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## Natural antibodies and the autoimmunity of atherosclerosis

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**Abstract** In recent years, the subject of natural antibodies has been revisited and the immunobiological roles of these humoral factors are being better defined. These antibodies are secreted by distinct sets of innate-like B cells, B-1 cells and marginal zone B cells, which arise early in development to become the sources of “natural immune memory”. Due to their interactions with a variety of self-determinants, natural antibodies have previously been postulated to play roles in the maintenance of host homeostasis. A central paradigm has recently been developed from the demonstration that oxidation derived epitopes on apoptotic cells and oxidized low-density lipoproteins are recognized by the phosphorylcholine-specific germline encoded B-1 cell natural antibody, T15, which has provided important insights into possible “house-keeping” functions under both normal and pathological conditions. In this review, the potential functions of natural antibodies in the pathogenesis and progression of the chronic inflammatory condition of atherosclerosis are discussed, as well as their capacities for apoptotic cell binding and clearance. These interactions of natural antibodies and oxidation-epitopes from phospholipids appear to provide a dynamic immunobiological connection linking host responses in infection, autoimmunity and atherosclerosis.

**Keywords** B-1 cell · Tolerance · Repertoire · Apoptosis · Host immunity

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### Introduction

Understanding the functional and structural properties of natural antibodies (NAbs) has been a topic of interest for several decades [26], but in recent years there has been a critical re-examination of the roles of these humoral factors in host defenses. In general, NAbs are defined as antibodies that spontaneously arise without prior infection or defined immune exposure. At birth, most serum immunoglobulins are of the IgM isotype, and these

levels may increase with age. More importantly, levels of IgM are not significantly depressed in mice raised under germ-free or antigen-free conditions [50, 134].

These IgM NABs are believed to predominantly derive from a distinct compartment of mature B-lineage cells, termed B-1 cells. While B-1 cells normally constitute only a minor fraction of peripheral lymphoid tissue, they represent a major fraction of B cells found in the peritoneal and pleural cavities of mice [54]. The antibody repertoire of B-1 cells, representing the major source of NABs [4], has been suggested to represent a primitive innate-like “layer” of the adaptive immune system to provide a primary line of defense against systemic infection from viral and bacterial pathogens [53]. In addition, by production of IgM NABs, the B-1 lymphocyte pool is believed to collaborate with marginal zone B cells in the spleen to recognize non-protein antigens (e.g., expressed on polysaccharides, glycoconjugates and phospholipids), and these immune responses do not have an absolute requirement for T cell involvement.

During immune development, the precursors for B-1 cells have been shown to arise in the fetal liver and omentum, to later colonize the linings of the peritoneal and pleural cavities, which provide a unique but poorly understood local supportive environment for this self-replenishing population [120]. These presumed developmentally regulated processes are still unclear, but there is evidence that B-1 cell clones may arise through a specialized selection pathway [22, 133]. While the accumulation of B-1 cells is essentially complete in the neonatal period, levels of certain NABs may subsequently be affected by exposure to exogenous antigens, such as gut flora [11].

In the adult, the activity of NABs depends on several specialized functions of the spleen. In addition to being an important site of IgM production, as described above, blood-borne antigens also encounter B cells in the spleen. Moreover, interactions with locally produced IgM NABs also commonly result in chemical modification of antigens with fragments of complement, which further enhances immunogenicity. Phagocytic cells in the spleen are also involved in the clearance of these resulting immune complexes from the circulation. Notably, deletion of the Hox-11 gene, or removal of the spleen, results in the permanent disappearance of peritoneal B-1 cells [141].

B-1 clones have been characterized that interact with a variety of self determinants, such as carbohydrates and glycolipids, but may also “cross-react” with bacterial and/or onco-fetal antigens. As different individual mice have been shown to express clonally distributed sets of B cells with the same (or very similar) antibody gene rearrangements [41, 113], it has also been postulated that there is a conserved B-1 cell (and perhaps marginal zone B cell) repertoire, which has been selected during evolution for its contribution to host defenses from infectious agents. Moreover, NABs may be encoded by non-mutated germline  $V_H/V_L$  gene rearrangements [18, 66, 114]. The autoreactivity of the expressed repertoire of B-1 cells has also been described as low affinity and multireactive [47, 74], but it can also be argued that the true antigenic ligands are unknown. Although direct evidence is still limited, it is now generally believed that the BCR of B-1 cells in particular (and perhaps all mature B cells in general) are responsible for autoantigen-mediated clonal selection [127]. In the best characterized system, a B-1 cell clone was shown to be selected *in vivo* by determinant(s) associated with the glycoconjugate on T cell-associated Thy-1 surface molecules [51, 52].

The “natural immune memory” associated with B-1 cells and splenic marginal zone B cells has also been postulated to contribute to the homeostasis of the internal milieu [83]. For instance, the binding of NABs with membrane protein determinants exposed in dam-

aged red cells, facilitates C3b deposition via the alternative pathway, which may then aid the clearance of senescent red cells [78]. Other clones have also been described that react with phosphatidylserine, which may become expressed in the external leaflet of the cell membranes of protease-damaged cells [3, 48]. Hence, there has been evidence of pathways in which NAbs may contribute to the elimination of autoantigens exposed during stress, tissue damage, or even conventional cell turnover. However, based on extensive evidence that there are also functionally overlapping or redundant clearance mechanisms involving receptors of the innate immune system, the relative importance of these antibody-dependent systems has been uncertain.

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### **Autoimmune responses in atherosclerosis**

Atherosclerosis is a chronic inflammatory disease of the vascular wall that results in a variety of clinical manifestations, including coronary artery disease, peripheral vascular disease, and stroke [42]. Although multiple risk factors have been identified, atherosclerosis is clearly initiated and sustained by hypercholesterolemia and associated elevations of serum low-density lipoproteins (LDL) [122]. Once trapped in the artery wall, LDL may undergo modifications, including oxidation (forming oxidized LDL, OxLDL), and the resultant products can activate inflammatory responses that further promote atherogenesis [90, 123]. Our interest in NAbs arose from investigations that in recent years atherosclerosis and, in particular, responses to OxLDL have been shown to be associated with features akin in some respects to pathogenic autoimmune responses, while other aspects of the associated immune responses are non-pathogenic or even protective.

The oxidation products of LDL can affect atherogenesis in many ways [21, 128]. Exposure to OxLDL activates endothelial cells and macrophages. OxLDL can also induce the chemotactic recruitment of monocytes into the artery wall, and cause cytotoxic damage to vascular cells (both necrotic and apoptotic), which further promotes the pro-coagulant properties of vascular cells, and induces smooth muscle cell migration and proliferation. Monocytes are particularly important for atherogenesis, as mice deficient in one of several molecules involved in monocyte recruitment and differentiation show decreased atherosclerosis when bred onto an atherosclerosis-prone background [10, 29, 43]. Moreover, once monocyte/macrophages are activated, these cells further promote the oxidation of LDL that is rapidly taken up via specific scavenger receptors, leading to the formation of lipid-laden foam cells, the hallmark cells of early atherosclerotic lesions (i.e., fatty streaks) [36, 42, 124, 143]. In fact, OxLDL uptake by the scavenger receptors, SR-A or CD36, represents another rate-limiting step in disease progression, because hyperlipidemic mice deficient in either of these receptors also have significantly reduced atherosclerosis [33, 129].

Specific immune mechanisms may modulate the progression of the disease process. Atherosclerotic lesions contain activated T cells [45, 64, 107, 126], and specific cytokines along with immunoglobulins are deposited in the pathological lesions, atherosclerotic plaques [44, 144]. Of great clinical importance, the unstable regions of these vascular plaques, which are prone to rupture and initiate acute clinical events, are particularly rich in activated immune cells [112]. Further evidence of the potential modulatory roles of the adaptive immune system in atherogenesis has also been demonstrated in apolipoprotein E-deficient (ApoE<sup>-/-</sup>) or LDL receptor-deficient (LDLR<sup>-/-</sup>) mice, which were crossed onto a

recombinase-activating gene-deficient ( $RAG^{-/-}$ ) background to generate mice susceptible to atherosclerosis but without functional B or T cells [27, 28, 105, 121]. In diets resulting in only mild elevations in plasma cholesterol and/or only limited atherogenesis, the immunodeficient mice displayed significantly reduced vascular lesions, suggesting that in general the involvement of lymphocytes yields a net pro-atherogenic effect [28, 121]. In  $ApoE^{-/-}$  mice, the presence of oligoclonal expansions of T cells in these vascular lesions suggests that these responses are in part antigen driven [101]. Furthermore, Th1-biased T cell responses, and certain Th1 cytokines, were found to play a disease-promoting role (reviewed in [6, 44, 46]).

By contrast, emerging evidence indicates that B cells can convey an atheroprotective function. Major et al. [79] generated B cell-deficient  $LDLR^{-/-}$  mice, by reconstitution of irradiated  $LDLR^{-/-}$  mice with the bone marrow from B cell-deficient mice, which when they were fed a high fat diet developed increased atherosclerosis, compared to  $LDLR^{-/-}$  mice that had functional B cells. In addition, splenectomy of  $ApoE^{-/-}$  mice, which was shown to decrease anti-OxLDL antibody titers, also resulted in increased atherosclerosis, while this effect could be reversed by infusion of B cells (but was also decreased by T cells) from aged  $ApoE^{-/-}$  mice [16]. Importantly, infusion of splenic B cells into  $ApoE^{-/-}$  mice with unimpaired immune systems also resulted in atheroprotection [16]. In this regard, it is noteworthy to reiterate that the spleen is central to the generation and maintenance of the B-1 cell pool [141] and a major source of the NAb products. Thus, it is possible that the atheroprotective function in these murine studies was conveyed to some degree by B-1 cell derived NABs.

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### Candidate autoantigens and atherosclerosis

Atherogenesis has been shown to be associated with active immune responses in which a range of candidate antigens are specifically recognized, and these include microbial antigens, endogenous heat shock proteins,  $\beta$ -2glycoprotein I, and modified LDL, especially OxLDL. As described above, LDL enters the intima of the artery wall, where it undergoes progressive stages of oxidation, leading to the formation of a variety of oxidation products derived from the triglyceride, cholesterol, and phospholipid moieties of LDL. Components of oxidatively modified LDL are highly immunogenic, and some of the best characterized of these immunogenic epitopes are the products of phospholipid oxidation. For example, when the polyunsaturated fatty acids of phospholipids undergo peroxidation, breakdown products, such as malondialdehyde (MDA) and 4-hydroxynonenal are formed, which are highly reactive and in turn can form covalent adducts with the lysine residues of the protein of LDL, apolipoprotein B (apoB). Similarly, the remaining core-aldehydes of oxidized phospholipids can also form covalent adducts with apoB [56]. In the case of the abundant PC-containing phospholipid 1-palmitoyl-2-(arachidonoyl)-*sn*-glycero-3-phosphorylcholine (PAPC), the reactive oxidation product 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine (POVPC) is formed, which in addition to being present on the lipid phase of modified LDL, can also derivatize apoB, and the resulting newly formed lipid-protein adduct retains the intact phosphorylcholine (PC) headgroup. It is important to keep in mind that in vivo generated OxLDL contains a wide spectrum of many different oxidation-specific epitopes that have not yet been defined. To study specific immune responses in vitro, two model oxidation-associated antigens have been especially well characterized: (1) MDA-LDL, which is made by direct derivatization of LDL with MDA, giv-

ing rise to MDA-lysine epitopes, and (2) CuSO<sub>4</sub>-oxidized LDL (CuOx-LDL), which was found to contain many different oxidation-specific epitopes, including oxidized phospholipids with the accessible and immunodominant form of the PC head group.

Studies in humans and animal models of atherosclerosis indicate that these oxidation-specific neo-antigens may be recognized by OxLDL-specific T cell-dependent (TD) and/or T cell-independent (TI) immune responses. For example, up to 10% of CD4<sup>+</sup> T-cells isolated from human atherosclerotic plaques displayed MHC class II-restricted specificity for OxLDL [125], but the exact T cell epitopes recognized have not been defined. Antibodies are also induced that recognize some of these well-characterized breakdown products of lipid peroxidation, and these oxidation-specific epitopes may be found in both the lipid as well as protein moieties of OxLDL or other proteins. Marked titers of IgG and IgM antibodies against oxidation-specific epitopes of OxLDL have also been documented in the plasma, and immunoglobulin-containing immune complexes with OxLDL are present in plasma [7, 137] and found in the vascular lesions of humans and animals with atherosclerosis [55, 144]. Moreover, the titers of OxLDL-specific antibodies have been shown to correlate with the extent of atherosclerosis [55, 99]. In fact, clinical epidemiological surveys have shown that these IgG antibody titers often (but not always) correlate with classical risk factors for cardiovascular disease and with clinical measures of disease severity [32, 61, 62, 109, 136]. On the other hand, patients or subjectively healthy individuals with documented carotid artery disease are also reported to display lower plasma anti-OxLDL IgM levels, which may suggest that IgM titers against OxLDL instead play a protective role [61, 62, 67]. However, much more work needs to be done to clarify this relationship.

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### **Specificity and immunobiological origins of NABs to OxLDL**

Fulfilling expectations for an NAb, naïve non-hypercholesterolemic C57BL/6 mice display detectable (but low) titers of IgM antibodies to OxLDL and MDA-LDL [97, 100], while the titers of these IgM antibodies are spontaneously increased in atherosclerosis-prone hyperlipidemic mice [97, 98]. To further investigate the structural, functional, and genetic origins of murine NABs to oxidation-associated determinants on OxLDL, the spleens of non-immunized ApoE<sup>-/-</sup> mice on high-fat diets were used to clone B cell lines [96]. The resultant monoclonal antibodies were selected for their binding to epitopes of OxLDL (e.g. CuOx-LDL and MDA-LDL) and were termed EO antibodies. All of these cell lines were found to produce IgM, and genetic analysis of the antibody genes encoding the prototypic IgM anti-CuOx-LDL antibody, EO6, revealed an unexpected finding. These V<sub>H</sub> and V<sub>L</sub> genes were completely identical to sequences previously shown to encode for a classical B cell clone, termed TEPC15 (T15), which was from an IgA-expressing plasmacytoma shown to specifically bind the phospholipid head group, PC [116]. The gene sequences encoding T15/EO6 are also without evidence of hypermutation, suggesting that these B cells did not pass through a germinal center reaction. Notably, these particular antibody gene sequences display a pattern consistent with primary sequence-directed rearrangements, without N insertions, which occur predominantly in early development during B cell neogenesis in the fetal liver [35].

B cell clones expressing the T15-defining antibody genes arise at high frequency in many inbred mouse strains, even without prior immune exposure, and they are also highly represented in germ-free mice [118]. In fact, beginning from about the first week of life,

T15 antibodies dominate *in vivo* antibody responses to PC-containing determinants, which have been shown to be the immunodominant moiety in the cell wall polysaccharide (C-PS) of *pneumococci*, in which the PC moiety is not present as the headgroup of the phospholipid, but is instead directly conjugated to lipoteichoic acid or teichoic acid, which are carbohydrates [119]. The immunological accessible PC moiety is also expressed on other microbial pathogens [49, 119]. Of all anti-PC antibodies, T15 antibodies are reported to be the most protective to systemic infection with *pneumococci* [13, 88], in part due to efficient clearance of these microbes from the blood [85]. Moreover, adoptive transfer studies have rigorously documented that T15 B cells exist predominantly (or solely) as part of the CD5<sup>+</sup> B-1 pool (i.e., B-1a) [84]. Despite the immunobiological importance of the T15 clonotype, the mechanism(s) responsible for the *in vivo* dominance of T15 clonotypic B cells was a mystery.

Our studies showed that T15 and EO6 were genetically and structurally identical, and also functionally equivalent in their capacity to recognize the PC moiety as a minimal motif prominently expressed on oxidized phospholipids, like OxLDL but not on native LDL [56]. PC is also expressed on certain apoptotic host cell membranes [19]. However, in the membranes of healthy host cells or native LDL, PC associated with non-oxidized phospholipids represents a cryptic epitope on phosphatidylcholine. Therefore, immunological exposure/accessibility of PC only occurs when native phospholipids from LDL or in cell membranes become oxidized [6]. Thus, the T15 clonal set of antibodies exemplify the NAb response that is directed against oxidation-specific epitopes that expand during atherogenesis and are arrayed on microbes, although there are likely other sets of NAb that recognize other types of OxLDL specific neo-epitopes.

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### **OxLDL-containing immunogens can provide clinical benefit**

In earlier studies, exogenous immunization of rabbits with model immunogens that include homologous OxLDL (e.g., MDA-LDL) was shown to strongly reduce atherosclerosis [96]. Following this initial demonstration, the atheroprotective effect of immunization with models of OxLDL was confirmed in several studies using atherosclerosis-prone rabbits [1, 92] or mice [7, 38, 40, 146]. In these mice, immunizations with homologous MDA-LDL decreased atherosclerosis by up to 50%. Later, Zhou et al. [146] reported that the protective effect deriving from MDA-LDL immunization correlated with the induced levels of TD IgG anti-MDA titers. In a recent study, we demonstrated that immunization of LDLR<sup>-/-</sup> mice with MDA-LDL induced a robust IgG1 predominant anti-MDA-LDL response and concurrent secretion of large amounts of IL-5, which is consistent with strong Th2-biased TD responses specific for MDA-LDL. In contrast, non-immunized cholesterol-fed mice generated autoantibodies to MDA-LDL predominantly of the Th1-dependent IgG2a isotype [7]. Therefore, while it is not yet clear as to whether there is a specific functional role of the IgG1 response alone that affects atherosclerotic lesion formation, these findings suggest that the specific isotype of IgG responses (and/or associated cytokine responses) correlates with different, even opposing, effects, which are akin to studies characterizing antibody responses in pathological autoimmune anti-red cell responses [37].

Despite the fact that the MDA-LDL immunogen, as prepared, does not contain exposed PC epitopes, these immunizations also induced antibodies to CuOx-LDL to levels that far exceeded those found to naturally arise in non-immunized mice fed an atherogenic high-

fat diet [7, 38], and these anti-PC responses were shown to predominantly derive from T15 clonotypic B cells [7]. A very likely explanation is that the co-induced IL-5, which is an important factor in the maturation and Ig secretion of B-1 cells [73, 89, 132, 145], also mediated the non-cognate expansion of protective T15 IgM in these mice. In support, naïve IL-5<sup>-/-</sup> mice had no measurable levels of T15 Ig and immunization of these mice with MDA-LDL could not induce these antibodies. To further assess the *in vivo* role of IL-5 in this induction of T15 clonotypic IgM, we generated LDLR<sup>-/-</sup> mice deficient in IL-5 by transfer of bone marrow from IL-5<sup>-/-</sup> mice. Indeed, when IL-5<sup>-/-</sup>LDLR<sup>-/-</sup> chimeric mice were fed a high-fat diet, much lower titers of T15 clonotypic antibodies were demonstrated than in the IL-5-competent controls. Moreover, these mice also had lower levels of circulating immune complexes formed by T15 antibodies and apoB, and had significantly more atherosclerosis. Hence, the induction of specific T cell responses to atherosclerosis-specific oxidation antigens can also be associated with a protective effect, presumably due in part to cytokine-mediated co-induction of NAb responses. Specifically, our data indicate that IL-5 appears to be important for the natural expansion of T15 IgM during atherogenesis. Other Th2 cytokines such as IL-9 [138] and IL-10 [63, 93, 94], and NKT-cell derived IL-4 [17], have been reported to contribute to B-1 cell development and/or function. Although the exact role of these factors has not yet been evaluated in the context of the host response to atherogenesis, it is noteworthy that IL-10 has also been shown to mediate atheroprotection [81, 102].

Furthermore, it is also possible that the process of immunization, possibly as a consequence of local responses to injury, may contribute to the generation of oxidation-specific neo-epitopes that in turn react with OxLDL. In fact, injections of LDLR<sup>-/-</sup> mice with complete Freund's adjuvant (CFA) alone can give rise to anti-MDA-LDL IgM (but not IgG antibodies), and such treated mice also have decreased atherogenesis [8]. CFA appears to have MDA epitopes, as two different MDA-specific monoclonal antibodies react with it [8], and it is possible that such epitopes are further generated following injection, as a result of oxidation of this lipid-rich product. It is also possible that the CpG component of the mycobacterium plays a role in the resulting specific B cell stimulation in a thymus-independent type II response. Although a topic of active interest, the cellular/clonal sources of anti-MDA antibodies have not yet been identified.

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### **Protective role of pneumococcal immunizations that induce anti-phospholipid NABs**

Based on the above-described rationale, we wondered whether *in vivo* induced PC-specific responses to a microbial antigen could be functionally equivalent to responses to (modified) LDL, and whether the experimental induction of higher levels of T15 clonotypic NABs could provide benefits in murine models of atherosclerosis. To address this question, cholesterol-fed LDLR<sup>-/-</sup> mice were immunized with heat-inactivated PC-bearing pneumococci known to specifically induce and expand T15 IgM in a TI manner [8]. This pneumococcal immunization was shown to induce high titers of anti-OxLDL IgM predominantly of the T15 clonotype, confirming the functional equivalence of anti-pneumococcal and anti-OxLDL responses. Despite exceedingly high plasma cholesterol levels (i.e., more than 1,500 mg/100 ml), this near mono- (or oligo)clonal expansion of T15 clonotypic IgM NABs was also associated with significantly reduced vascular lesion formation. Hence, these results demonstrate that atheroprotection was mediated by the clonal

expansion of one single antibody with specificity for an “oxidation-specific” epitope on OxLDL, namely PC. In addition to increased T15 IgM titers, pneumococci immunized mice also displayed an increased *in vivo* formation of circulating IgM immune complexes with apoB-containing particles, suggesting that the induced IgM antibodies bound minimally oxidized LDL. These studies established functional *in vivo* molecular mimicry of immunodominant pneumococcal determinants and those on OxLDL and atherosclerotic vascular lesions [8]. However, while a protective role for T15 IgM was documented, the exact mechanism(s) have not been clearly defined.

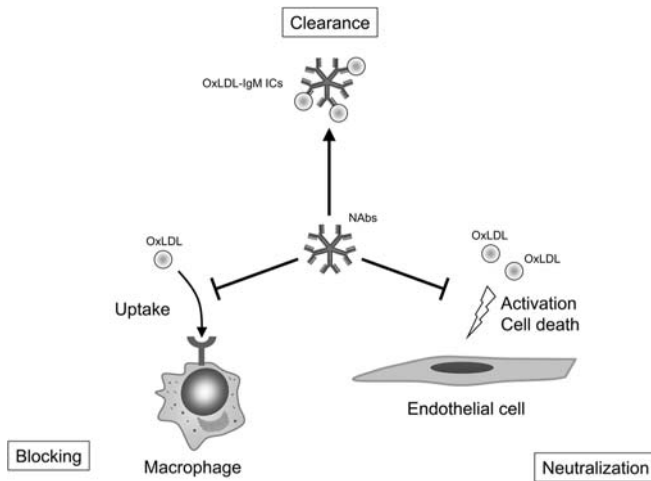
Relevant to these animal model systems, we have shown that the sera of patients recovering from pneumococcal pneumonia contain IgM antibodies to pneumococcal C-PS with titers that significantly and directly correlated with levels of anti-OxLDL IgM antibodies in the same serum sample. However, there was no correlation between the levels of these two types of IgG antibodies. These findings suggest that, akin to that documented in mice, humans also have the PC-specific IgM antibodies with microbial/OxLDL cross-reactivity, while it is possible that the development of certain IgG responses may instead be blocked due to immunological tolerance mechanisms. Notably, while preliminary clinical epidemiological studies suggest that human IgM antibodies to OxLDL also correlate with a protective role [61, 67], the exact specificity and cellular origin of these human antibodies has not yet been characterized.

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### **Possible mechanisms and controversies regarding the roles of NAb**

A number of potential protective mechanisms for NAb have been proposed (Fig. 1). For example, *in vitro* studies have shown that both the entire IgM molecule and Fab fragments of T15/EO6 antibodies can inhibit the scavenger receptor-mediated binding and uptake of OxLDL by macrophages in a dose-dependent manner [56]. If such a mechanism operates *in vivo*, the efficient blocking of OxLDL uptake would be predicted to prevent the formation of detrimental foam cells, an event that is rate limiting in atherogenesis [34, 129]. Consistent with this notion, compared to control mice the plasma of pneumococci immunized mice significantly inhibited the *in vitro* binding of OxLDL to macrophages [8]. Another protective effector function of PC-specific natural IgM antibodies, such as T15, could be found in an ability to neutralize the many pro-atherogenic effects of oxidized phospholipids of OxLDL and minimally modified LDL (which both display PC-containing oxidized phospholipids). In this case, the simple sequestration of the biologically active oxidized phospholipids from the cellular target may be sufficient to exert a protective effect. In addition, evidence that T15/EO6 IgM inhibited the ability of apoptotic blebs to induce monocyte adhesion by endothelial cells [60] suggests that apoptotic and oxidized membrane blebs contain the same biologically active oxidized phospholipids present in minimally modified LDL. Interestingly, such shed cell membrane microparticles are also generated in atherosclerotic plaques [82] and found at elevated levels in the peripheral circulation of patients with acute coronary syndrome [12, 80]. These microparticles can cause endothelial dysfunction and have procoagulant activity [12, 82]. Hence, many of the pro-atherogenic effects mediated by OxLDL and its products may be neutralized by specific natural IgM antibodies. These effects include the induction of certain chemoattractants (e.g., MCP-1), adhesion molecules (e.g., VCAM-1), or tissue factor expression, or the promotion of apoptotic and necrotic death of endothelial cells [21, 122]. Thus, it could be hypothesized that natural anti-OxLDL antibodies promote the clearance of pro-athero-





**Fig. 1** Possible protective functions of NAb in atherogenesis. (1) Blocking: T15/EO6 IgM antibodies can block the uptake of OxLDL by macrophages, thereby preventing formation of lipid-laden foam cells. (2) Immune complex formation and clearance: T15/EO6 IgM antibodies form circulating (intravascular) immune complexes with minimally oxidized LDL, thereby preventing it to act on the vessel wall and promoting its removal at anatomical sites distant from the lesion prone sites. (3) Neutralization: T15/EO6 IgM can neutralize the proinflammatory properties of oxidized phospholipids present in apoptotic blebs or OxLDL, thereby preventing endothelial cell activation, tissue factor expression, induction of cell death, etc (NAb natural antibodies, OxLDL oxidized low-density lipoproteins)

genic minimally oxidized LDL away from disease prone vascular sites to anatomically distant sites, such as the spleen. In support, IgM-apoB immune complexes have been documented to form *in vivo* [8]), and such apoB-IC can contain T15 NAb [7]. Certain complement components would likely be involved in aiding their clearance, and recently it was shown that C3-deficient LDLR<sup>-/-</sup> mice have increased atherogenesis, suggesting such a protective role for complement [15]. In humans, it has been recently shown that following percutaneous angioplasty, which disrupts atherosclerotic plaques, there is an abrupt fall in autoantibody titers to OxLDL epitopes, while at the same time a rise in immune complexes with apoB [137].

Despite the above-described correlative *in vivo* and *in vitro* evidence, the biological relevance of these postulated mechanisms for protective NAb remains controversial. To directly address this question, Reardon et al. [106] studied the kinetics of *in vivo* clearance of native human LDL or human OxLDL that had been infused into mice, by applying immunochemical methods to quantitate levels of oxidized human LDL with antibodies to human apoB-100, and a T15 clonotypic IgM antibody to recognized associated oxidation-specific phospholipid neo-epitopes. However, despite the detection of the formation of *in vivo* circulating immune complexes, there were equivalent apparent rates of clearance in immunocompetent ApoE-deficient mice and in ApoE-deficient mice that were also deficient in RAG-2, which therefore lacked mature T cells, B cells and antibodies. While these studies provided little if any support for a mechanistic function of anti-OxLDL antibodies in clearance of atherogenic LDL, it is also possible that the formation of circulating immune complexes without immediate clearance may already provide a protective effect, by retaining potentially pro-atherogenic OxLDL particles largely intravascular, and pre-

venting their interaction with cells of the vessel wall. However, apoptotic lymphocytes, generated during physiological daily cell turnover, are also a major source of oxidation-specific epitopes (see below) that compete for binding by the same T15 clonotypic antibodies. Hence, the absence of lymphocytes in these RAG-2-deficient mice may also have affected the apparent *in vivo* rate of clearance in these studies, which suggests that this topic should be revisited in the future.

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### **Isotype and immune function of NAb**

How could a change in specific isotype of an anti-OxLDL antibody response convey very different implications for cardiovascular pathology? It has been hypothesized that anti-OxLDL IgG via binding of OxLDL enables uptake of the complex by local phagocytic cells via membrane-associated Fc- $\gamma$  receptors (Fc- $\gamma$ Rs), contributing to foam cell formation [71] and inflammatory responses [139]. Patients with insulin-dependent diabetes mellitus, who have accelerated atherosclerosis, are reported to display titers of IgG anti-OxLDL antibodies that greatly exceed those for IgM [139]. Posing a seeming paradox, infusions of intravenous hyperimmune immunoglobulin (i.e., IVIg) into ApoE<sup>-/-</sup> mice have been reported to result in reduced atherosclerosis [91]. IVIg, which is derived from the pooled plasma of healthy human donors, has been shown to contain NAb of the IgG isotype [68], including IgG anti-OxLDL antibodies [142]. Although the mechanisms responsible for the outcome of these high-dose treatments are undefined, a benefit may derive either from the supra-physiological doses of IgG anti-OxLDL, or be due to inefficient interactions with the cross-species Fc $\gamma$ R, which may contribute to a very different outcome than if much lower levels of specific native IgG antibodies were induced. There is still much to learn regarding the impact of Ig isotype of antibody responses to OxLDL, and also of mixed isotypes, on *in vivo* atherosclerotic lesion formation.

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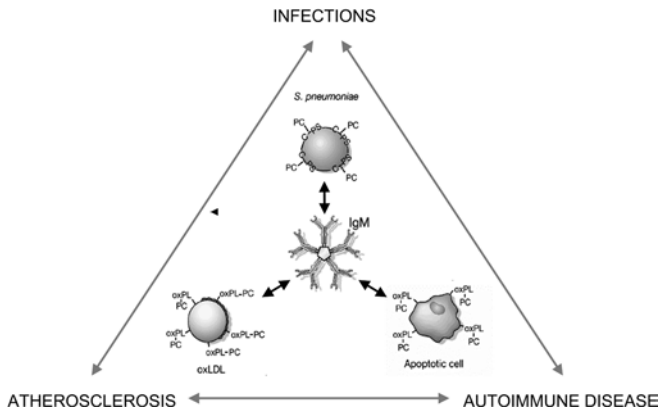
### **OxLDL and apoptotic cells share immunogenic determinants**

The changes associated with the lipid peroxidation of LDL that occur during atherogenesis display many parallels with those attributed to oxidative stress affecting the phospholipids of the membranes of cells undergoing apoptotic death. In fact, the finding that OxLDL can inhibit the uptake of apoptotic cells by macrophages [9] suggests the presence of common ligands on OxLDL and on apoptotic cells. From this perspective, these oxidation-derived ligands may be considered a type of apoptotic cell-associated molecular patterns (ACAMPs) that can mediate recognition by phagocytes [110]. Therefore, similar to the generation of thrombospondin binding sites and the display of phosphatidylserine ligands on the external membrane leaflet on apoptotic cell membranes, OxLDL-like moieties on apoptotic cells represent additional ligands that contribute to the recognition and subsequent removal of apoptotic cells by phagocytes. As referred to above, the presence of oxidation-specific epitopes on apoptotic cells was also specifically documented in the demonstration that murine monoclonal EO IgM antibodies, which are specific for epitopes of OxLDL (see above), also specifically bind apoptotic but not viable cells [19]. Thus, analogous to OxLDL, cells undergoing apoptosis express such “oxidation-specific” epitopes. Moreover, these IgM also inhibited the uptake of OxLDL by macrophages. Specifically, PC-containing oxidized phospholipids seem to be present on the surface of apoptotic cells,

because they are specifically bound by the natural IgM antibody T15/EO6 [19]. This epitope is important as T15/EO6 partially inhibited the uptake of apoptotic cells by macrophages, as did OxLDL and more specifically BSA derivatized with the oxidized phospholipid POVPC [19]. In fact, Podrez et al. [103] recently demonstrated that several different PC-containing oxidized phospholipids are also ligands for CD36, which is also a receptor for apoptotic cells.

Evidence that a different murine monoclonal EO IgM antibody, EO14, from ApoE<sup>-/-</sup> mice, recognizes MDA-LDL and MDA-lysine, and binds apoptotic cells, indicated that MDA-epitopes represent another type of oxidation-specific epitope on apoptotic cells [19]. Moreover, EO14 also inhibited apoptotic cell uptake by phagocytes. Importantly, during atherogenesis, ApoE<sup>-/-</sup> mice and LDLR<sup>-/-</sup> mice also develop very high antibody titers to MDA-LDL, and immunization with homologous MDA-LDL leads to atheroprotection. Therefore, apoptotic cells and OxLDL share “oxidation-specific” epitopes that are recognized by OxLDL-specific IgM antibodies, and it is likely that in fact apoptotic cells are the principle antigenic ligands responsible for the *in vivo* selection of the B cell clones responsible for these NAb responses [5, 69]. The *in vivo* importance of these common ligands on OxLDL and apoptotic cells was demonstrated in a recent study, in which lupus-prone mice with disturbed clearance of apoptotic cells were backcrossed into atherosclerosis-prone ApoE<sup>-/-</sup> mice that have increased accumulation of OxLDL. These newly generated mice exhibited a much more pronounced defect in apoptotic cell clearance, which was presumed to be due to competition of apoptotic cells and OxLDL for phagocyte uptake [2].

Traditional theories on apoptosis typically regard the “corpses” of apoptotic cells as noninflammatory and nonimmunogenic. Nevertheless, there is increasing evidence that under certain conditions and stages of apoptosis, apoptotic cells can promote inflammation [76, 77, 110]. An accelerated formation of apoptotic cells and/or their defective clearance have been hypothesized to be partially responsible for autoimmune responses [108]. Indeed, surface membrane blebs from virus-induced apoptotic cells that have been demonstrated to contain virus-derived determinants may contribute to the breach in self-tolerance for antigens on apoptotic cells and lead to immune activation. Moreover, Cocca et al. [23, 25] demonstrated that during apoptosis, ligands are generated that consist of complexes between phosphatidylserine and  $\beta$ 2GPI, which are also recognized by certain autoantibodies, that also bind to oxidized moieties [57, 65]. In support of the notion that apoptotic cells can serve as a trigger of autoimmune responses, mice immunized with syngeneic apoptotic cells have been shown to generate IgM autoantibodies to apoptotic cells and cellular components, including ssDNA and cardiolipin [87]. Notably, cardiolipin, which is a component of cellular mitochondria, is also an important phospholipid of LDL, and it is likely that immune responses to cardiolipin are actually directed against oxidized cardiolipin, which is found in OxLDL [58]. Therefore, it is tempting to speculate that oxidation-specific epitopes on membranes of apoptotic cells may be further examples for such immunogenic determinants, and PC of oxidized phospholipids represents a membrane component that could challenge tolerance [115]. In analogy to the murine IgM T15 clonotypic antibodies, these antibodies have been shown to cross-react between a variety of microbes, e.g., dental plaque bacteria, and OxLDL [111] (see Fig. 2).



**Fig. 2** Natural IgM antibodies link OxLDL, apoptotic cells, and microbes. The natural IgM T15/EO6 binds to the PC moiety of the capsular polysaccharide of *Streptococcus pneumoniae* and other microbes, as well as to PC headgroup of oxidized phospholipids in membranes of apoptotic cells and OxLDL. This common immune response suggests a potential link between infection, atherosclerosis, and autoimmune disease (PC phosphorylcholine)

### Many antibodies to PC are inherently autoreactive

Evidence that T15 clonotypic antibodies recognize determinants on OxLDL, and also recognized PC-inhibitable determinants in atherosclerotic plaques and on apoptotic cells [117], led us to investigations that demonstrated that PC-specific responses are functionally equivalent in OxLDL and pneumococcal extracts [8] (discussed below). We therefore wondered whether the cross-reactivity between PC determinants on microbial antigens, and as expressed on apoptotic cells, OxLDL and in atherosclerotic plaques, is a property restricted to T15 anti-PC antibodies, or whether autoreactivity is a property inherent to all anti-PC antibodies. To address this question, we studied a panel of IgM and IgA anti-PC antibodies related to T15, termed Group I anti-PC antibodies, which are believed to all represent examples of NAb that derived from clones of B-1 cells and marginal zone B cells [115]. These Group I anti-PC antibodies all use the same S107.1  $V_H$  gene segment, but with variations in  $V_H$  CDR3 and L chain usage. These antibodies display similar functionality as they all share the same fine specificity, with recognition of free PC as a salt, as a determinant in pneumococcal C-PS, and for PC chemically conjugated to protein carriers. For comparison, we assessed two IgG antibodies that had been derived from mice immunized with conjugates of PC to keyhole limpet hemocyanin. These IgG anti-PC antibodies, termed Group II, display evidence of hypermutation and diverse antibody gene usage, and are functionally distinct as they do not recognize the free PC head group or C-PS, but require PC to have an alkyl bond for molecular recognition [14].

Surprisingly, all of these anti-PC antibodies displayed reactivity with self ligands, whether in oxidized LDL, in atherosclerotic plaques by immunohistology, or on apoptotic cells by flow cytometry, and the fine specificity for PC determinants was confirmed in inhibition studies. Moreover, in general these binding activities were roughly proportional to their binding reactivities with PC conjugates in ELISA. Hence, we found that all anti-PC antibodies may be inherently capable of autoreactivity. In retrospect, these findings were also consistent with previous unexplained observations in mice transgenic for the expres-

sion of immunoglobulin genes encoding for anti-PC reactivity. The peripheral lymphoid compartments in each of these different Ig transgenic strains all displayed arrested B cell development, suggestive of *in vivo* B lymphocyte encounter and recognition of autoantigens. These findings therefore provided circumstantial evidence consistent with the hypothesis that antigenically active PC-containing self ligands are widely expressed *in vivo*. It is also notable that such Ig transgenic mice commonly display expansions of transgene-expressing B cells in their B-1 or marginal zone compartments, consistent with their postulated autoreactivity. Murine B cells expressing transgenes for Group I anti-PC antibodies have previously been reported to display altered activation, trafficking and peripheral accumulation [70], which is consistent with the hypothesis that these Ig transgene-expressing B cells were being selected by an autoantigen. However, until the demonstration that PC neo-determinants on apoptotic cells and on atherosclerosis-associated oxidation-specific antigens are functionally equivalent, the true nature of such postulated selecting self antigens was unsuspected.

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### **Evidence of two types of membrane-associated PC neoantigen on apoptotic cells**

Our studies of anti-PC antibodies also provided the unexpected observation of the sequential expression of a range of different membrane-associated PC neo-determinants that appear to be revealed during the course of dexamethasone-induced apoptosis [115]. We found that Group I anti-PC antibodies, which represent S107.1 V<sub>H</sub> gene-expressing NABs, preferentially reacted with apoptotic thymocytes during the earlier phases of apoptosis. In fact, binding interactions were enhanced in apoptotic thymocytes treated with the pan-caspase inhibitor, Z-VAD, which delayed the execution phase of apoptosis. In contrast, Group II anti-PC antibodies, which require a covalent alkyl bond for recognition of PC, preferentially recognized thymocytes at later stages in the process. We confirmed the specificity of PC binding to the apoptotic cells by showing that cellular recognition by the Group I anti-PC antibody was inhibited by PC-chloride or by a PC-protein conjugate, while only the PC-protein conjugate inhibited recognition of the apoptotic cells by the Group II anti-PC antibodies.

The Group II anti-PC antibodies appeared akin to the reactivity of IgG autoantibody, 3H9, a classic lupus IgG autoantibody to native DNA, that also recognizes phosphatidylserine, a distinct phospholipid determinant expressed on lymphocytes at advanced stages of apoptotic death [23, 24]. The 3H9 antibody also has replacement mutations that enhance PS binding affinity. Like the Group II anti-PC antibodies, 3H9 recognition of apoptotic cells is also impaired if the cellular targets are induced in the presence of a pan-caspase inhibitor, presumably because caspase-dependent late apoptosis events are required to generate or reveal the neo-self-phospholipid ligands recognized by this autoantibody.

Potentially relevant to our immunochemical findings, recent compositional analyses of thymoma cell lines undergoing glucocorticoid-induced apoptosis were shown to accumulate PC-containing monoesters early, appearing to peak 12 h after exposure to the pro-apoptotic stimulus [135]. In contrast, lysophosphatidylcholine (lyso-PtC) was shown to peak only at later stages of apoptotic death (e.g., 24–32 h after induction). This pattern may be explained by the generation of lyso-PtC through the action of phospholipase A2, a caspase-dependent enzyme. In fact, an example of a Group I anti-PC antibody was shown to not recognize synthetic lyso-PtC or other phospholipids containing sn1 long-chain fatty acids, as recognition required phospholipid moieties with sn2 chains with oxidized fatty

acids containing an aldehyde that interacted with a peptide to yield a Schiff base, or the sn2 oxidized fatty acid that had undergone aldehyde self condensation [39].

The importance of PC-containing apoptosis-associated determinants has also been independently confirmed by Elkon and coworkers [72], who demonstrated that activation of soluble cytoplasmic phospholipase A2 activation during apoptosis led to the generation of lyso-PtC from membrane phospholipids of apoptotic cells, leading to epitope exposure and their recognition by IgM in the sera of healthy human adult donors. This allowed the recruitment of complement components (e.g., C1q) on the apoptotic cell surface leading to activation of C3, which in turn would promote the clearance of these opsonized cells. Importantly, lyso-PtC has long been known to be a major phospholipid component of OxLDL, which acts as a chemoattractant for monocytes and T cells [104] and—at high concentrations—can convey cytotoxicity for vascular cells [59], all of which are believed to contribute to atherosclerotic plaque formation and progression. Notably, non-metabolizable analogues had no chemotactic effect, suggesting that later breakdown products in fact have most or all such biological activities [86]. More recently, lyso-PtC produced in apoptotic cells was shown to also act as a lipid attractant for phagocytes [75] that could lead to a local tissue damage. Moreover, lyso-PtC has recently been shown to induce endothelial cells to release IL-6 and IL-8 [130], which may in part explain the apparent connection between *in vivo* levels of OxLDL and C-reactive protein, a receptor of the innate immune system shown to bind PC-containing ligands in microbes, apoptotic cells and OxLDL [20]. Similar to Group I and Group II anti-PC antibodies, C-reactive protein also has a binding specificity for PC, which enables recognition in the context of plate-bound—structurally distorted—LDL, but not for soluble LDL in its natural configuration [20]. Thus, during apoptosis and OxLDL formation there may be subtle differences in the variations of the specific PC epitopes products, which reflect the stage of apoptotic cell death or the biological context of the tissue that express PC as haptens.

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## Conclusions

Based on recent reports, there is increasing evidence that natural IgM antibodies may at times provide housekeeping mechanisms that protect from adverse immune activation by efficient removal of apoptotic cell debris. Furthermore, such IgM may have important regulatory properties as a consequence of cognate binding activity. For example, in mice engineered to be deficient in secreted IgM, but which can still class switch and secrete IgG antibodies, the absence of serum IgM was accompanied by higher levels of IgG autoantibodies and the development of autoimmune disease [30]. Deficiency in serum IgM has also been associated with a concurrent expansion in the representation of splenic marginal zone B cells, and peritoneal B-1 cells, while these cellular expansions were reversed upon infusions with polyclonal IgM [31]. These studies may be interpreted as evidence that IgM NAbs aid in the clearance of autoantigens, and thus reduce the selection/expansion of autoreactive B cells, and perhaps thereby lessen the severity of autoimmune pathology. We speculate that these pathways are also relevant to the pathogenesis of accelerated atherosclerosis, especially in patients with autoimmune diseases. Future studies will therefore need to better assess the functional role of B-1 cells and natural IgM antibodies in the recognition of defined “oxidation-specific” epitopes on apoptotic cells. In particular, these

studies will need to determine whether PC-specific and/or MDA-specific IgM antibodies make a major contribution to apoptotic cell clearance and maintenance of homeostasis.

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