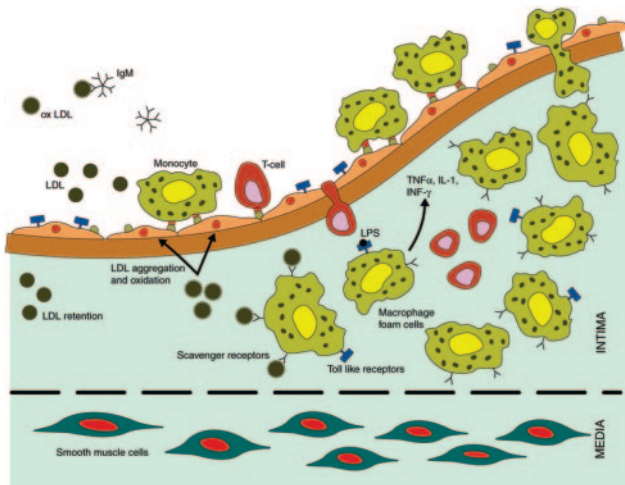


Figure 1. Innate immune responses to retention of modified lipoproteins in the arterial intima. LDL particles adhering to intimal extracellular matrix proteoglycans aggregate and become oxidized in response to enzymatic modification or oxidative stress. Activation of endothelial adhesion molecule expression results in infiltration of monocytes and T cells. Monocytes differentiate into macrophages expressing SRs and TLRs. They remove oxidized LDL from the extracellular space and release proinflammatory cytokines that promote further leukocyte recruitment. LDL with minor oxidative modification may be removed by IgM in the circulation.

marily involves proinflammatory Th1 cells and that these promote disease development.^{3,4} Accordingly, adjuvants that favor a shift toward an anti-inflammatory Th2 response, such as Alum and Freund’s incomplete adjuvant, may be more effective than adjuvants favoring Th1 responses. Alternatively, it may be possible to inhibit activation of Th1-mediated immune responses through induction of tolerance by mucosal administration with or without adjuvants such as CTB.³

Antigen Activation of Immune Responses in Atherosclerosis

There is compelling evidence from experimental studies that the arterial inflammation that precedes plaque development is caused by accumulation of LDL in the extracellular matrix of the vessel (Figure 1).^{24–26} These LDL particles are modified by a number of mechanisms, including enzymatic degradation, aggregation, and oxidation, resulting in induction of a number of proinflammatory genes, including leukocyte adhesion molecules and chemokines, which collectively help to recruit, retain, and activate inflammatory cells in the vessel wall.^{27–31}

Once resident in the arterial intima, monocytes will differentiate into macrophages expressing an array of SRs.^{32–35} These mediate removal of modified lipoproteins, apoptotic cells, and some microorganisms through the endocytic pathway, which not only results in degradation but may lead to antigen presentation and activation of adaptive immunity.³⁶ Macrophages also express TLRs that may be activated by lipopolysaccharide, Hsps, and other microbial antigens and induce intracellular signaling through the activator protein-1 and nuclear factor κB pathways, further promoting the inflammatory response and modulating subsequent adaptive

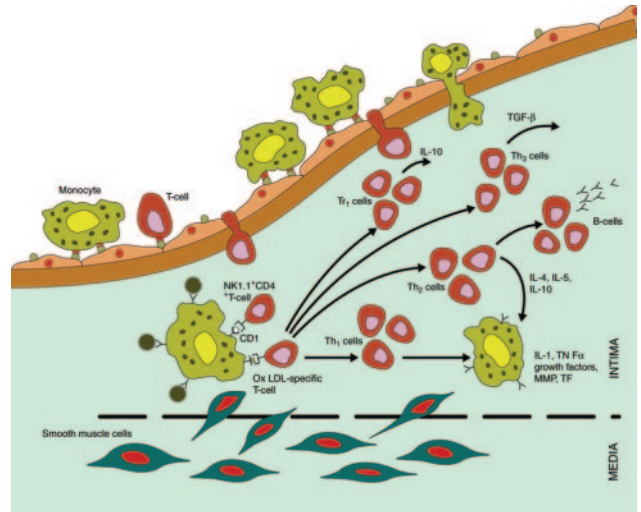


Figure 2. Adaptive immune responses to oxidized LDL and Hsps. Oxidized LDL and microbial Hsp antigens are presented by macrophage MHC class II proteins for recognition by antigen-specific CD4⁺ T cells. Activated CD4⁺ T cells may differentiate into proinflammatory Th1 cells, Th2 cells promoting antibody production, Tr1 cells that suppress antigen-induced activation of other CD4⁺ T cells, or TGF-β-producing Th3 cells. Presentation of lipid antigens by the macrophage class I-like molecule CD1 results in activation of NK1.1⁺ CD4⁺ cells promoting Th1 and Th2 responses. The balance between proinflammatory and anti-inflammatory T-cell subsets has a major influence on disease activity and progression. Regulation of this balance remains to be fully understood but is in part determined by cytokines.

immune responses (Figure 1).^{37–39} Recent studies have shown that TLRs are expressed in human and murine atherosclerotic lesions and may be induced by modified lipoproteins.^{40–43} Interruption of TLR-mediated innate immune signaling through genetic ablation of key genes has been shown to be atheroprotective in murine models.^{44,45}

Uptake and Processing of Oxidized LDL by Macrophages Represent a Link Between Innate and Adaptive Immunity

After its uptake by SRs, oxidized LDL is processed by macrophages and probably also by dendritic cells, and epitopes derived from the LDL particle are presented by MHC class II proteins for recognition by specific CD4⁺ T cells (Figure 2).^{32,46} When T cells encounter their specific antigens on an MHC class II molecule, an adaptive immune response is activated including clonal proliferation of the T cell and production of cytokines and immunoglobulins.

There are 2 major subsets of CD4⁺ cells: Th1 cells that secrete IFN-γ and TNF-α, and Th2 cells that produce IL-4, IL-5, and IL-10.⁴⁷ In general terms, Th1 cells promote macrophage activation and inflammation, whereas Th2 cells mediate antibody production and allergic reactions (Figure 2). Both types are required for complete protection against various forms of infections, but Th1 cells are usually associated with initiation or aggravation of organ-specific autoimmune diseases.⁴⁸ Analysis of the cytokine expression in atherosclerotic plaques suggests a dominance of Th1 cells.^{49,50} During the early stages of plaque development in apolipoprotein E^{-/-} (apoE^{-/-}) mice, circulating IgG

against oxidized LDL is mainly of the IgG_{2a} subtype characteristic for a Th1 immune response.⁵¹ However, at more advanced stages of the disease, a shift toward expression of Th2-specific IgG has been observed in severely hypercholesterolemic animals,⁵¹ suggesting that the immune system is adapting to evolve into a less proinflammatory and proatherogenic response.

Studies in apoE^{-/-} mice lacking functional T and B cells have shown a reduction of atherosclerosis with up to 70% suggesting that the net effect of adaptive immunity is proatherogenic.⁵² Reconstitution of such mice with functional CD4⁺ T cells accelerates disease almost to the level of the fully immunocompetent apoE^{-/-} mice. This implies that the dominating effect of CD4⁺ T cells on atherosclerosis is to aggravate the disease process.

During recent years, it has become clear that the subdivision of CD4⁺ T cells into Th1 and Th2 cells is too simplified. The action of these subtypes is checked by Tregs, which produce inhibitory signals through immunosuppressive cytokines.⁵³ Tregs are characterized by constitutive CD25 expression and can be isolated as CD4⁺CD25⁺ cells in peripheral blood. They inhibit the activation of other T cells by producing immune inhibitory cytokines; functional subsets within the Treg population can be identified because of their profile of cytokine expression. Cells that produce high levels of IL-10 but only minor amounts of IL-4 have been termed Tr1 (T regulatory 1) cells and suppress antigen-induced activation of other CD4⁺ T cells by secreting IL-10, which is a "cytokine secretion inhibiting cytokine."⁵⁴ Bystander activation of Tr1 cells was found recently to reduce atherosclerosis in a mouse model,⁵⁵ and there is ample evidence for an atheroprotective role of IL-10 in mouse models of atherosclerosis.⁵⁶⁻⁵⁸

Immunosuppression is also accomplished through secretion of TGF- β . Several different cell types can inhibit Th1-type immune responses by secreting TGF- β , including macrophages and vascular cells. TGF- β -producing Treg and Th3 cells can provide antigen-specific immunosuppression by acting on other T cells. Abrogation of TGF- β signaling to T cells (by a genetic strategy using a dominant-negative TGF- β receptor under the CD4 promoter) results in a dramatic exacerbation of atherosclerosis on the apoE^{-/-} background.⁵⁹ This suggests that proatherogenic T cells under normal conditions are inhibited by TGF- β . Antigen-specific activation of TGF- β - or IL-10-producing Tregs could be an attractive means of inducing protective immunity against atherosclerosis.

A further subset of T cells that could be involved in atherosclerosis is the NK1.1⁺ CD4⁺ cells that express the NK 1.1 receptor usually found on natural killer (NK) cells. These cells recognize lipid antigens presented by the class I-like molecule CD1,⁶⁰ which is expressed by macrophages of atherosclerotic lesions.⁶¹ Activation of such NKT cells results in strong Th1 and Th2 responses and was found recently to aggravate early atherosclerosis in apoE^{-/-} mice.⁶²

Together, these studies suggest that adaptive immunity has an important but complex role in atherosclerosis. There is considerable evidence that activation of Th1 immune responses contribute to a more aggressive progression of the

disease but counteracted several immunoregulatory loops. If this concept is correct, it should be possible to influence disease activity by selective activation or inhibition of specific immune responses. In support of this interesting possibility, immunization of hypercholesterolemic animals with oxidized LDL has been found to inhibit atherosclerosis.⁶³⁻⁶⁷ These data provide important evidence for atheroprotective adaptive immune responses. Evidence suggesting that 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.⁶⁸ These observations suggest that an atheroprotective immunity develops during the course of disease and support the idea that a vaccine could be developed for prevention or treatment of atherosclerosis.

Immune Responses Against Microbes and Hsps

Other antigens than those present in oxidized LDL have also been implicated in atherogenesis. Infections agents such as *Chlamydia pneumoniae* have been detected in human atherosclerotic plaques.⁶⁹ It is possible that immune responses against viral and bacterial antigens in plaques may aggravate disease and destabilize plaques by activating TLRs, but this remains to be shown. A particularly interesting family of microbial antigens in this respect are the Hsps, such as the mycobacterial Hsp 65 and the chlamydial Hsp 60.⁷⁰ Both of these proteins show considerable mimicry with human Hsp 60,⁷¹ suggesting that immune responses against microbial Hsps could cross-react with Hsps expressed by stressed arterial cells. In line with this, immunization of LDL receptor (LDLR)^{-/-} mice⁷² and hypercholesterolemic rabbits⁷³ with Hsp 65 was found to promote atherosclerosis. Moreover, Lamb et al⁷⁴ found an association between the magnitude of the immune response to Hsp 65 after bacillus Calmette-Guérin immunization and development of atherosclerosis in rabbits. It has also been reported that antibody levels against Hsp 65 correlates with progress of carotid disease in humans.⁷⁵

Immunization Strategies and Atherosclerosis

Experimental studies evaluating effects of immunization on atherosclerosis have focused on 2 different types of targets. The first type of target is pre-existing immune responses believed to be part of the disease process, such as immune responses against oxidized LDL epitopes and Hsp 60. The second types of target are endogenous proteins believed to promote atherogenesis such as cholesteryl ester transfer protein (CETP) and TNF. For the first type of target, the aim is to stimulate those immune responses that are per se protective but of insufficient magnitude unless boosted by a vaccine, initiate similar and possibly more effective immune responses, or to induce tolerance against unwanted auto-immune responses. For the second type of target, the aim is to produce neutralizing antibodies inhibiting the effect of the targeted antigen.

Immunization Using Intact Oxidized LDL or Defined Oxidized LDL-Associated Antigens

Oxidized LDL is believed to play a key role in atherosclerosis by causing intimal inflammation and foam cell formation.

Polysaturated 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.^{76–78} LDL oxidation also leads to degradation of apoB-100 into numerous peptide fragments. These modifications target oxidized LDL for recognition by the immune system.^{28,29} Subsequent clinical studies have revealed that autoantibodies against oxidized LDL are common in healthy subjects and in patients with cardiovascular disease.^{79,80} IgM titers are usually high, suggesting a strong T cell-independent B-cell response. Several studies have reported presence of increased antibody levels in subjects with cardiovascular risk factors,^{81–83} clinically manifested cardiovascular disease,^{84–88} and a more rapid progression of disease.⁸⁹

To study the functional role of these immune responses, experimental animals were immunized with oxidized LDL. Unexpectedly, this was found to result in a 40% to 70% reduction of plaque development in rabbits and mice.^{63–67} These results need to be interpreted with some caution because mixed-breed animals were used in some of the studies and variable effects seen in different parts of the aorta in 2 of the studies,^{64,65} yet they clearly support the existence of atheroprotective immune responses against epitopes present in oxidized LDL. The levels of autoantibodies against oxidized LDL are low in mice on chow diet but increase dramatically in response to a cholesterol-rich diet.⁹⁰ These antibodies are primarily of IgM type, whereas immunization induces a shift toward IgG.⁶⁷ After immunization, there is also an association between the increase in specific IgG levels and the extent of inhibition of plaque formation.⁶⁷ Together, these observations demonstrate the existence of oxidized LDL autoimmune responses in man and suggest that such responses may have atheroprotective effects. They also suggest the possibility of developing new therapeutic approaches based on selective activation of immune responses against oxidized LDL antigens. However, because oxidized LDL is a complex and poorly characterized particle containing numerous different epitopes that could potentially 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 possible toward development of an immunization-based therapy for atherosclerosis. 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 antigen inhibits atherosclerosis, the immune responses demonstrate clearly separate characteristics.

MDA-ApoB-100 Peptide Antigens

We have used a library of 302 20-aa-long polypeptides covering the complete apoB-100 sequence to construct a corresponding number of MDA-apoB-100 peptide antibody ELISAs.⁹¹ After screening of these ELISAs with pooled

human plasma, >100 specific antibodies binding to different MDA-apoB-100 peptide sequences were identified. 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, suggesting that these antibodies may function by clearing oxidized LDL from the circulation. However, recent studies demonstrating that the absence of B cells does not influence the clearance of oxidized LDL from plasma in mice argue against this possibility.⁹² 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 that developed acute myocardial infarction or sudden cardiac death within 5 years.⁹¹ 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 attributable to an atherogenic effect of the immune response or reflected on association between severity of disease and activation of protective immune responses.

The functional role of these immune responses was studied in apoE^{-/-} mice immunized with the same apoB-100 peptide sequences that were identified to induce autoimmune responses in humans. Immunizations with several of these apoB-100 peptides were found to reduce atherosclerosis up to 70% as well as to decrease macrophage and increase collagen contents of remaining plaques.⁹³ 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. IgG expression also changed from IgG_{2a} to IgG₁, suggesting activation of a Th2 response. However, it was not associated with a decreased expression of Th1 cytokines in atherosclerotic plaques or in the spleen.

Together, these data suggest an important but complex role for immune responses against MDA-modified apoB-100 peptide sequences in atherosclerosis. The predominating immune response to oxidation of LDL in tissues appears to be production of IgM. The level of this immune response correlates with disease severity^{91,94} but does not appear to provide sufficient protection against development of atherosclerosis. Atheroprotective immunization with apoB-100 peptides in mice is associated with expression of IgG. Schiopu et al⁹⁵ produced recombinant human IgG against the same apoB-100 peptides. Treatment of apoE^{-/-} mice with these antibodies reduced plaque size and macrophage content, suggesting that the atheroprotective effect to some extent is carried by generation of specific IgG. In man, 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.

Oxidized Phospholipid Antigens

The presence of oxidized phospholipid antigens in oxidized LDL was demonstrated by Hörkkö et al,⁹⁶ who established a panel of B-cell hybridomas from naive (nonimmunized) apoE^{-/-} 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 oxidized phospholipids, primarily 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine.^{97,98} The antibodies recognized epitopes in the lipid moiety of oxidized LDL, as well as in delipidated, modified apoB-100, suggesting that the antigen can exist as a free lipid as well as an adduct to apoB-100. Epitopes recognized by this antiphospholipid IgM were also identified on the surface of apoptotic cells, and antibody binding was shown to inhibit SR-mediated uptake of oxidized LDL and apoptotic cells in macrophages.^{97,99} 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.¹⁰⁰ T15 antibodies also provide protection against several common infectious agents, including *Streptococcus pneumoniae*.¹⁰¹ Binder et al¹⁰² used this information to study the functional role of antiphospholipid antibodies in atherosclerosis by immunizing LDLr knockout mice with *Streptococcus pneumoniae*. This treatment was found to result in induction of high levels of oxidized LDL-specific IgM, reduced serum cholesterol level, 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, 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 an innate-like, B cell-dependent synthesis of IgM. Both types of immune responses appear to have atheroprotective effects, but it remains to be determined whether they are additive.

Are the Effects of Immunization With Oxidized LDL Antigens General or Site-Specific?

It still remains to be fully clarified whether the atheroprotective effect of immunization with oxidized LDL is the same at different locations of the arterial system. In a study performed on hypercholesterolemic rabbits, Ameli et al⁶⁴ demonstrated inhibition of atherosclerosis in the abdominal but not in more proximal parts of the aorta. Studies performed in mice have consistently shown that immunization with oxidized LDL inhibits plaque development in the aortic root,^{65,67,103} whereas the only study determining plaques in remaining aorta found no significant effect.⁶⁵ Also, induction of immune responses against oxidized LDL phospholipids by immunization with *Streptococcus pneumoniae* primarily reduced aortic root plaques.¹⁰² In contrast, immunization of mice with aldehyde-modified apoB-100 peptides reduced plaque size in the

descending aorta but not in the aortic root (although aortic root plaques demonstrated a more stable phenotype).⁹³ Although these inconsistencies may reflect biological variations of the models used, further studies of the effects of immunization with oxidized LDL antigens on different parts of the arterial tree are clearly required.

The fact that immunization with oxidized LDL and oxidized LDL-specific antigens has been performed in young animals with minimal pre-existing atherosclerosis, and that the studies have been focused primarily on prevention of early lesions, represents a potential limitation from a clinical perspective. Although prevention of atherosclerosis by immunization at early age remains a fascinating long-term goal, it would be preferable if immunization also could stabilize or even induce regression of existing plaques. Observations from some studies that immunizations may decrease plaque inflammation and increase collagen content bring some hope that this may be possible, but further studies addressing this important question are required.

Immunizations Using Hsp and Other Infectious Antigens

Many infections are associated with activation of immune responses against Hsp of the invading microorganisms. In contrast to the findings with oxidized LDL-associated antigens, parenteral immunization with mycobacterial Hsp 65 is strongly atherogenic.^{72,73} This is believed to be because of induction of autoimmunity against native Hsp 60 in stressed vascular cells. Clinical studies by the same group have shown an association between antibodies against Hsp 65/60 and the severity and progression of carotid atherosclerosis.⁷⁵

Interestingly, the route of Hsp60 immunization may have drastic consequences for atherosclerosis. As discussed above, mucosal administration frequently generates tolerance, suppresses proinflammatory Th1 cells, and decreases organ-specific inflammation in several animal models of autoimmune disease.^{104,105} Harats et al¹⁰⁶ exposed LDLr^{-/-} mice to Hsp 65 through oral administration, and Maron et al¹⁰⁷ used oral and nasal administration of Hsp 65 in the same strain of mice. Both studies reported a significant decrease in development of atherosclerosis associated with decreased levels of Hsp antibodies and suppressed T-cell reactivity against Hsp. There was also a suppression of plaque inflammation and a reduced expression of Th1 cytokines. The possibility of inhibiting atherosclerosis by suppressing autoimmune responses against Hsp is of considerable interest and needs to be studied further. It also needs to be clarified whether this will influence the protection against infections.

It is not uncommon that the occurrence of an acute myocardial infarction is preceded by an upper respiratory infection. Two recent case-control studies have suggested that influenza vaccination is associated with a markedly reduced short-term risk of myocardial infarction.^{108,109} In support of this, Nichol et al¹¹⁰ reported that the incidence of hospitalization for cardiac disease in a community-dwelling cohort of 288 238 persons ≥ 65 years old was 19% lower in those who received influenza vaccination. In a pilot randomized trial on 301 patients with existing coronary disease, a significant reduction in cardiovascular death was observed at 6 months

follow-up after influenza vaccination.¹¹¹ It has been suggested that influenza increases the risk for cardiovascular disease by triggering destabilization of already present vulnerable plaques rather than by initiating or aggravating development of atherosclerosis.¹¹² However, the role of influenza vaccination in prevention of cardiovascular disease remains to be fully established, and Jackson et al¹¹³ failed to identify a protective effect of vaccination in a younger cohort of survivors from a first myocardial infarction.

Immunization Using Other 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. Ritterhaus et al¹¹⁴ immunized hypercholesterolemic rabbits with a peptide containing a region of CETP, an enzyme known to be responsible for transferring cholesteryl esters from high-density lipoprotein (HDL) to very-LDL and LDL. This was found to result in inhibition of CETP activity, increased HDL cholesterol, and reduced development of atherosclerosis. A first clinical trial using a CETP vaccine is ongoing.¹¹⁵ Attempts to inhibit the effect of TNF- α by immunization with a recombinant TNF- α molecule has failed to reduce atherosclerosis in apoE⁰ mice,¹¹⁶ whereas treatment with antibodies against CD40 ligands has been shown to inhibit atherosclerosis in LDLr^{-/-} mice.¹¹⁷ However, early clinical trials using CD40 blockade were terminated because unwanted side effects.

Summary

Modulation of immune responses involved in atherosclerosis using vaccines, passive immunization by antibody treatment, and induction of tolerance represent a new generation of therapies for prevention and treatment of cardiovascular disease. Development of such therapies has advanced more rapidly in other areas such as cancer, type 1 diabetes, and rheumatoid arthritis. Initial animal studies have also generated promising results in atherosclerosis, but our understanding of the complex role of immunity in this disease remains incomplete as well as our knowledge about safety issues. However, there is likely to be a rapid development in this field during the years to come, and results from the first clinical studies will hopefully be available within a few years. It should be kept in mind that the studies performed so far suggest that immunization can reduce development of atherosclerosis by \approx 50% to 60%, but it will not completely prevent development of the disease, as in the case with immunization against many infectious diseases. Accordingly, if it becomes possible to develop an effective atherosclerosis vaccine, it is likely to complement other well-established treatments, such as statins and other risk-modifying interventions.

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