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Review

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Rethinking oxidized low-density lipoprotein, its role in atherogenesis and the immune responses associated with it

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Summary

Atherosclerosis is a chronic inflammatory disease, resulting from hyperlipidemia and a complex interplay of many environmental, metabolic, and genetic risk factors. The unregulated macrophage uptake of cholesterol and lipids through modified forms of low-density lipoprotein (LDL), such as "OxLDL", transforms macrophages into "foam cells" to form the initial morphological lesion (the fatty streak). The modification of LDL not only enhances its uptake by macrophages, but also changes the natural structures of these otherwise ubiquitous molecules to generate a variety of modified lipids and proteins that represent highly immunogenic neo-determinants. For example, in ApoE$^{-/-}$ mice, autoantibody titers to epitopes on OxLDL are correlated with the extent of atherosclerosis. Similarly, oxidative stress on cellular membranes could also give rise to "oxidation-specific" epitopes and common autoantibodies. However, OxLDL is not uniform, but rather contains complex structures, ranging from a small conformational change in surface lipids to the breakdown of the peptide chain. Therefore, the immune responses to the variety of OxLDL and their association to atherosclerosis progression are very different. For example, phosphorylcholine (PC) is a natural component of phospholipids and exists in LDL and plasma membranes. "Natural" antibodies against PC can distinctively react to PC on bacteria, OxLDL and apoptotic cells, but not to those on unoxidized phospholipids, native LDL and viable cells, which suggests the broader role of such autoantibodies in maintaining the homeostasis of the host. While malondialdehyde-modified structures resemble more the exogenous changes and associate with advanced stage of lesion, they are more likely to associate with adaptive immunity.

Key words: **oxidized LDL • MDA-LDL • atherosclerosis • immune response • anti-phosphorylcholine • inflammation**

Abbreviations: **OxLDL** – oxidized low-density lipoprotein, **MDA-LDL** – malondialdehyde-modified LDL, **PC** – phosphorylcholine, **ApoE$^{-/-}$** – apoprotein E deficient, **LDLR$^{-/-}$** – LDL receptor deficient

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INTRODUCTION

Atherosclerosis is the leading cause of morbidity and mortality in the western world. The pathogenesis of atherosclerosis is complex and involves the impact of many well-documented "classical" risk factors, yet other important risk factors still need to be unidentified. Among those, hypercholesterolemia clearly plays a dominant role in the initiation and progression of the fatty streak, the earliest morphological atherosclerotic lesion. Indeed, hypercholesterolemia induces fatty streaks in humans as early as during fetal development[37] and seems to be essential for lesion development in all animal models so far studied. Fatty streaks consist of macrophages predominantly filled with lipid droplets, which typically appear as "foam-cells" under the microscope. Fatty streaks then progress to more complicated atheromatous plaques containing a necrotic core rich in highly thrombogenic lipids with mechanically weak regions in the overlying fibrous cap. Once it forms, an atherosclerotic lesion possesses the characteristics of chronic inflammation.

Like all other inflammatory diseases, factors from both innate and adaptive immunity play important roles in the initiation and progression of atherosclerosis. While initial interest in the oxidation of low-density lipoprotein (LDL) stemmed from its ability to cause foam cell formation, it is now clear that many changes in LDL, including oxidation, and its many resultant molecules are pro-inflammatory and promote the progression of atherosclerosis by many different mechanisms. For example, oxidized products from minimally modified (oxidized) LDL (mm-LDL) or fully oxidized LDL (OxLDL) directly or indirectly recruit T cells and monocytes to the intima and promote differentiation of monocytes into macrophages[66]. In addition, OxLDL has been reported to affect vascular cell growth, differentiation, and/or induction of apoptosis and cell death. Even early forms of OxLDL, e.g. mm-LDL, cause changes in gene expression (e.g. activating NFκB-like factors) of vascular wall cells leading to the initiation and maintenance of an inflammatory response[3]. The varying molecules of OxLDL which affect so many different properties are under active study. However, it is likely that we have only begun to understand the many complex ways in which these oxidized/modified products are pro-inflammatory and influence atherosclerosis and the mechanisms by which these effects occur.

The original focus of our study is to investigate the immune response to oxidation-specific epitopes on OxLDL and their impact on the progression of atherosclerosis. Although most of the immune response seems to be pro-atherogenic, some of our findings contradict this notion. For example, immunization of Watanabe Heritable Hyperlipidemic (WHHL) rabbits with autologous LDL modified with malondialdehyde (MDA), a model epitope of OxLDL, resulted in high titers of antibodies to MDA-LDL and led to a 34% reduction in the extent of atherosclerosis[49]. This result was confirmed by other groups[20, 70]. More recently, a similar experiment was repeated in LDL receptor-deficient ($LDLR^{-/-}$) mice and it was found that even in mice immunized with native LDL a certain degree of lesion reduction resulted[19], indicating that even subtle change in mild conditions could lead LDL to present some immune determinants and result in protective immune response. Another finding is that autoantibodies to various epitopes of OxLDL have been cloned from spleens of apoprotein E-deficient ($ApoE^{-/-}$) mice that had been on a high-cholesterol diet but had never been immunized exogenously[40]. These monoclonal antibodies had IgM isotype, which could be developed in a T cell-independent fashion and divided into two groups; one group bound to copper-induced OxLDL (Cu-OxLDL), while another group bound strongly to MDA-LDL. All of the EO autoantibodies immunostained rabbit and human atherosclerotic lesions and several bound to epitopes on circulatory human LDL[40]. Importantly, many of them, e.g. EO6, inhibit the uptake of OxLDL by macrophages and, similarly, the phagocytosis of apoptotic cells[8, 30], demonstrating that the modified structures recognized by them not only serve as immuno determinants for such autoantibodies, but also as ligands mediating binding to macrophage scavenger receptors. It is therefore hypothesized that autoantibodies to OxLDL could modulate atherosclerosis by blocking uptake of OxLDL via scavenger receptor pathways to prevent foam cell formation or by promoting enhanced plasma clearance of early forms of OxLDL in circulation, thus diverting such LDL from the vascular wall. This raises our notion that at least one type of immune response, which mainly associates with a more primordial branch of immunity, can be anti-atherogenic, suggesting the broader role of such autoantibodies in maintaining the homeostasis of the host. On the other hand, the IgG antibodies to OxLDL, which are derived from T cell-dependent immune response ether by exogenous immunization or by neo-self determinants generated during extensive modification of lipids or protein, seem more likely to associate with the narcotic core of advanced lesions.

PROS AND CONS OF ADAPTIVE AND INNATE IMMUNITY

Immunity can be roughly divided into innate and adaptive responses. Innate immunity is evolutionari-

ly more ancient and utilizes receptors that are of germline origin and are products of natural selection. It provides a rapid, first-line defense against deadly pathogens; therefore it is preserved through evolution and can be transferred from one generation to the next. However, since each receptor is directly from a germline gene, the number of such receptors is limited. It is estimated that there are only 100 such receptors, and of necessity these are focused on highly conserved motifs found in many pathogens. Typically, a given receptor binds more than one motif, albeit with lower affinity than occurs with receptors of adaptive immunity. These receptors are termed pattern-recognition receptors (PRRs) and can be categorized into those that signal, those that are secreted, and those that are endocytic. In turn, the conserved motifs on pathogens to which they bind are termed pathogen-associated molecular patterns (PAMPs), and examples include bacterial lipopolysaccharide (LPS), lipoteichoic acid, mannans, and bacterial DNA. The cellular mediators of innate immunity are macrophages, dendritic cells, mast cells, neutrophils, and certain primordial B cells, such as B-1 cells. Because of their ability to present antigens or release various cytokines, these cells also serve to link innate with adaptive immunity. In addition, so-called "natural antibodies", which arise in naive hosts without immune exposure, are also integral mediators of innate immunity[26, 27].

Adaptive immunity results from the rearrangement of a set of germline genes and somatic mutations occurring in T and B cells, leading to the generation of an estimated 10^{18} T cell and 10^{14} B cell receptors, respectively. The huge amount of receptors provides a virtually unlimited number of unique structural features that supply great diversity and high specificity. Although this aspect of immunity provides highly discriminatory responses to a given immunogen, its onset is delayed, and the acquired information cannot be passed on to the next generation. Therefore, each generation is at risk of being attacked by same deadly pathogens, to which its parents had been immunized.

Some innate immune responses, such as natural antibodies, are effective and necessary when fighting limited pathological situations, such as acute viral and bacterial infections[39]. However, cellular PRRs are crucial components for most of the innate immune reactions, as, for example, CD14 recognizes LPS variants of virtually all Gram-negative bacteria and initiates an array of inflammatory responses. In a situation where the inciting pathogen persists and inflammation becomes chronic, immune responses can produce complex results that, although initially protective, can eventually lead to tissue damage and cause localized or even systemic disease. Atherosclerosis is possibly the most common chronic inflammatory disease, and markers of inflammation, such as C-reactive protein and interleukin 6, have recently been shown to be strong and independent risk factors[35]. Indeed, microbial pathogens as well as OxLDL have been proposed as important pathogens, triggering and sustaining this inflammatory response. In this review, I will focus on how subtle changes in OxLDL will associate with different branches of immunity and the possible consequences for atherogenesis that result from these immune responses.

OxLDL and atherosclerosis

It was initially anticipated that macrophage uptake of LDL was via a "classic LDL receptor" pathway, which might give rise to foam cells, but this did not occur, as uptake via this pathway led to compensatory down-regulation of LDL receptors. The description of a separate "scavenger" receptor provided the explanation for the initial paradox. Brown et al.[7] and Goldstein et al.[21] discovered that chemical modification of LDL, achieved by acetylation of lysine residues of LDL, led to a modified LDL that was now avidly recognized by an "acetyl-LDL receptor" with resultant rapid uptake, failure of down-regulation, and cholesterol accumulation. Other chemically modified forms of LDL such as MDA-LDL were also recognized by the same receptor. Subsequent studies showed that incubation of LDL with cultured endothelial cells (ECs), smooth muscle cells (SMCs), or macrophages converted LDL into a modified form that was taken up by macrophage scavenger receptors due to the ability of ECs and other cells to initiate lipid peroxidation in the LDL (reviewed in[2, 58, 67]).

Since the discovery of the acetyl LDL receptor (now known as SR-A), a variety of scavenger receptors capable of recognizing modified forms of LDL, including OxLDL, have now been described. These include SR-A (I, II, III), CD36, macrosialin (CLA-1, CD68), MARCO, LOX-1, and others[59]. While many of these receptors undoubtedly play important roles in other cellular functions, such as adhesion, they redundantly bind to OxLDL, suggesting the generalized biological importance of clearance of oxidized lipoproteins and lipids (as occurs in cellular debris, bacteria, and apoptotic cells). For example, gene targeting of the SR-A (I and II) receptor led to a reduction in atherosclerosis when the SR-A$^{-/-}$ animals were crossed into a ApoE$^{-/-}$ background (a genetic model of hypercholesterolemia that spontaneously develops severe atherosclerotic lesions within the first 12–24 weeks of life)[63]. Furthermore, Silvertein's laboratory

reported that deletion of the important CD36 receptor had a dramatic impact in protecting hypercholesterolemic mice against atherosclerosis[18, 47]. These studies demonstrate the central important role of macrophage uptake of OxLDL in the atherogenic process and form an important basis of our understanding that the presence of such autoantibodies that interact with the immune determinants which also serves as the ligands for the scavenger receptors could play a role in modulating the atherogenic process.

The specific biological properties of OxLDL, usually studied following oxidation of native LDL *in vitro*, depend on the extent of modification. This can range from mm-LDL, in which the LDL particle can still be recognized by LDL receptors[4, 46], to extensive oxidation, in which the ApoB component is fragmented and lysine residues are covalently modified with reactive breakdown products of oxidized lipids[59]. The full-OxLDL particles are defined as those not binding to the LDL receptor, but rather only to several so-called scavenger receptors expressed on macrophages and SMCs.

OxLDL seems to be involved in every stage of atherogenesis. The mm-LDL is thought to be inflammatory and to stimulate the expression of the adhesion molecules on the surface of ECs, which are responsible for the recruitment of monocytes to lesion-prone sites. Vascular cell adhesion molecule-1 (VCAM-1) was one of the first such molecules to be identified, based on its increased expression on ECs over lesion-prone areas, its preferential recruitment of monocytes, and its pattern of regulation by pro-inflammatory stimuli[13]. Studies to confirm a role of VCAM-1 in atherosclerosis in the mouse have been complicated by the fact that systemic deletion of the VCAM-1 gene results in early embryonic lethality. E selectin and P selectin appear to play quantitative roles in monocyte entry since ApoE$^{-/-}$ mice lacking both genes resulted in a 40–60% decrease in atherosclerosis[16]. Other adhesion molecules possibly involved in macrophage recruitment include intercellular adhesion molecule (ICAM-1)[12] and very late antigen-4 (VLA-4) integrin[55].

Migration of monocytes into the artery wall is also likely to be stimulated in part by OxLDL, which can directly attract monocytes[58] and can also induce the expression of chemotactic molecules by ECs, such as monocyte chemotactic protein 1 (MCP-1)[38]. Intriguingly, monocyte expression of CCR2, the receptor for MCP-1, is stimulated by hypercholesterolemia, and monocytes derived from hypercholesterolemic patients exhibit increased chemotactic responses to MCP-1[22]. Disruption of the MCP-1 or CCR2 genes markedly reduces the development of atherosclerosis in an ApoE$^{-/-}$ or ApoB over-expressing context.

The formation of morphological lesion is marked by development of macrophage "foam cells" that contain massive amounts of cholesterol esters. Cholesterol accumulation in these cells is thought to be mediated primarily by uptake of fully oxidized LDL via scavenger receptors[57]. Recognition of OxLDL by scavenger receptors is mediated, at least in part, by oxidized phospholipids (OxPLs) in both the lipid phase and as covalent adducts on ApoB[30]. Scavenger receptors A (SR-A) and CD36 have been demonstrated to play quantitatively significant roles since they are the principal receptors responsible for the uptake of OxLDL leading to lipid loading in macrophages[33]. OxLDL-derived cholesterol brought into the macrophage via scavenger receptors consists of both free cholesterol and cholesterol esters that are hydrolyzed in lysosomes.

Immune response to OxLDL

Early studies by Witztum et al. found that many subtle modifications of autologous LDL, including glucosylation, methylation, and carbamylation, conferred distinct immunogenicities to the modified products[60]. These observations led to the hypothesis that oxidative modification of LDL would generate a variety of immunogenic neoepitopes. The original hypothesis of the oxidation of LDL assumed that it started with the peroxidation of polyunsaturated fatty acids (PUFAs) in phospholipid (PL) moieties. Although the detailed structures have never been verified, it seems this can be accomplished *in vitro* by incubation of LDL with copper ions. *In vivo* it is more likely that the initiation of LDL oxidation occurs by exposure to vascular cells. This could occur by the gradual seeding of the LDL particle with lipid hydroperoxides derived from the cells. For example, 15-lipoxygenase can introduce lipid hydroperoxides into the PUFAs of cellular PLs and this in turn leads to the seeding of hydroperoxides in extracellular LDL[1]. Indeed, recent studies demonstrate that deletion of the 12/15-lipoxygenase gene in mice results in a dramatic reduction in the extent of atherosclerosis in ApoE$^{-/-}$ mice made homozygous for this deletion[14]. In addition, cells may induce oxygenation of LDL by other mechanisms related to the secretion of superoxide, other reactive oxygen species, release of thiols, or myeloperoxidase-related mechanisms[2].

Once formed, lipid hydroperoxides in PUFAs decompose to promote lipid peroxidation in all adjacent moieties, creating a variety of breakdown prod-

ucts which are highly reactive, such as MDA and 4--hydroxynonenal (4-HNE). They in turn can form covalent adducts with lysine residues of associated proteins and/or with amino groups of lipids, such as phosphatidylethanolamine. In addition PLs, containing an unsaturated fatty acid in the *sn2* position, can undergo decomposition, generating short-chain aldehydes in the *sn2* position, such as an aldehyde (5-oxyvaleroyl). Consequently, the intact PL containing the reactive 5-carbon aldehyde can form an adduct with the associated ApoB of LDL, leading to OxPL – ApoB adducts (Fig. 1). Such modifications that occur when LDL undergoes oxidation lead to major structural changes in the modified LDL. Thus, a wide variety of modified molecules, including lysophosphorycholine (lysoPC), oxidized cholesterol and cholesteryl esters, OxPLs, and a variety of modified lipid--protein adducts, could be formed when LDL undergoes oxidation. When such enhanced lipid peroxidation occurs in the artery wall, similar modifications may occur to a variety of structural proteins, such as extracellular matrix, as well as a variety of cellular membranes. These oxidatively induced modifications of autologous proteins convert ubiquitous molecules into neoantigens, which subsequently stimulate immune response, and this centralized our earlier understanding of the molecular basis of oxidative modifications and possible immune response associated with them. Indeed, immunologic evidence of LDL oxidation is observed even in human fetal arteries prior to the presence of macrophages[37].

These newly formed structures were once termed "oxidation-specific" neoepitopes, and it was demonstrated that they are immunogenic and occur *in vivo*. The presence of anti MDA-LDL and anti-OxLDL autoantibodies in sera of animals and humans provides one line of such evidence[40, 43]. For example, autoantibodies to OxLDL had been cloned from ApoE$^{-/-}$ mice never having been immunized with such antigen and they were shown (e.g. EO6) to bind to OxPLs *in vitro* and in lesions as well[40]. Much evidence has also suggested the involvement of immunological factors in the progression of atherosclerosis. Atherosclerotic lesions contain relatively large quantities of immunoglobulins and various complement components[51, 68] as well as activated T lymphocytes[24]. Sera from atherosclerotic patients and animal models contain autoantibodies against epitopes of OxLDL[43]. Furthermore, LDL isolated from animal and human atherosclerotic lesions contains tightly bound IgG that recognizes epitopes of OxLDL[68, 69]. These data indicated that the immune response and autoantibodies against OxLDL may play an important role in atherogenesis.

Although there is much evidence that there is immune activation of cells within atherosclerotic lesions, the antigens involved have only recently begun to be identified[24]. While a number of candidates have been proposed, including bacterial and viral proteins, heat shock proteins, and modified matrix proteins, most data support a central role for neo-self-determinants of OxLDL in mediating these immune responses. For example, studies by Hansson et al.[24] demonstrated that 10% of CD4$^+$ T cells cloned from human carotid plaques responded to OxLDL by proliferation and interferon γ secretion and this response was dependent on autologous antigen-presenting cells and restricted by HLA-DR[61]. As will be discussed later, OxPLs appear to be a prominent immunogenic epitope on OxLDL, as well as on

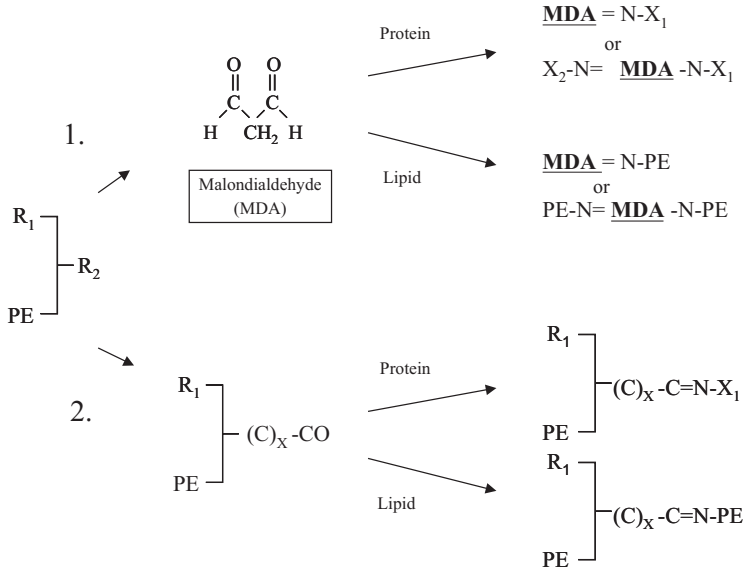

Figure 1. Proposed scheme to explain how oxidation of PUFA of a PL leads to the formation of oxidation-derived neoepitopes with associated proteins and lipids. Pathway 1 – decomposition of peroxidized PUFA leads to the generation of many reactive compounds, such as MDA. In turn, these can bind to lysine residues of associated proteins, or to amino-PLs, such as PE. Pathway 2 – as a result of b scission, reactive aldehydes may form and remain bound to the glycerol backbone and further bind to protein or amino-PLs. R1 – sn-1 fatty acid, R2 – sn-2 fatty acid, X1 – protein 1, X2 – protein 2 (from Hörkko et al.[30]).

apoptotic cells and necrotic cells. These and other model epitopes, such as MDA-LDL, all occur in lesions and therefore could be dominant immunogens in atherosclerotic lesions. Therefore, there are many data that support the concept that a vigorous humoral and cellular immune response occurs to OxLDL.

NATURAL ANTIBODIES TO OXIDATION EPITOPES IN ATHEROSCLEROSIS MOUSE MODELS

In order to clone some of these autoantibodies, spleens from two ApoE$^{-/-}$ mice that had been on a high cholesterol diet, but had never been immunized exogenously, were used for fusions to generate hybridomas[40]. OxLDL, as well as several models of oxidation-specific epitopes, such as MDA-LDL, were used for selection. From a single initial cloning, 1,536 viable Ig-secreting hybridomas lines were obtained. Supernatants from every 2 wells were pooled after 10 days and tested for binding to native LDL, as well as OxLDL and other models. Of the 768 pooled samples, 494 were positive for one or more epitopes of OxLDL. Thus, between 25 and 50% of all antibody-secreting hybridomas were secreting antibody binding to epitopes of OxLDL! This very large number of positive clones undoubtedly reflects the extraordinarily active immune response to the various oxidation-specific epitopes being generated in the ApoE$^{-/-}$ mice, which have extensive atherosclerosis and enhanced lipid peroxidation[14, 42]. From these hybridomas we eventually cloned 13 monoclonals to various epitopes of OxLDL (termed "EO" autoantibodies). Of interest was that all isotyped as IgMs. In general it was noted that one group of antibodies bound to Cu-OxLDL, while another group bound strongly to MDA-LDL. Of significance, each of the EO monoclonal antibodies immunostained rabbit and human atherosclerotic lesions[40]. These data demonstrated that a profound immunological response occurred to a large number of different oxidation-specific epitopes of OxLDL *in vivo* in the face of marked atherosclerosis.

The fact that the autoantibodies from ApoE$^{-/-}$ mice recognize similar oxidation-specific epitopes on OxLDL, as well as on apoptotic cells[8, 30], suggests that the original antigens responsible for the generation of these antibodies might therefore have been from apoptotic cells and/or even membranes from necrotic cells, as well as from OxLDL. In addition, we speculate that similar oxidatively induced damage to bacterial membranes, for example, might also lead to the generation of similar epitopes. The reiteration of similar "oxidation-specific epitopes" might then lead to a strong immune response. This would imply that many of the antigen-specific B cells would recognize common epitopes, as occurs in the atherosclerotic lesion, leading to a clonal expansion and enhanced antibody production. Because one isotype, IgM, was so prominent in our original cloning, and because such antibodies are known to be a part of "innate" immune responses and, in particular, to be a prominent component of "anti-lipid antibodies", we sought to determine in greater detail the genetic origins of the variable regions.

In a recent publication, Hörkko et al.[29] demonstrated that all of the EO antibodies that were selected for binding to OxLDL also specifically bound to oxidized 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (OxPAPC) but did not bind to unoxidized PAPC. Furthermore, these EO antibodies specifically bound to the oxidation product POVPC, in which the *sn2* fatty acid has been decomposed to a 5 carbon aldehyde[29]. Because POVPC contains a reactive aldehyde group, it is capable of forming a Schiff's base with amino groups of lysine. The EO antibodies also bound a POVPC-lysine or POVPC-BSA adduct. These data suggested that the antibody could recognize the OxPL when present as an isolated lipid or as a covalent adduct to protein.

DEMONSTRATION THAT EO AUTOANTIBODIES TO OXLDL ARE CLASSIC T15 ANTI-PC ANTIBODIES

The further characterization of such autoantibodies now relies on genetically analyzing their variable genes. Each of the EO hybridomas prepared from the ApoE$^{-/-}$ mice was immunochemically screened and determined to be of IgM isotype and to contain κ light chains exclusively.

The nonbiased cloning and sequencing of the variable regions of the EO6 autoantibody that bound to OxLDL revealed that it had 100% homology to a classic anti-PC antibody, T15. In addition, a similar analysis on other hybridomas secreting EO antibodies which bound OxLDL, including EO3, EO4, and EO7, found that they all had the same sequences for both heavy-chain variable region (VH) and light-chain variable region (VL). When these were independently sequenced, we were surprised to find that they represented only one unique functional VH gene rearrangements, which was an unmutated sequence deriving from the S107 family. This gene rearrangement is 100% identical to a previously reported functional V1-DFL16.1-JH1 rearrangement used by the T15 clonal set. Using the unique upstream leader sequence of either the S107 V1 gene or V11 gene further confirmed the genetic origin of the VH genes expressed by the EO6 cell line indeed

to be identical to s107 with V1 leader. From careful analysis of reported murine Vκ genes, 2 highly promiscuous Vκ gene FR1-targeted oligonucleotides were designed and each of these sense primers were paired independently with 3 different antisense oligonucleotides that target distinct Jκ gene-specific sequences. These data revealed the VL of EO6 rearranged Vκ22 family gene termed Vκ1-C to a Jκ1 gene segment. These gene rearrangements are consistent with primary homology-directed recombination, which means that no terminal dideoxylnucleotide transferase-mediated somatic mutation ever happens during the generation of these antibodies.

These data clearly show that EO3, 4, 6, and 7 each have the same primordial gene usage consistent with the T15 set, and the secreted antibody of each hybridoma binds exclusively to OxLDL. What is remarkable is that each of the individual EO monoclonal antibodies originally cloned for binding to OxLDL, each of which binds specifically to POVPC and POVPC-BSA, has the same genotype for both VH and VL in the variable regions. Although we cannot absolutely rule out that each clone represents the progeny of a single fusion event that was expanded during cloning, given the fact that 25–50% of the original 1536 viable hybridomas reacted with epitopes of OxLDL it seems far more likely that each is a separate fusion event.

The T15 cell line was first isolated over 30 years ago from pristane-conditioned peritoneal cells and specifically binds the PC moiety that is immunodominant in the teichoic acid cell wall polysaccharide (C-PS) of pneumococci and certain other microbial pathogens[25]. Monoclonal antibodies expressing this idiotype provide optimal protection to mice against certain lethal strains of pneumococci[6]. Indeed, it was recently shown that gene targeting of the S107V1 gene renders mice highly susceptible to bacterial infection with pneumococci[36]. The T15 antibodies are produced by a special subset of B-1 cells which are present at high frequency in mice several days after birth and even occur in mice raised in germ-free environment and without immune exposure[10]. Because T15 is prominently represented in the early B-1 cell response, we hypothesized that due to OxLDL-mediated antigen-driven expansion it constitutes a prominent fraction of proliferating B cells that form successful fusions yielding viable hybridomas. To validate that the different EO antibodies did indeed express the T15 idiotype at the protein level, we tested the ability of a series of anti-idiotypic antibodies previously generated to bind to the various EO antibodies[15]. Antibody T139.2 is directed against the unique light-chain of T15 (Vκ22-1c), antibody T139.2 is directed against the unique heavy-chain of T15 DFL16.1-JH1, and antibody AB1-2 is directed against the idiotype that is made up of both the VL and VH regions. Note that EO3, 4, 6 and 7 all express the T15 idiotype, while EO12 and 14, which bind to MDA-LDL, do not[53].

Because of the sequence homology in the variable region between T15, which is an IgA, and EO6 (as an example of the EO antibody binding to OxLDL), which is an IgM, we sought to demonstrate that they shared similar antigen recognition. As expected, EO6 bound to OxLDL, and to POVPC-BSA. In addition it also bound to the classic T15 antigen, PC conjugated to BSA (PC-BSA) and to PC-KLH. In turn, T15, besides binding to PC-BSA, and PC-KLH as expected, also bound to OxLDL and to POVPC-BSA, similar to EO6. Neither antibody bound to native LDL nor to another model epitope of OxLDL, MDA--LDL. In reciprocal competition studies, the binding of EO6 to OxLDL was inhibited by PC-KLH, or PC alone, and, conversely, the binding of T15 to PC--KLH was inhibited by OxLDL. In further studies we directly demonstrated that T15 competed effectively for the binding of biotinylated EO6 to OxLDL, whereas a control murine IgA did not. This suggests that these two monoclonal antibodies are directed against an immunologically closely related if not identical epitope on OxLDL. Further confirmation that EO6 binding to PC-KLH was by use of the T15 VL/VH paratope was the demonstration that the anti-idiotype antibody T139.2 competed very effectively for binding of EO6 to PC-KLH. Finally, we directly demonstrated that both EO6 and T15 bound to polysaccharide from the pneumococcal cell wall (C-PS), which has been previously demonstrated to contain PC and is a strong immunogen for anti-PC antibodies[25]. However, autoantibodies selected for binding to MDA-LDL, such as EO14, did not bind to C-PS. These data indicate that an epitope for both EO6 and T15 on OxLDL has a similar structure to those found on the cell wall of certain bacteria. As described above, it is well known that anti-PC antibodies and the T15 B cell subset play a vital role in the "innate" defense against pneumococcal infection. This raises the interesting possibility that pneumococcal infection could lead to an anamnestic response, with a variety of isotype antibodies that could cross-react with OxLDL and thereby influence atherogenesis. For example, IgM and IgA antibodies might bind to OxLDL and block uptake by macrophages as well as effect plasma removal of LDL particles bearing OxPL epitopes. Indeed, EO6 epitopes are present on plasma LDL[40]. Conversely, an IgG response, mostly resulting from adaptive immune response, might actually lead to an enhance-

ment of OxLDL IgG uptake by macrophages mediated via Fc receptor mechanisms.

As shown several years ago, EO6 stains macrophage filled lesions intensely[40]. Because the T15 anti-idiotypic antibody AB1-2 recognizes the paratope on T15/EO6, we were able to immunostain murine lesions and demonstrate that T15/EO6 antibodies were present in murine atherosclerotic lesions[53]. This also directly implies that this autoantibody could play a role in modulating the atherogenic process, since this antibody that interacts with the ligand of scavenger receptors is physically deposited in atherosclerotic lesions. Indeed, we recently immunized mice with heat inactivated R36A (an *Streptococcus pneumoniae* strain), which contains the PC moiety that is recognized by EO6/T15 in the bacterial cell wall. We found that the titers to OxLDL rose substantially in animals immunized with R36A compared with adjuvant alone and this inhibited atherogenesis[5]. These observations and others suggest that it might be possible to modulate the development of atherosclerosis by immunization or other immune-based interventions (review in[23, 28]). For example, elimination of T cells with cyclosporin or immunization of experimental animals with antigens that are homologous to endogenously expressed proteins present in lesions, such as certain heat shock proteins, have been found to accelerate lesion formation. In contrast, interruption of CD40 ligands, intravenous administration of polyspecific immunoglobulin, or hyperimmunization of hypercholesterolemic rabbit and murine models with homologous OxLDL decreases atherosclerosis[19, 28, 41].

TWO GROUPS OF THE ANTI-PC ANTIBODIES BEHAVE DIFFERENTLY IN THEIR BIDING TO OXLDL

Another exciting discovery that would help our understanding of immune response to OxLDL came from our recent study of dual reactivity of anti PC antibodies. The antibodies to PC can be divided into 2 groups. Group I are the so-called natural antibodies to PC. Most of them are generated from B-1 cells in a T cell--independent manner and can even form in a germ-free environment. Group II anti-PC antibodies, however, are derived from secondary immune responses to a T cell-dependent PC-protein conjugate, such as PC-KLH. While both groups of anti-PC bind to PC--protein conjugate, their binding to the oxidation-specific neo-determinants are different. We tested 6 group I anti-PC antibodies, EO6, T15, H8, M167, M511, and M603, which all utilize VHS107.1 rearrangements and bear related idiotypes[11, 48]. They all displayed strong and specific binding interactions with protein adducts of POVPC, an immunodominant oxidation-specific PC-containing epitope in atherosclerotic lesions, and also with copper OxLDL; binding was specifically inhibited by simple PC-Cl salt. Significantly, the relative level of these reactivities was also roughly proportional to that for the recognition of determinants in atherosclerotic vascular lesions.

Unlike group I antibodies, the group II anti-PC antibodies tested do not recognize PC alone, although they generally have high affinity for the derivatized PC compounds, especially those with phenyl diazo-linkages such as in nitrophenyl PC[9]. These antibodies are also unlike group I antibodies as they do not utilize a limited set of V gene elements[62], and therefore their antigen-binding sites are also more structurally diverse. However, group II anti-PC antibodies also recognized neo-self determinants on OxLDL and stained atherosclerotic lesions. Also akin to the properties of group I antibodies, these reactivities were relatively proportional to that for POVPC-BSA (and Cu-OxLDL).

Although both group I and group II anti-PC antibodies displayed strong reactivity for neo-determinants on apoptotic thymocytes, they exhibited distinct differences in their preferences for the binding of PC--epitope determinants exposed during apoptotic death, suggesting that during the course of induced cell death a range of different membrane-associated PC neo-determinants may be generated, or revealed. The group I antibodies T15 and M603 reacted primarily with thymocytes that were PI-dim or negative (and 7-AAD dim or negative), indicating their recognition of dying cells that still maintained the integrity of their cell membrane. Moreover, these group I antibodies also preferentially recognized cells with little or no reactivity with Annexin V, which is specific for phosphatidyl serine that has flipped to the outer membrane during apoptotic death[50]. In contrast, the group II antibodies PCG1-14 recognized only a subpopulation of cells that were also Annexin V-high and 7-AAD-high, representing a set of dying thymocytes distinct from those recognized by the group I anti-PC antibodies. Moreover, co-staining with Annexin V did not interfere with binding by the group II antibodies, suggesting that the antibody recognizes a PC-containing determinant that is co-expressed with membrane-accessible phosphatidyl serine on dying thymocytes. Hence, while the group I antibodies recognized apoptotic thymocytes at a relatively early stage of programmed cell death, the PCG1-14 antibodies primarily recognized thymocytes at later stages of programmed cell death[52].

There was evidence that immune globulin-expressing B cells encountered and were being selected by an auto-antigen; but until our recent demonstration of

PC neo-determinants on apoptotic cells and on atherosclerosis-associated antigens, the true identity of such postulated selecting self-antigens was unsuspected[56, 65]. The demonstration of the auto-reactivity of the group II antibodies also adds a further dimension to this notion. PCG1-14 and M3C65, which were originally generated by immunization with a form of PC chemically conjugated via a diazophenyl linker to the carrier protein, had gene usage from diverse families with non-templated DNA insertions at the V-D-J splice sites, suggesting that these rearrangements first arose in adult bone marrow. Moreover, the genes for these group II antibodies also exhibit hypermutations that are the hallmark of the recruitment of conventional B cells (also called follicular or B-2 cells) into T cell-dependent germinal center reactions, which are also the sources of memory B cells. Our findings therefore also suggest that autoreactivity with oxidation-associated neo-self PC-containing determinants clearly does not prevent clonal entry into the peripheral B cell pools, even for follicular B cells.

Programmed cell death results from a specific external signal triggering a complex sequence of molecular pathways that leads to a "clean" death, as phagocytic cells are recruited to efficiently clear cell debris without affecting neighboring cells. Although cells undergoing apoptosis may proceed according to different pathways, mitochondrial membrane alterations leading to the release of oxidatively reactive species are often relatively early events, while the full activation of the cascade of cysteine proteases, or caspases, are generally later events that rapidly lead to cytolysis. Our results demonstrated that when dexamethasone-induced apoptotic thymocytes were treated with caspase inhibitor (Z-VAD), two types of anti-PC antibodies displayed distinct patterns of reactivity. In specific, the group I antibodies displayed enhanced reactivity with these Z-VAD-treated thymocytes. By contrast, the inhibition of the caspase pathway diminished the reactivity of these cells with group II antibodies. Hence, PC-containing neo-self auto-antigen(s) on different stages of OxLDL or apoptotic cells, recognized by two types of anti-PC antibodies, display different molecular features and cellular distributions. Notably, recent studies have demonstrated an enrichment of oxidized PC-containing PLs in such apoptotic cells[31], suggesting that neo-self PC-antigens develop as part of the oxidative changes affecting membrane PL moieties in cells undergoing apoptosis. Alternatively, these determinants may become accessible for antibody recognition due to apoptosis-associated phospholipase activity[32]. Therefore, the induced variations in the representation of oxidatively modified membrane components, and perhaps associated changes in the accessibility or conformation of the associated PC-headgroup, would then likely explain the differential reactivity of the group I and group II anti-PC antibodies, suggesting that either the density or the structural features of PC-containing determinants may vary greatly during different stages of apoptosis, as well as on OxLDL in different stages of atherosclerotic lesion development.

ANTIBODIES TO MDA-LDL ARE GENETICALLY DIVERSE

Unlike autoantibodies to OxLDL or PC, antibodies to MDA-LDL, another model epitope thought derived from oxidative modification, display a very wide genetic and functional diversity. Most of the antibodies studied thus far possess the character of T cell-dependent immune response. One of the first mouse monoclonal antibodies to MDA-LDL cloned was MDA2, which has an IgG1 isotype and has been extensively characterized *in vitro*. MDA2 has specificity to MDA-LDL but not native LDL, HDL, or VLDL. It also recognizes MDA-lysine epitopes on other modified proteins, such as MDA-BSA. Immunocytochemistry has shown that MDA2 recognizes atherosclerotic lesions with both lipid rich foam cells and extracellular lipid deposits that contain abundant MDA-specific epitopes, but does not stain normal arterial tissue[45].

As mentioned above, a group of autoantibodies to MDA-LDL were also isolated from ApoE$^{-/-}$ mice. The immunological characterization of this group of antibodies showed less uniform binding patterns[40]. For example, EO14 and EO17 bound to reduced and non-reduced acrolein-modified LDL, with a preference for the non-reduced form, and to 4-HNE-LDL. However, the relative binding to these antigens, as well as the recognition of LDL modified by arachidonic or linoleic acid oxidation products, was different for each antibody. EO13 recognized both MDA--LDL and Cu-OxLDL and bound particularly to acrolein-LDL. EO14 and EO12 recognized both moderately modified MDA-LDL and acrolein-LDL, and also bound to LDL and BSA modified by fatty acid peroxidation products. The binding structures of these antibodies that selected to MDA-LDL, ranging from MDA-polylysine, MDA protein conjugates and MDA-like structures such as adducts formed with the 3-carbon aldehyde acrolein, suggest that the epitope recognized is more complex than simply MDA-lysine. Later, all of these EO antibodies to MDA-LDL were sequenced in my lab and they, despite IgM isotype, all had V-D-J regions that contained the non-template insertion, which is the hallmark of T cell-dependent semiotic mutation (unpublished data by author).

It is likely that immunogenic structures formed during the more extensive oxidation of LDL stimulate the production of these antibodies in a T cell-dependent pathway. When fatty acids undergo lipid eroxidation, a variety of highly reactive breakdown products are generated, such as MDA and 4-HNE, which in turn can modify prime anime groups in lysine and other residues of ApoB. The products formed from even these simple aldehydes are highly complicated and many different structures are generated[17], which consequently raises significant plasma titers of IgG and IgM autoantibodies against such epitopes of OxLDL, in particular against MDA-LDL and 4-HNE--LDL, in a variety of experimental animals[42, 43]. For example, in $LDL^{-/-}$ mice fed a high cholesterol diet, there was a fourfold rise in autoantibody titers to MDA-LDL, and the titer correlated with the extent of atherosclerosis[44]. In a recent study, when $ApoE^{-/-}$ mice were immunized with MDA-LDL, MDA-VLDL, IgG antibodies were developed against both protein and lipid components of OxLDL[70]. This implies that T cell help was provided for B cell responses and resulted in immunoglobulin isotype switching. Such a switch is generally on booster immunization with protein antigens, since antibodies to lipid antigens often develop in a non-T cell-dependent manner. However, their finding of IgG anti-oxidized cardiolipin suggests that T cells recognize oxidized cardiolipin or molecular structures associated with it.

In preliminary studies in humans, the titers of autoantibodies to these epitopes were higher in subjects with increased cardiovascular disease or clinical manifestations of atherosclerosis[34, 64]. To further study their genetic immunological properties, a panel of human monoclonal Fab antibodies against epitopes of OxLDL has been cloned from a phage display library which was made from a patient of by-path surgery who had very high plasma titers to MDA--LDL and which consisted of up to 10^8 combinations of monoclonal Fab. Three strong binders to MDA--LDL were identified and designated as IK3, IK17, and IK103, with dissociation constants (Kd) to MDA--LDL of 2.9×10^{-8}, 3.7×10^{-8} and 2.1×10^{-8} M, respectively. Also, importantly, the plasma of the patient was able to inhibit the binding of these antibodies to MDA-LDL. IK17 also showed strong binding to Cu-OxLDL and was chosen for further studies. The binding of IK17 to MDA-LDL was effectively competed by both MDA-LDL and Cu-OxLDL, but not by native LDL. IK17 binding to MDA-LDL was proportionally inhibited by both the delipidated protein and the lipid moiety of Cu-OxLDL. In contrast, neither the protein nor lipid moiety of native-LDL competed. These data were confirmed by a Western blot analysis that showed that IK17 bound extensively to the protein moiety of Cu-OxLDL, MDA-LDL, and MDA-HDL. The data indicate that the epitope recognized by IK17 includes MDA-modified lipids as well as MDA-lipids covalently bound to the protein backbone of LDL. IK17 was also able to inhibit the uptake of OxLDL and phagocytosis of apoptotic cells by macrophages, implying that the epitopes defined by IK17 played a role as ligands mediating the uptake of OxLDL and phagocytic clearance of apoptotic cells[54].

However, staining of atherosclerotic lesions in human and rabbit arteries indicated that the epitopes, e.g. MDA-LDL, recognized by IK17 occurred mostly in the necrotic core. Macrophage-rich early lesions and shoulder areas of transitional lesions generally showed very little IK17 staining. Only a few early lesions in arteries showed weak cellular staining (Fig. 2A). In contrast, strong IK17 staining was found in necrotic areas of advanced lesions of human coronary arteries (Fig. 2B) and in the core of classic atheromas in human brain arteries (Fig. 2C). Similarly, in WHHL rabbit aortas, IK17 strongly stained necrotic areas, whereas only weak staining of early lesions and superficial macrophages was detected (Fig. 2D). These results appear qualitatively different from the staining patterns that we previously obtained with antisera and monoclonal antibodies against other oxidation-specific epitopes, e.g. Cu--OxLDL/PC, which consistently showed strong staining in macrophage-associated and diffuse extracellular early lesions of humans, rabbits, and mice, but relatively weaker staining in necrotic areas[40, 43]. The difference was further demonstrated in immunostaining side by side of a large macrophage- and lipid-rich lesion from a balloon-catheterized cholesterol-fed New Zealand White rabbit. Figure 2E shows the IK17 staining, whereas Fig. 2F illustrates the pattern seen with EO3, a representative monoclonal antibody binding to OxPL adducts of OxLDL. One other clinical significance of this human antibody is the demonstration that *in vivo* injection of ^{125}I-IK17 into $LDLR^{-/-}$ mice resulted in specific accumulation of ^{125}I-IK17 in atherosclerotic lesions. However, this is far from the scope of this review.

The genetic analysis of the cloned human monoclonal antibodies was done by sequencing their VL and VH genes and aligning with the human immunoglobulin data base. The results revealed that the VH genes of IK103 and IK3 were likely derived from B cells in the same clonally related set and had 91.3–97.8% homology. The antibody genes of IK17 are derived from completely unrelated clonal origins. These findings, with comparable levels of hypermuta-

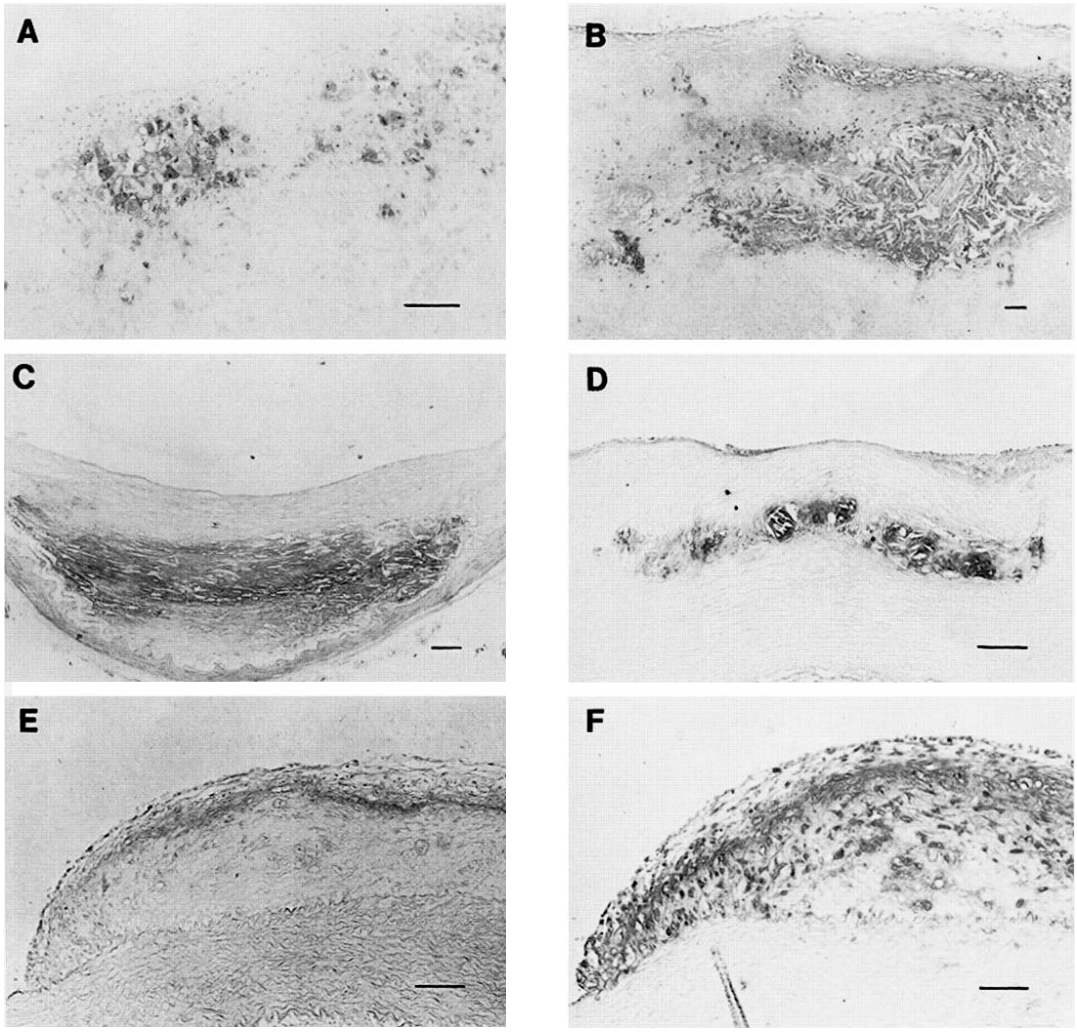

Figure 2. Immunostaining of atherosclerotic lesions by antibodies to MDA-LDL and Cu-OxLDL/PC. Epitopes recognized by biotinylated-antibodies are indicated by dark gray; the nuclei are counterstained with methyl green. **A** – section of human coronary lesion stained with IK17. **B** and **C** – IK17 staining necrotic areas of advanced lesions of human coronary artery (**B**) and in the core of classic atheromas in human brain artery (**C**). **D** – IK-17 staining in necrotic areas of WHHL rabbit aorta. **E** – large macrophage- and lipid-rich lesion from a balloon-catheterized cholesterol-fed New Zealand White rabbit stained with IK17. **F** – adjacent section stained with EO3, which is identical to EO6 in its variable regions and staining patterns, as seen with most of our other oxidation-specific epitopes. Bars = 100 μm (adapted from Shaw et al.[54]).

tion of both heavy and light chains, are consistent with the T cell-dependent adaptive immune response and features previously described for IgG-expressing B cell clones.

CONCLUSIONS

We now should have a new understanding of the oxidative modification of lipoprotein and the immune responses associated with the different processes (illustrated in Fig. 3). There are at least two types of modification which happen to lipoproteins *in vivo* when they are under oxidation pressure. The first type, which is usually referred to as "minimally--oxidized" LDL or "oxidized" LDL, mostly only reflects conformational changes. These changes involve the rearrangement of the molecular structures on the surface of LDL and/or cellular membranes during the apoptotic process, but do not necessarily involve the break-up of the intra-molecular covalent bonds and Schiff's base formation. This type of changes can happen in either natural/physiological conditions or the early stage of disease, resulting in the presentation of some molecular structures, such as phosphorylcholine, which mimic the structure displayed on pathogenic bacteria. These changes typically do not "create" the neo-immunogens, as described before, to stimulate the specific T cell--dependent adaptive immune response; they rather induce or expand the intrinsic innate immune response associated with such pathogen-associated molecular pattern to produce natural antibodies,

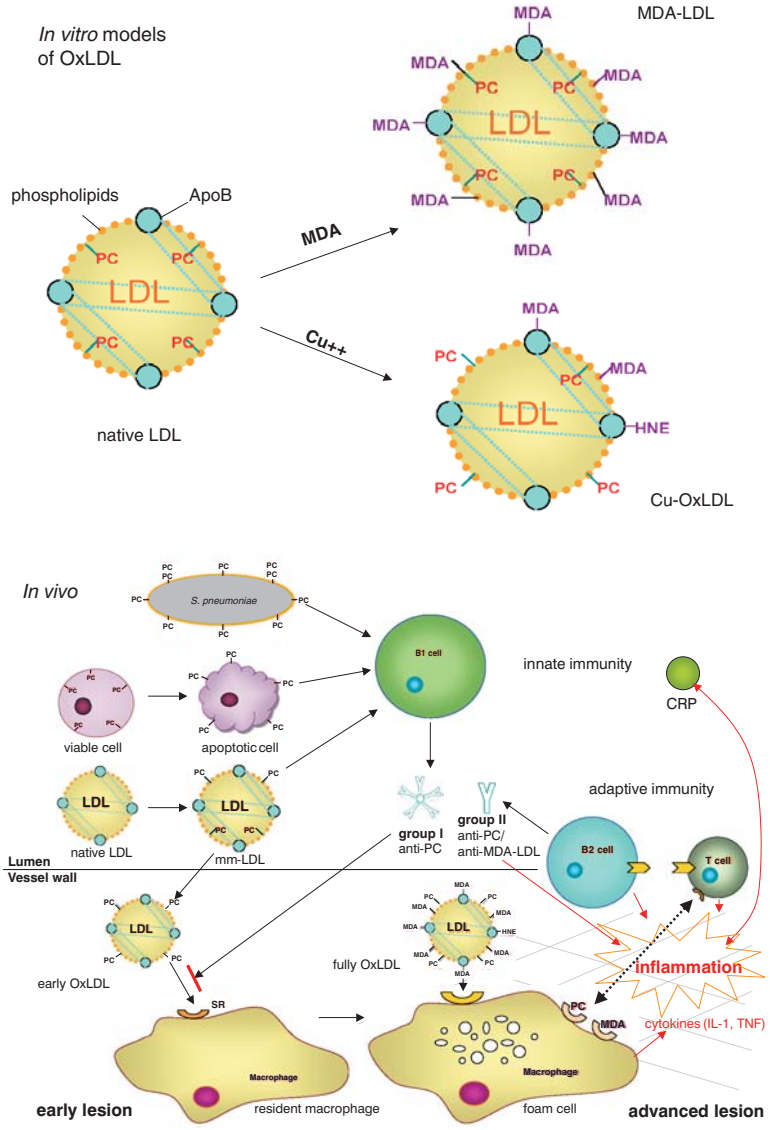

Figure 3. Schematic representation of oxidative modification of LDL and the immune response associated with such modifications. There are two types of commonly used *in vitro* models of OxLDL: MDA-LDL and Cu-OxLDL. The MDA-LDL is a more uniform structure, in which the reactive MDA is attached to LDL through lysine residues in ApoB as Schiff's base or directly form covalent bond to lipids. The Cu-OxLDL has rather complex structures, which include modified PLs, such as presentation of PC, and adducts formed from breakdown products of oxidation of polyunsaturated fatty acids. *In vivo*, the molecular mimicry occurs when LDL or a viable cell undergoes limited oxidative modification to present the PC antigens like those expressed on *Streptococcus pneumoniae*, which stimulate B1 cells to produce the protective EO6/T15 autoantibodies. These autoantibodies to PC may block the uptake of OxLDL through scavenger receptors, promote the clearance of apoptotic cells, or neutralize the infecting bacteria. When further oxidation occurs, particularly in inflammatory settings, the more extensive oxidation will be predominant to produce MDA-like immuno determinants, which in turn, along with some PC antigens, are presented by antigen-presenting cells such as macrophage to stimulate the T cell-dependent immune response to produce IgG antibodies. These antibodies, along with other pro-inflammatory cytokines produced by foam cells and C-reactive protein (CRP), in turn accelerate lesion progression, resulting in an even more oxidative environment.

such as T15. This gives the host a signal, and the natural antibodies work as a check-point for these changes. Under physiological conditions, the modified cell membranes or lipoproteins that display pattern recognition signals can be cleared by a regulated pathway, so host homeostasis is established. However, when such changes accelerate to a certain degree, as in atherosclerosis, such balance is broken, resulting in the elevated level of this type of OxLDL in the circulation and in atherosclerotic lesions, as well as the expansion of such autoantibodies. Therefore, this type of OxLDL is more likely to reflect the disease status, especially in the early stage of lesion development. Thus they can be used as a clinical tool to monitor the disease progression. As an example, Dr. Tsimikas recently used autoantibody EO6 to measure OxLDL in the circulation and to predict the degree of atherosclerosis (unpublished data).

The second type of modification is exemplified by MDA-LDL, which is actually formed when polyunsaturated fatty acids have undergone fragmentation and yield the reactive molecule, such as MDA. This type of modification presumably changes the natural structures of lipoproteins and plasma membranes by chemically forming some additional adducts which expose to the host as foreign-like structures and initiate a typical T cell-dependent immune response to generate antibodies with higher specificities. This type of change usually happens during the later stage of atherosclerosis development, since the micro-environment is much more isolated from normal physiological conditions. Stronger oxidation pressure is present in those areas, resulting in the fragmentation of intra-molecular double bonds and the formation of the Schiff's base, etc. In turn, these antibodies are usually more proatherogenic and tightly associate

with the advanced stage of lesions. From a clinical point of view, the antibodies resulting from such adaptive immunity are more useful as imaging reagents or for drug delivery since they directly interact with lesions and plaques; such a method of using an antibody to MDA-LDL for non-invasive imaging is being developed by us. With this illustration, we hope to have a better understanding of the immune responses to *in vivo* modified structures and their role in the natural situation and also in atherosclerosis.

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