thematic review

*Thematic review series: The Immune System and Atherogenesis*

# The role of natural antibodies in atherogenesis

**Christoph J. Binder,[1,*] Peter X. Shaw,[*] Mi-Kyung Chang,[*] Agnès Boullier,[*] Karsten Hartvigsen,[*] Sohvi Hörkkö,[*] Yury I. Miller,[*] Douglas A. Woelkers,[†] Maripat Corr,[*] and Joseph L. Witztum[*]**

Departments of Medicine* and Reproductive Medicine,† University of California, San Diego, La Jolla, CA

**Abstract Atherosclerosis is now widely recognized as a chronic inflammatory disease that involves innate and adaptive immune responses. Both cellular and humoral components of the immune system have been implicated in atherogenesis. Natural antibodies can be considered humoral factors of innate immunity, and their functional role in health and disease has been reexamined in recent years. Natural antibodies exhibit a remarkably conserved repertoire that includes a broad specificity for self-antigens. For this reason, they are believed to be a product of natural selection and have been suggested to play an important role in "housekeeping" functions. Recent evidence has revealed that oxidation-specific epitopes are important and maybe immunodominant targets of natural antibodies, suggesting an important function for these antibodies in the host response to consequences of oxidative stress, for example, to the oxidative events that occur when cells undergo apoptosis. This review will focus on these recent findings and discuss the emerging evidence for an important role of natural antibodies in atherogenesis.**—Binder, C. J., P. X. Shaw, M-K. Chang, A. Boullier, K. Hartvigsen, S. Hörkkö, Y. I. Miller, D. A. Woelkers, M. Corr, and J. L. Witztum. **The role of natural antibodies in atherogenesis. *J. Lipid Res.* 2005.** 46: **1353–1363.**

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## INTRODUCTION TO THE ROLE OF IMMUNE MECHANISMS IN ATHEROGENESIS

Atherosclerosis is a disease of the vascular wall that leads to myocardial infarction, heart failure, peripheral vascular disease, and stroke (1). Although multiple risk factors have been identified that contribute variably to lesion formation, the growth of the atherosclerotic lesion is both initiated and sustained by increased levels of LDL and low and/or dysfunctional HDL. Atherosclerosis develops over decades and is believed to progress from intimal thickening to ever more complex lesions involving the accumulation of cells derived from the circulation, proliferation of inherent vascular wall cells, and elaboration of extracellular matrix and lipid accumulation, both extracellular bound to matrix and intracellular, within macrophage foam cells. Macrophage cholesteryl ester formation is believed to be attributable in large part to enhanced and unregulated uptake of oxidized, aggregated, and variously otherwise modified LDLs and possibly other lipoproteins and disturbed cellular responses that are unable to export the accumulated cholesterol load. As the lesions progress, many of the lipid-filled cells undergo apoptosis but are not sufficiently cleared, leading to an abnormal accumulation of apoptotic cells in the lesion (2). Under these conditions, apoptotic cells may undergo secondary necrosis, yielding the acellular gruel characteristic of the advanced atherosclerotic plaques. Smooth muscle cell proliferation and secretion of a thick collagen cap may stabilize the lesion, but eventually vulnerable areas of the plaque erode or rupture, leading to thrombosis, ischemia, and clinical events or even death.

Recent clinical trial data clearly document that decreasing plasma LDL levels is a potent way to reduce the risk of clinical events, and these trials all suggest that the clinical adage, "the lower the LDL level the better," is correct (3, 4). Yet, it is also clear that among individuals with apparently similar LDL levels or exposed to the same therapeutic interventions, the expression of clinical events varies widely, indicating that many other factors are involved in the response of the artery wall to a given LDL level. It is for this reason that so much effort is being made to understand the fundamental mechanisms involved in lesion formation.

It is now widely recognized that the evolving atherosclerotic lesion has all the characteristics of a slowly smoldering inflammatory disease (5), including the presence of immune cells such as monocytes/macrophages, dendritic cells, T-cells, natural killer T-cells and natural killer cells, and mast cells. Neutrophils are curiously absent, and

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[1] To whom correspondence should be addressed.
e-mail: cbinder@ucsd.edu

B-cells have only rarely been described, although they are found in the immediate adventitia surrounding lesions and in draining lymph nodes. In addition, noncellular components of immunity are prominently found in established lesions, including immunoglobulins, in part bound to specific disease-related antigens as immune complexes, as well as complement and C-reactive protein (CRP). That immune mechanisms play an important role in disease progression is now attested to by scores of examples in which manipulations of specific aspects of immune function have been altered, resulting in significant modulation of the course of atherogenesis. The introduction to the current Thematic Series on the Role of the Immune System in Atherogenesis (6) provides an overview of the now large body of evidence supporting an important role for many different immune mechanisms in atherogenesis. Individual aspects are covered in detail in the different papers in this Thematic Series and are summarized as well in several excellent recent reviews (7–9). In this review, we will describe emerging evidence for an important role for innate immune responses in atherogenesis and, specifically, the role of natural antibodies (10, 11).

## ANTIGEN RECOGNITION IN ADAPTIVE AND INNATE IMMUNITY

To put in perspective the role of natural antibodies, it is important to first provide a brief overview of the organization of the immune system. It is traditional to think of the immune system as divided into two layers: adaptive and innate immunity. The essence of adaptive immunity is the generation of T-cell receptors (TCRs) and B-cell receptors (BCRs) via somatic generation through varied V-gene recombinations to produce a vast number of unique and diverse receptors that provide great specificity and high affinity against selecting pathogens. This provides both cellular (via T-cells) and humoral (via secreted immunoglobulins from B-cell-derived plasma cells) immunity. Adaptive immunity thus provides a tailored, high-affinity, and hopefully definitive response to a pathogen, but the time needed to allow the selection and maturation of T- and B-cell responses results in a considerable delay in the deployment of optimal responses, which typically take days to weeks. Furthermore, once selected by antigen exposure, for example, a given selected clone is not transferable from one generation to the next, so in essence, the selection of an optimal clone must occur anew in subsequent generations. Classical components of adaptive immunity include conventional B-cells (or B-2 cells), T-cells, and dendritic cells, and the maturation of these responses occurs primarily in the germinal center of follicles.

In contrast, innate immunity uses the natural selection of receptors (12). Because the components of innate immunity are in essence preformed and present at birth and/or matured via positive selection during the neonatal period or shortly thereafter, they are available for almost immediate defense against a perceived pathogen. Thus, whereas adaptive immunity provides specific but delayed responses, innate immunity represents the first line of defense and provides immediate and early broad protection to supply host defenses until adaptive responses mature. Although adaptive immunity provides an almost limitless number of specific receptors (estimated at $10^{18}$ TCRs and $10^{14}$ BCRs), each selected to bind to and inactivate individual pathogens in a highly efficient manner, innate receptors of necessity are focused on highly conserved motifs that are present on multiple pathogens as well as many neoepitopes and even apparent self-epitopes. The receptors of innate immunity are termed "pattern recognition receptors" (PRRs). In turn, the "conserved" patterns on various pathogens to which they bind are termed "pathogen-associated molecular patterns" (PAMPs). These PRRs are thought to be far more restricted in number (in the hundreds).

Examples of primitive innate immune PRRs include acute phase proteins such as CRP, which we will show not only bind to infectious pathogens but also to neoself-epitopes generated on modified lipoproteins and dying cells. The complement system is another example of such preformed proteins, as are various natural antibacterial factors. Natural antibodies are another essential and unique layer, providing humoral immunity primarily as IgM antibodies, which at least in the mouse are in large part secreted by a special compartment of primitive, self-replenishing B-cells, termed B-1 cells. The generation of natural antibodies occurs in the complete absence of external antigenic stimulation and takes place in a tightly regulated manner. Until recently, information regarding the biology and role of B-1 cells and the natural antibodies they generate has been limited. However, new insights have recently been made in B-1 cell biology and in the crucial role of natural antibodies in immediate host defenses against pathogens (13–15). Of particular relevance, our discovery that natural antibodies bind to oxidation-specific epitopes present on oxidized LDL (OxLDL) and on apoptotic cells as well as the molecular mimicry between such neoself-antigens and certain microbial antigens has provided new insights into a novel class of PAMPs (16). These observations suggest mechanisms that lead to their selection and expansion and insights into the functions of natural antibodies in both health and disease.

The monocyte/macrophage is the central cell of innate immunity. Through a variety of endocytic PRRs, it detects and engulfs pathogens bearing PAMPs, and through signaling PRRs it secretes cytokines and growth factors that provide both targeted and generalized responses that help orchestrate a coordinated response to the pathogen. The familiar scavenger receptors that bind OxLDL, such as CD36 and SR-A, are prime examples of endocytic PRRs of macrophages, and these receptors bind a wide variety of other substances as well, such as FFAs, advanced glycation end products, fucoidin, anionic phospholipids, etc. (17). The Toll family of receptors are examples of PRRs that signal to the host the presence of pathogens, with many profound consequences (18). For example, TLR4, which binds lipopolysaccharide leading to nuclear factor κB activation and the secretion of a variety of proin-

flammatory cytokines, also binds minimally modified LDL (19), which also results in the activation of proinflammatory gene expression (20). Finally, and of great consequence, the macrophage serves to link innate immunity with adaptive immunity. For example, antigens endocytosed by PRRs or engulfed by other pathways are processed within the cell, and epitopes presented in the context of appropriate presenting molecules initiate T-cell activation and thus engage arcs of adaptive immunity. Natural antibodies, which mediate specific humoral immunity in the innate defense layer, are secreted in large part by innate-like B-1 cells, primarily in the spleen. In contrast to B-2 cells, the maturation and secretion of natural antibodies by B-1 cells is thought to be largely thymus-independent and does not require cognate T-cell help. In addition, marginal zone B-cells may also be responsible for the generation of some natural antibodies.

The primary focus of this review will be on these natural antibodies and the B-1 cells that make them and their relationship to atherogenesis. We will also review surprising new data that different components of innate immunity also bind to some of the same common oxidation-specific neoepitopes that the natural antibodies recognize. Thus, as a result of oxidative stress, oxidation-specific epitopes constitute one category of "altered self," which represents "danger signals" (i.e., PAMPs) that are recognized and defended against by multiple arcs of innate immunity (**Fig. 1**).

## ROLE OF B-1 CELLS AND NATURAL ANTIBODIES IN INNATE IMMUNITY

Natural antibodies are usually defined as antibodies that are found in normal individuals in the complete absence of any exogenous antigenic stimulation. They have an important role in providing a first line of defense against invading pathogens and as such represent a nonredundant component of the humoral immune system (21). Natural antibodies are predominantly IgM, which is in part established by the fact that serum IgM levels are similar in mice raised under germ-free or antigen-free conditions compared with mice housed under conventional conditions (22–24). In contrast, serum IgG and IgA levels in these mice are depressed, indicating the greater dependence of these isotypes on antigenic stimulation (25). Natural antibodies exhibit a remarkably conserved repertoire, which has been suggested to represent a primitive layer of the immune system (21, 26). They are typically regarded as "polyreactive" in that they bind to a number of self or foreign antigens. This pattern of broad reactivity of a preformed pool of antibodies is required for the rapid and immediate recognition and protection against invading pathogens. On the other hand, natural antibodies may also play a role in the recognition and removal of senescent cells, cell debris, and other self-antigens and thereby possess another function in protecting from autoimmunity. In fact, it can be hypothesized that this physiological "housekeeping" role is even more important and has contributed in large part to their evolutionary selection. This housekeeping role of natural antibodies to recognize altered self may become especially important under conditions that lead to increased production of stress-induced self-antigens, such as occurs in atherosclerosis (27, 28).

Natural IgM antibodies are predominantly produced by a small subset of long-lived, self-replenishing B-cells, termed B-1 cells (29, 30). In fact, B-1 cells have been shown to contribute 80–95% of serum IgM in noninfected mice (31). B-1 cells differ from conventional B-2 cells in many ways, including phenotypic and developmental differences, anatomic localization, as well as distinct activation requirements and signaling pathways (32).

One of the classic phenotypic markers of B-1 cells is the expression of CD5. However, a second B-1 cell population with similar characteristics has been identified that does not express CD5 and is termed B-1b, in contrast to $CD5^+$ B-1a cells. Thus, the expression of CD5 alone seems inadequate to identify B-1 cells, and they are best identified by

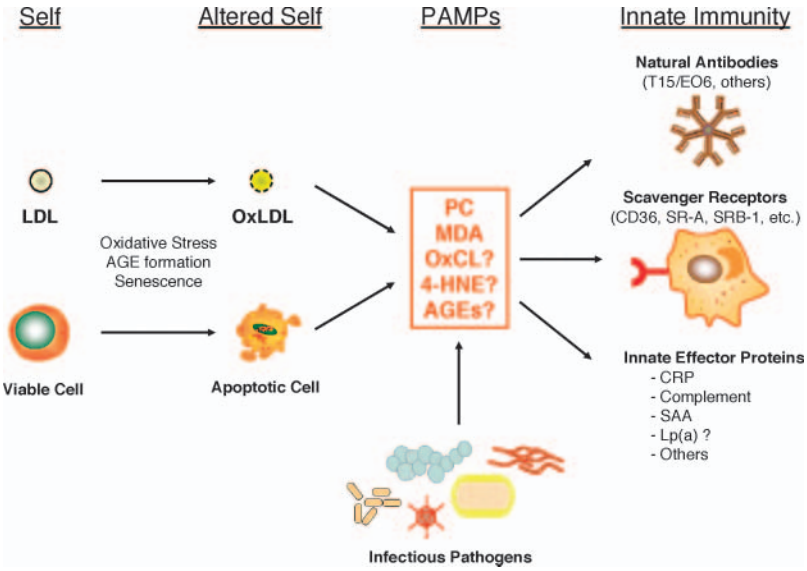


**Fig. 1.** Oxidation-specific epitopes are a class of pathogen-associated molecular patterns (PAMPs) that are recognized by natural antibodies and other innate immune receptors. Physiological and pathological stress can lead to the generation of oxidation-specific epitopes (altered self) on membranes of lipoproteins as well as cells (self), which are subsequently recognized by natural antibodies, scavenger receptors, and other innate effector proteins via these motifs. In many, if not all, cases, molecular mimicry exists between oxidation-specific epitopes of self-antigens and epitopes of infectious pathogens. AGE, advanced glycation end product; CRP, C-reactive protein; 4-HNE, 4-hydroxynonenal; Lp(a), lipoprotein [a]; MDA, malondialdehyde; OxCL, oxidized cardiolipin; OxLDL, oxidized LDL; PC, phosphorylcholine; SAA, serum amyloid A.

the combination of typical phenotypic and anatomic localization criteria. In the peritoneal cavity, B-1 cells express $IgM^{hi}$ and $IgD^{lo}$ and are Mac-$1^+$, $CD23^-$, and $CD5^+$ (in the case of B-1a cells) (32). B-1 cells arise during fetal and neonatal development and typically reside in the peritoneal and pleural cavities, which provide a unique but poorly understood local supportive environment, where they persist as a self-replenishing population thereafter (30, 33). Very few B-1a cells develop from progenitors in adult bone marrow, whereas significant numbers of B-1b cells can be derived from bone marrow.

Because different individual mice have been shown to frequently exhibit the same (or very similar) heavy- and light-chain Ig rearrangement (34), it has been postulated that there is a conserved B-1 cell repertoire that has been selected during evolution for its contribution to host defenses from infectious agents or, as suggested above, to various altered self-antigens generated as a result of oxidative stress, such as that generated by inflammation. Moreover, natural antibodies are frequently encoded by germline $V_H$ and $V_L$ genes that lack N insertions (35–37), which are nontemplated nucleotides inserted at V-D-J splice sites by the enzyme terminal deoxynucleotide transferase (TdT). B-2 cells express TdT and thus generate N insertions when antibody gene rearrangement occurs. This enzyme is not expressed in B-1 cells; thus, their expressed antibodies lack N insertions, giving rise to antibodies that have so-called germline gene expression of their $V_H$ and $V_L$ genes. In contrast to the rearrangement in conventional B-cells, the generation of B-1 cells is largely restricted to the fetal and neonatal period, and B-1 cells seem to be positively selected by their ability to bind to self-antigens, unlike B-2 cells, which are negatively selected. For example, in mice carrying an Ig transgene specific for the T-cell surface antigen Thy-1, the specific B-1 cells were shown to be selected in vivo by the recognition of their cognate antigen (38, 39). Therefore, it is now generally believed that the BCRs of B-1 cells in particular are responsible for "autoantigen"-mediated clonal selection (40). In addition, the development of B-1 cells may also be dependent on different sensitivities to signaling pathways through various receptors (32), and certain selection/signaling events during fetal immune development appear to result in the preferential inclusion of certain V genes that predominate in the B-1 cell repertoire (41). These presumed developmentally regulated processes are still unclear, but there is evidence that B-1 cell clones may arise through a specialized selection pathway (42, 43). Although surface IgM cross-linking is crucial for the development and selection of B-1 cells, it is not sufficient to trigger B-1 cell antibody responses. Nevertheless, certain antigenic stimuli [e.g., phosphorylcholine (PC)] in the context of an appropriate carrier can induce robust antibody responses by B-1 cells. These responses, however, do not lead to the generation of typical B-cell memory or affinity maturation. In addition, B-1 cells show a particularly good responsiveness to "nonspecific" stimuli such as lipopolysaccharide and certain cytokines.

In addition to their specificity for microbial determinants, antibodies produced by B-1 cells have been shown to contain "autoreactivity" by binding what appears to be a variety of self-determinants, although as we will show below, these may include previously unrecognized common self-induced changes attributable, for example, to oxidatively induced alterations in conformation or structure. Examples include cell membrane components such as PC (44) as well as carbohydrates and glycolipids (21) and intracellular molecules such as single-stranded DNA (45) or cell surface molecules on T-cells such as Thy1(CD90) (39). Many of these self-epitopes are also present on or closely related to structures that are on pathogens, which explains the high degree of cross-reactivity of these autoreactive antibodies with microbial antigens (29, 32, 46). Thus, many natural antibodies may react with both cognate self-antigens and microbial pathogens. In fact, it is hypothesized that the entire repertoire of natural antibodies is composed of autoantibodies with this dual reactivity. There is increasing evidence that this inherent self-reactivity has beneficial consequences in protecting from autoimmunity and therefore may be autoreactive by design (47, 48). Mice unable to secrete IgM but still able to express membrane-bound IgM and thus able to mature and secrete other Ig isotypes have been shown to be more susceptible to systemic infection (49). Interestingly, when bred onto the lupus-prone MRL-lpr strain, these mice had an accelerated incidence of autoimmune disease (50). Moreover, it has been reported that with increasing age, even the regular serum IgM-deficient mice had an increased tendency to produce anti-double-stranded DNA IgG autoantibodies (51). These data suggest that natural IgM antibodies have a protective role in preventing autoimmunity. It has been hypothesized that these effects are mediated through their role in clearing apoptotic debris and cells aided by certain complement components. For instance, the binding of natural antibodies to epitopes on membranes of damaged red cells facilitates C3b deposition via the alternative pathway, which may then aid the clearance of senescent red cells (52). Other antibodies have also been described that react with phosphatidylcholine on the cell membranes of protease (bromelain)-damaged red cells (53, 54), and other natural antibodies may bind to lysophospholipids formed on apoptotic cells, effecting removal by similar complement-dependent mechanisms (55).

Thus, natural antibodies have been postulated to contribute to the elimination of autoantigens exposed during stress, tissue damage, or even conventional cell turnover. Because there are a number of redundant receptor-mediated pathways that provide a similar function, the physiological importance of this antibody-mediated pathway is unclear. However, under certain pathological conditions that involve increased accumulation of stress-induced self-structures [e.g., the increased generation of oxidation-specific epitopes that occurs in atherosclerosis (7)], antibody-mediated clearance may become increasingly relevant.

## GENERATION OF "OXIDATION-SPECIFIC" EPITOPES

OxLDL is present in atherosclerotic lesions and contains a wide variety of lipid peroxidation products, which we have shown represent "neoself determinants" recognized by specific innate and adaptive immune responses (7, 8). LDL contains cholesterol as well as cholesteryl esters and phospholipids, which contain polyunsaturated fatty acids. Under appropriate conditions, all of these can undergo oxidation reactions, and we have documented autoantibodies to oxidized cholesterol, cholesteryl esters, and phospholipid moieties in hypercholesterolemic, atherosclerotic murine models (56–58). However, most of our effort has focused on oxidized phospholipids (OxPLs). Typically, peroxidation of the abundant phospholipid phosphatidylcholine is initiated at the oxidation-prone *sn*-2 polyunsaturated fatty acid. Decomposition of the oxidized fatty acid generates a wide spectrum of reactive molecular species, such as malondialdehyde (MDA) and 4-hydroxynonenal (59), as well as the "core aldehyde" of the residual OxPL backbone, yielding 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC), which contains the PC head group (60). These reactive aldehydes can modify autologous molecules, including both the protein moiety of LDL, apolipoprotein B (apoB), and other lipid molecules, such as amine-containing phospholipids (e.g., phosphatidylserine). Thus, altered lipids as well as oxidized lipid-protein adducts are formed, yielding, for example, MDA-modified lysines on proteins as well as aminophospholipids, as well as OxPLs and OxPL-protein/lipid adducts. When LDL is subjected to copper-mediated oxidation in vitro, up to 70 mol of apoB lysines can be modified with PC-containing OxPL adducts (61). LDL is a very large particle (molecular mass $2 \times 10^6$ kD), 200 nm in diameter, and thus can bear multiple modifications at the same time. Theoretically, hundreds of such different modified structures can occur, and we have named these epitopes "oxidation-specific epitopes." Unlike native LDL, this oxidatively modified LDL is now recognized by scavenger receptors and therefore can give rise to foam cells. Moreover, by forming multiple copies of similar adducts on its surface, we speculate that it becomes an excellent immunogen, not only capable of stimulating classic adaptive immune responses but also able to stimulate thymus-independent type 2 antibodies, which typically require multivalent ligands (62).

## OXIDATION-SPECIFIC EPITOPES ARE PAMPS AND ARE RECOGNIZED BY A VARIETY OF PRRS

The early work with oxidatively modified LDL also demonstrated that although they occur in OxLDL particles, such oxidation-specific epitopes could also be found in other proteins and phospholipids. Furthermore, OxLDL (and/or similar immunogenic structures formed on other oxidized lipoproteins or similarly modified structures, such as cell membranes) induced humoral immune responses in vivo (63, 64), and both IgG and IgM autoantibodies specifically binding to these oxidation-specific epitopes could be found in plasma of animal models of atherosclerosis as well as in human plasma (56, 57, 65, 66). Modifications of LDL render it particularly immunogenic, and indeed, we had shown many years ago that even subtle chemical modifications of homologous LDL made it highly immunogenic (modifications such as glycation, methylation, ethylation, acetylation, and carbamylation) (67). Of considerable interest was the observation that the antisera generated by such immunizations bound not only to the modified LDL but to a variety of other proteins on which the same subtle epitope was found. Thus, antisera generated against methylated LDL, for example, would recognize a variety of similarly methylated proteins. These data suggest that autoantibodies, once formed in vivo in response to modified LDL, would be capable of binding to a variety of similarly modified endogenous proteins. Indeed, we showed that sera from diabetic patients had autoantibodies that bound not only to glycated LDL but to a large number of other glycated autologous proteins (68).

Cholesterol-fed apoE-deficient (apoE$^{-/-}$) mice have very high autoantibody titers, particularly IgM, to a wide variety of oxidation-specific epitopes (57). Indeed, this enabled us to clone a large panel of B-cell hybridomas from the spleens of these mice that had specificity for epitopes of OxLDL or MDA-LDL (65). The monoclonal autoantibodies secreted by these hybridomas were all IgM and immunostained atherosclerotic lesions of mice and humans. Each of the OxLDL-specific IgMs bound to both the lipid and apoB moieties of OxLDL, but not of native LDL. Subsequent studies revealed that each of these bound specifically to OxPL containing the PC head group, such as POVPC, but did not bind to native unoxidized PC phospholipids (69). Indeed, detailed studies using synthetic OxPL models revealed that the specific epitope on OxPL was the PC head group (70). Thus, these antibodies, such as the prototypic EO6, bound to the PC of OxPL but not to the PC of unoxidized PL.

Of substantial interest were the observations that EO6 bound to OxLDL as well as to its oxidized lipid and apoB and inhibited their uptake by macrophage scavenger receptors (69), specifically by CD36 and scavenger receptor class B type I (SR-BI) (71, 72). In more recent studies, it has been shown that similar to the binding to EO6, the PC moiety of OxPL was sufficient to mediate binding to CD36 (73). These data demonstrate that the PC moiety of OxPL is a PAMP, recognized by the PRRs CD36, SR-BI, and natural antibodies such as EO6. This further suggested the hypothesis that such IgMs may inhibit the uptake of OxLDL in vivo and in that way decrease macrophage uptake and foam cell formation.

As noted in the introduction, innate immunity is conserved by natural selection. What, then, could be the "natural epitopes" providing this selective pressure? It does not seem likely that atherogenesis would exert such selective pressure. Steinberg and colleagues (74, 75) reasoned

some time ago that an OxLDL might simply resemble an oxidatively damaged red blood cell (RBC) or even an apoptotic cell, which is known to undergo marked oxidative changes as part of the apoptotic program. Indeed, they demonstrated that oxidized RBC, as well as OxLDL, could compete for the uptake of apoptotic cells by macrophages (74, 75). These studies demonstrated that there were common recognition ligands on OxLDL and apoptotic cells recognized by macrophage scavenger receptors. Indeed, Chang et al. (76) then demonstrated that a number of the different oxidation-specific IgM monoclonal antibodies that bound OxLDL, such as EO6, and EO14, which binds to MDA-LDL, bound to cell surface determinants on apoptotic cells but not viable cells. Furthermore, each of these antibodies could inhibit the uptake of apoptotic cells by macrophages in an additive manner. In recent studies, we have demonstrated together with Judith Berliner that there is an enrichment of PC-containing OxPL in membranes isolated from apoptotic cells (64). Thus, we speculate that these oxidation-specific neoepitopes are indeed PAMPs representing "eat me" signals to innate immunity, as manifested by macrophage scavenger receptors and natural antibodies. Indeed, we speculate that there are likely to be many other such neoepitopes generated as a result of stress-induced alterations in native structure. These epitopes can be generated by adduct formation between reactive lipid moieties and proteins or other lipids, generating entirely novel structures, such as MDA-lysine adducts on LDL. Alternatively, such neoepitopes can be as subtle as conformational changes in one part of a molecule caused by alterations in another part. For example, it appears that the oxidation of the *sn2* side chain of PC-containing PL in OxLDL, or in the plasma membrane of apoptotic cells, "exposes" the PC head group so that it is now recognized by several aspects of innate immunity (i.e., it is now a PAMP). These ideas are depicted in Fig. 1, which suggests the possibility that there are likely to be many more such oxidatively derived PAMPs.

## NEW INSIGHTS GAINED FROM DETERMINATIONS OF THE GENETIC ORIGIN OF THE OXIDATION-SPECIFIC MONOCLONAL ANTIBODIES

Because all of these cloned autoantibodies were IgMs, which are thought in large part to represent natural antibodies in uninfected mice, we sought to determine their genetic origin. Shaw et al. (16) sequenced the $V_H/V_L$ of the complementary determining regions from four hybridomas secreting IgM to OxLDL. To our surprise, all four were revealed to be 100% homologous through 350 bp in both their $V_H$ and $V_L$ genes, and furthermore, each was shown to be genetically and structurally identical to a well-characterized B-1 cell clone, T15, that had been described more than 30 years ago. The T15 natural antibodies bind to PC covalently linked to the pneumococcal cell wall polysaccharide and not present as a PL (77). T15 has been among the most studied of antibodies because it provides optimal protection to mice from lethal infection with *Streptococcus pneumoniae* (78). In vitro binding assays confirmed that the classic T15 antibody (IgA) specifically bound to OxLDL and POVPC, whereas our oxidation-specific monoclonal antibody (IgM) bound to the cell wall polysaccharide. These studies demonstrated molecular mimicry between the PC of OxPL present on OxLDL and apoptotic cells on the one hand and the PC moiety present on pneumococci and many other infectious pathogens on the other. As noted above, this dual specificity has been described as a characteristic of natural antibodies (21).

The fact that the same T15 natural antibody that blocked the uptake of OxLDL by macrophages also bound the PC of common microbial pathogens suggested that the natural IgM antibodies may ameliorate atherosclerosis. To test this hypothesis, we immunized cholesterol-fed LDL receptor-deficient ($LDLR^{-/-}$) mice with heat-inactivated PC-containing pneumococci (79), which are known to specifically induce and expand T15 natural antibodies. This pneumococcal immunization induced high titers of anti-OxLDL IgM (predominantly of the T15 clonotype) and significantly reduced atherosclerosis as measured at the aortic valve site. Plasma of these mice had an enhanced ability to inhibit the uptake of OxLDL by macrophages. We also demonstrated that the sera of patients recovering from pneumococcal pneumonia contained IgM antibodies to pneumococcal cell wall polysaccharide that significantly correlated with levels of anti-OxLDL IgM antibodies in the same serum sample. These findings suggest that humans also have the PC-specific IgM antibodies with microbial/OxLDL cross-reactivity. Notably, although preliminary, clinical epidemiological studies suggest that human IgM antibodies to OxLDL also correlate with a protective role (80, 81), the exact specificity and cellular origin of these human antibodies have not yet been characterized.

## INTERLEUKIN-5 LINKS ADAPTIVE AND INNATE IMMUNITY THROUGH THE STIMULATION OF NATURAL ANTIBODY PRODUCTION

To better understand the role of OxLDL-specific immune responses in general, we originally immunized hypercholesterolemic rabbits with homologous MDA-LDL as a model epitope of OxLDL. This immunization led to the induction of high-titered IgM and IgG antibodies to MDA-LDL and strongly reduced atherosclerosis (82). In subsequent studies, we and others confirmed the atheroprotective effect of immunization with models of OxLDL in both atherosclerosis-prone rabbits (83, 84) and mice (58, 85–88).

Recently, we observed that immunization with MDA-LDL also induced T15/EO6 antibodies to levels that exceeded those found in nonimmunized mice fed an atherogenic high-fat diet (87) and remarkably were actually one-third as high as titers achieved by pneumococcal immunization. MDA-LDL does not contain PC-exposing OxPL and thus should not stimulate direct thymus-independent expansion of T15/EO6. In detailed analyses of the induced immune response, however, we found that

this immunization led to a strong Th2-biased response specific for MDA-LDL that was characterized by the robust production of interleukin-5 (IL-5) sufficient to increase plasma levels. IL-5 in turn is an important factor in the noncognate maturation and Ig secretion of B-1 cells (89–92) and mediated the expansion of T15/EO6 IgM in these mice. This is supported by the observation that naive IL-$5^{-/-}$ mice had no measurable levels of T15 Ig and that immunization of these mice with MDA-LDL could not induce these antibodies. To further assess the in vivo role of IL-5, we generated $LDLR^{-/-}$ mice deficient in IL-5 by transfer of bone marrow from IL-$5^{-/-}$ mice. When IL-$5^{-/-}LDLR^{-/-}$ chimeric mice were fed a high-fat diet, much lower titers of T15 clonotypic antibodies and circulating T15/EO6-apoB immune complexes were observed than in the IL-5-competent controls. These data suggest that IL-5 is important for the natural expansion of T15/EO6 IgM during atherogenesis. Importantly, the absence of IL-5 led to significantly more atherosclerosis.

Therefore, the induction of T-cell responses to atherosclerosis-specific oxidation epitopes can also be associated with an atheroprotective effect, presumably attributable in part to cytokine-mediated stimulation of natural antibody responses. Importantly, our data demonstrate how the induction of certain adaptive Th2 responses can stimulate more primitive innate-like "layers" of the immune response. These results were further corroborated in studies of $LDLR^{-/-}$ mice that were deficient for the Th1 lineage-specific transcription factor T-bet (93). When fed an atherogenic diet, these mice developed significantly less atherosclerosis. Moreover, these mice exhibited a profound switch of atherosclerosis-specific immune responses toward Th2, which was accompanied by a >2.5-fold increase in serum levels of T15/EO6 IgM antibodies. Indeed, other Th2 cytokines, such as IL-9 (94) and IL-10 (95–97) and natural killer T-cell-derived IL-4 (98), have also been reported to contribute to B-1 cell development and/or function. Although the exact role of these factors has not been evaluated in the context of the host response to atherogenesis, it is noteworthy that IL-10 has also been shown to mediate atheroprotection (99, 100).

## POSSIBLE EFFECTOR MECHANISMS OF NATURAL ANTIBODIES IN ATHEROSCLEROSIS

Little is known about the mechanisms by which natural antibodies, such as T15/EO6, could provide atheroprotection. These protective properties would result from the ability of T15/EO6 to recognize OxPL, OxLDL, and/or apoptotic cells and thus interfere with rate-limiting steps of atherogenesis. Simple binding of OxPLs present in minimally modified LDLs or apoptotic cells by IgM antibodies could potentially neutralize most of their proinflammatory properties, which promote atherogenesis through the activation of endothelial cells, induction of tissue factor, etc. In support of this, we have recently shown that apoptotic cells that contain OxPL have the ability to activate endothelial cells to bind monocytes and that this could be inhibited by T15/EO6 IgM (64). In addition, as described above, a number of in vitro studies have suggested that these IgM antibodies block the uptake of OxLDL by macrophages and thus could prevent foam cell formation in vivo (69, 79). Finally, the formation of circulating (intravascular) immune complexes of these IgMs with minimally OxLDL alone may already have protective properties by preventing LDL from entering vulnerable sites of the artery wall. Whether this would promote its accelerated removal is questionable, as recent studies by Reardon et al. (101) showed that the apparent clearance rates of infused OxLDL did not differ between immunocompetent $apoE^{-/-}$ mice and $apoE^{-/-}$ mice that lacked any antibodies as a result of an additional deficiency of recombinase activating gene-2. This was the case despite the fact that the formation of in vivo circulating IgM-apoB immune complexes could be demonstrated. Thus, a number of potential mechanisms by which natural antibodies could be protective have been suggested; however, it is still unclear which, if any, of these becomes relevant in vivo. On the other hand, in certain settings, natural antibodies with different specificities than for PC may also exert proatherogenic effects. For example, certain B-1 cell-derived IgMs have been shown to play a pathogenic role in intestinal ischemia/reperfusion injury (102). There is still much to learn about the effector mechanisms of these antibodies.

## APOPTOTIC CELLS ARE IMMUNOGENIC

The fact that the same receptors on macrophages that bound and internalized OxLDL were PRRs of innate immunity raised the question of the identity of the true natural ligands that such scavenger receptors had evolved to bind. Because the PRRs are believed to have been conserved as a result of natural selection, there should be some other set of ligands, because it is not likely that OxLDL and the attendant atherogenesis would exert such pressure. Sambrano and Steinberg (103) reasoned some years ago that OxLDL may simply mimic epitopes present on an apoptotic cell. Indeed, they showed that OxLDL could compete with apoptotic cells for uptake by macrophages. This strongly suggested that there were common oxidation-specific epitopes on OxLDL and apoptotic cells. Chang et al. (76) subsequently showed that many of the oxidation-specific autoantibodies that bound to OxLDL bound to the surface of apoptotic cells and inhibited their uptake by macrophages, just as they inhibited the uptake of OxLDL. Indeed, cells undergoing apoptosis are known to be subjected to enhanced oxidative stress as a result of the loss of mitochondrial membrane integrity and the release of redox-active cytochrome *c*. Indeed, Kagan and colleagues (104) have shown that nearly all of the phosphatidylserine present in the membrane of apoptotic cells is oxidized. Furthermore, Chang et al. (76) have recently shown that the membranes of apoptotic cells are enriched in oxidized PC phospholipids of the type that react with EO6, suggesting that apoptotic cells bearing such oxida-

tion-specific epitopes should be immunogenic. Indeed, Chang et al. (64) also showed that immunization of mice with syngeneic apoptotic cells led to high-titered antisera that reacted not only with the apoptotic immunogens but also to a panel of different oxidation-specific epitopes. Remarkably, among all IgMs induced that bound to the apoptotic cells, ~60–70% bound to oxidation-specific epitopes also found on OxLDL. These data strongly argue that such oxidation-specific epitopes are immunodominant epitopes on apoptotic cells, reflecting the generalized importance of the many neoepitopes that are generated as a result of oxidative stress. In this context, one can perhaps rationalize that oxidation of LDL (or indeed a variety of similar lipid-rich structures) recapitulates what occurs when cells undergo programmed cell death.

## IN VIVO MODEL TO ASSESS THE REPERTOIRE OF NATURAL ANTIBODIES SECRETED BY B-1 CELLS DERIVED FROM HEALTHY NAIVE MICE

It can be predicted that if one could examine the repertoire of B-1 cell-derived IgMs from naive mice, one would see an overrepresentation of natural antibodies devoted to the variety of oxidation-specific epitopes that are undoubtedly ubiquitously expressed in both health and disease. The proposed role of oxidation-specific natural antibodies to provide recognition of apoptotic cells, as well as other stressed/damaged membranes and oxidized lipoproteins, suggests that a significant portion of all such antibodies would have specificity for these epitopes. To test this hypothesis, we developed a murine model in which all of the plasma antibodies are natural antibodies derived from B-1 cells. To accomplish this, we isolate and purify B-1 cells from the peritoneum of normal uninfected and nonimmunized wild-type mice and transfer these self-replenishing cells into RAG-1$^{-/-}$ mice, which do not contain any functional T- or B-lymphocytes. Consequently, there is an exclusive reconstitution of natural antibody-secreting B-1 cells, and hence, the entire plasma antibody population consists of natural antibodies. Detailed characterization of the specificity of these natural antibodies showed that >35% of the total plasma IgM could be absorbed by antigens containing various oxidation-specific epitopes. Thus, an unexpectedly large proportion of B-1 cell-derived natural antibodies has been selected to bind oxidation-specific epitopes, reflecting the importance of defending against oxidative stress. It remains to be tested whether the simple reconstitution of natural IgM antibodies will be sufficient to decrease atherogenesis in an immune-deficient atherosclerosis mouse model.

## CRP ALSO BINDS TO AN OXIDATION-SPECIFIC EPITOPE

CRP is an acute phase protein that has been widely discussed as a marker of inflammation and is a powerful prognosticator with respect to cardiovascular disease, as discussed elsewhere in this Thematic Series (105). CRP was originally recognized for its ability to bind to the cell wall of *S. pneumoniae*, specifically to the PC moiety that is covalently linked to teichoic or lipoteichoic acid. It is worth noting that Chang et al. (106) recently demonstrated that CRP also binds to the PC of OxPL present in OxLDL or apoptotic cells but does not bind to PC of native phospholipid. CRP is the prototypic example of a primitive innate protein, and in this context, it is instructive that it too binds to an oxidation-specific epitope, as does natural antibody EO6 and scavenger receptor CD36. Although high-sensitivity CRP levels have been found to be independent risk factors for future cardiovascular risk, the functional role of CRP is still unknown. Despite a similar ability of T15/EO6 and CRP to bind to OxLDL, the immune complexes they form are recognized by different types of receptors and cells; therefore, they may have different or even opposing roles in atherogenesis. This again emphasizes the variety of different mechanisms that innate immunity has evolved to deal with the consequences of oxidative stress (Fig. 1).

## SUMMARY

In this review, we have emphasized the potential importance of natural antibodies in response to oxidation-specific epitopes and their possible protective role in atherogenesis. Because natural antibodies are conserved and presumably selected for their beneficial consequences, it seems reasonable that their presence serves to mediate the clearance of otherwise toxic pathogens, be they of infectious origin or attributable to modifications of the self. In this review, we have put forth the hypothesis that the generation of oxidation-specific epitopes as a result of oxidative stress represents a major pathogenic burden that has resulted in the conservation of a variety of innate immune responses to deal with the modified structures on which these epitopes are found. Thus, oxidation-specific epitopes constitute one category of "danger signals" (i.e., PAMPs), which identify "altered self" that occur as a result of oxidative stress and to which multiple arcs of innate immunity, using a variety of different PRRs, have converged to defend against. Viewed in this context, one would predict that the natural antibody arc of innate immunity would be heavily represented by antibodies directed to a variety of oxidation-specific epitopes that, in turn, would play a major role in the early detection and removal of structures that contain these epitopes. Thus, we predict that these antibodies likely play a major role in the removal of apoptotic and senescent cells, such as aged RBCs, as well as the clearance of similarly modified structures that may also be present on infectious pathogens. In turn, because oxidation-specific epitopes are abundantly present in atherosclerotic disease, these antibodies likely play an important role in modulating the course of atherosclerosis as well. We are only at the beginning of our study of the definition and importance of natural antibodies, even in murine models, and even less is known of

their role in humans. Along with other aspects of innate immunity, identifying these antibodies and understanding their role in health and disease are likely to yield an understanding of the basic mechanisms that evolved to deal with the consequences of oxidative stress. Such insights may lead to novel therapeutic approaches to deal with the consequences of oxidative stress, which, in turn, may have applications for the control of atherogenesis.

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