Oxidation-Specific Epitopes Are Danger-Associated Molecular Patterns Recognized by Pattern Recognition Receptors of Innate Immunity

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Abstract: —Oxidation reactions are vital parts of metabolism and signal transduction. However, they also produce reactive oxygen species, which damage lipids, proteins and DNA, generating “oxidation-specific” epitopes. In this review, we discuss the hypothesis that such common oxidation-specific epitopes are a major target of innate immunity, recognized by a variety of “pattern recognition receptors” (PRRs). By analogy with microbial “pathogen-associated molecular patterns” (PAMPs), we postulate that host-derived, oxidation-specific epitopes can be considered to represent “danger (or damage)-associated molecular patterns” (DAMPs). We also argue that oxidation-specific epitopes present on apoptotic cells and their cellular debris provided the primary evolutionary pressure for the selection of such PRRs. Furthermore, because many PAMPs on microbes share molecular identity and/or mimicry with oxidation-specific epitopes, such PAMPs provide a strong secondary selecting pressure for the same set of oxidation-specific PRRs as well. Because lipid peroxidation is ubiquitous and a major component of the inflammatory state associated with atherosclerosis, the understanding that oxidation-specific epitopes are DAMPs, and thus the target of multiple arcs of innate immunity, provides novel insights into the pathogenesis of atherosclerosis. As examples, we show that both cellular and soluble PRRs, such as CD36, toll-like receptor-4, natural antibodies, and C-reactive protein recognize common oxidation-specific DAMPs, such as oxidized phospholipids and oxidized cholesteryl esters, and mediate a variety of immune responses, from expression of proinflammatory genes to excessive intracellular lipoprotein accumulation to atheroprotective humoral immunity. These insights may lead to improved understanding of inflammation and atherogenesis and suggest new approaches to diagnosis and therapy. (Circ Res. 2011;108:235-248.)

Key Words: oxidation-specific epitopes | innate immunity | oxidized lipids
were capable of binding a much larger number of bacterial molecules to their normal states. If these mechanisms fail, then oxidation reactions also produce reactive oxygen species (ROS), which damage, often excessively, lipids, proteins, and DNA. To counteract the oxidative damage, complex antioxidative mechanisms reduce oxidized “bystander” molecules to their normal states. If these mechanisms fail, then oxidation-damaged molecules, molecular complexes, and cells must be removed from the tissue. This task is most often performed by macrophages, professional scavenger cells, and other components of the innate immune system.

In this review, we discuss the hypothesis that a major mechanism by which recognition of oxidation-damaged molecular complexes occurs is via the detection of “oxidation-specific epitopes” by “pattern recognition receptors” (PRRs) of innate immunity. The concept of PRRs was first introduced to explain how a limited number of macrophage receptors capable of binding a much larger number of bacterial ligands. Such PRRs were postulated to bind to structurally common pattern motifs on microbial products, termed “pathogen-associated molecular patterns” (PAMPs). Thus, a limited number of PPRs could provide to the host a broad-based innate defense against a variety of microorganisms. By analogy, we postulate that host-derived, oxidation-specific epitopes, which also constitute products of diverse classes of oxidative reactions, can be considered to represent “danger (or damage)-associated molecular patterns” (DAMPs). DAMPs, of course, refer to a much broader group of “inadvertent” modifications of lipids, carbohydrates, proteins, and DNA, aside from oxidative events, which pose danger to the functioning of an organism. Although we use the term DAMP to describe common oxidation-specific epitopes, as discussed in detail below, many such epitopes share molecular identity and/or mimicry with epitopes on microbes, thus often making the distinction between PAMPs and DAMPs a semantic issue rather than a real distinction.

Traditionally, one would argue that the need to recognize microbial PAMPs provided the evolutionary pressure for the emergence of PRRs. However, we would suggest that recognition of host-originated DAMPs, including oxidation-specific epitopes, may have provided equal if not more compelling evolutionary pressure. Here, we only refer to 2 arguments in favor of the DAMP hypothesis in the context of oxidation-specific epitopes, and we discuss this in more detail at the end of the review. Recognition and removal of apoptotic cells is an essential and very early process in embryonic development, usually occurring in an environment protected from infectious pathogens. Macrophage PRRs, such as CD36 and SR-A, which recognize oxidation-specific epitopes on apoptotic cell surfaces, such as oxidized phospholipids (OxPL) and malondialdehyde (MDA)-modified structures, are intimately involved in the process of apoptotic cell removal. Another example is mammalian toll-like receptor (TLR) and bacterial lipopolysaccharide (LPS). Although this is considered a classic PRR/PAMP pair, which provides one of the strongest and wide-ranging innate immune responses to a microbial ligand in mammalian cells, recent evidence from our laboratory and others suggests that oxidized lipids, such as oxidized cholesteryl esters (OxCE) and OxPL, appear to be ligands for TLR4. Indeed, the prototypical Drosophila Toll, although acting in a defensive role against microbes, is also a developmental gene, essential in dorsoventral axis formation. Furthermore, recent studies demonstrate that both zebrafish paralogs of mammalian TLR4, TLR4a, and TLR4b do not recognize bacterial LPS, although the ligands for zebrafish TLR4 are yet unknown.

In our immediate area of research, atherogenesis, the concept that oxidation-specific epitopes form a family of DAMPs recognized by innate PRRs has led to an improved understanding of “why” innate immunity is involved in the pathophysiology of atherosclerosis. Oxidation of low-density lipoprotein (LDL) is thought to contribute to atherogenesis by multiple mechanisms, including the formation of myriad oxidation-specific epitopes, which not only makes oxidized LDL (OxLDL) a ligand for multiple PRRs but leads to the generation of a variety of oxidized lipids that directly influence atherogenesis in multiple ways. In the first part of this

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article, we briefly review potential mechanisms of LDL oxidation in vivo, and evidence that oxidation-specific epitopes mediate binding to PRRs. In the second part, we discuss cellular and humoral PRR-mediated immune responses to oxidation-specific epitopes and consider their roles in atherogenesis. In both parts, we limit our discussion to moieties derived from oxidation of polynsaturated fatty acids (PUFAs) in PL or CE, as well as the moieties resulting from modification of proteins with these products of lipid oxidation. Most of these are found in various forms of OxLDL during the progression of atherosclerosis.

**Oxidized LDL**

Many lines of evidence suggest that LDL undergoes oxidation in vivo, particularly under conditions of hyperlipidemia and when retained in the vascular wall. A large body of work has defined a variety of enzymatic mechanisms that could mediate oxidation of LDL, including 15- or 12/15-lipoxygenase (LO), myeloperoxidase, and peroxidase-like activity of hemoglobin, although other modes of oxidation are important as well (reviewed elsewhere). Nonenzymatic, free radical–mediated oxidation depends on the presence of superoxide, hydrogen peroxide, and nitric oxide generated by NADPH oxidases and nitric oxide synthases, and the catalysis by transition metal ions, copper and iron, and hemin.

A widely used model of extensively OxLDL, leading to profound oxidative degradation, is LDL subjected to prolonged incubation with copper sulfate. This type of Cu$^{2+}$-catalyzed oxidative attack on the PUFAs in PL may lead to degradation of 40% of the phosphatidylcholine. The apoB of OxLDL already contains such moieties. In the presence of LO, an intracellular enzyme found in all vascular cells and CE. Such early products are formed in LDL modified by 15LO, an intracellular enzyme found in all vascular cells under inflammatory conditions. A mechanism by which cellular 15LO can lead to the oxidation of the PUFA of CE residing in the core of extracellular LDL has been suggested by Yoshimoto and colleagues. It involves binding of LDL to LRP-1, exchange of CE between LDL and the cellular membrane, recruitment of 15LO from the cytosol to the site of LDL binding, and then reverse transport of 15LO-oxygenated CE from the cell to the LDL. This mechanism agrees well with the known preferential oxygenation of CE by 12/15LO-expressing cells. Human 15LO and mouse 12/15LO have been proposed to play a major role in vivo LDL oxidation and the development of human and experimental murine atherosclerosis, as reviewed elsewhere.

In the absence of transition metals, CE hydroperoxides are relatively stable, particularly when shielded from water inside the lipophilic core of LDL. Accumulation of CE hydroperoxides has been documented in human atherosclerotic lesions and in the lesions of apoE$^{-/-}$ and LDLR$^{-/-}$ mice fed a high-fat diet. Using liquid chromatography–mass spectrometry analysis of mmLDL, we demonstrated polyoxygenated CE hydroperoxides in which the cholesterol moiety was not oxidized, but the arachidonate acyl chain contained a hydroperoxide at the 15th carbon, as well as 1 to 4 additional oxygen atoms. These CE hydroperoxides, which were also generated by direct incubation of cholesteryl arachidonate with 15LO (the product of this reaction is abbreviated as 15LO-CE), were demonstrated to induce a number of important biological responses in macrophages as described below.

**Innate PPRs that Detect Oxidation-Specific Epitopes**

Macrophages express the highest density of cell surface and intracellular PRRs, but other cell types express PRRs as well. In addition, there are important soluble innate PRRs including soluble variants of some cellular PRRs, pentraxins, and natural antibodies (NAbs), which are predominantly of IgM and IgA isotypes.

**CD36, SR-A, and Other Scavenger Receptors**

“Scavenger receptors” of macrophages were so named because they bind and internalized extensively OxLDL, but not native LDL, and to date the list includes CD36, SR-A1 and -A2, SR-BI, MARCO, LOX-1, PSO, and others. Although all were originally identified for their ability to bind oxidation-specific epitopes (DAMPs) of OxLDL (or models of OxLDL), it is now apparent that they bind a wide variety of different ligands, including many microbial pathogens (PAMPs). CD36 and SR-A have the highest affinity for OxLDL and acetylated LDL, respectively, and are responsible for up to 90% of their uptake by macrophages in vitro. OxPL in OxLDL have been shown to mediate CD36 binding. POVPc (1-palmitoyl-2-(5'-oxovaleroyl)-sn-glycero-3-phosphocholine) is an example of an OxPL that binds to CD36. There appear to be at least 2 different motifs on the OxPL that can confer recognition. First, we demonstrated that the nonmodified phosphocholine (PC) head group of OxPL, but not of native PL, is an epitope of POVPc recognized by CD36 (and SR-B1). This PC-dependent binding to CD36 was observed for both free POVPc, as well as for POVPc covalently linked to lysine residues of proteins (eg, apoB in OxLDL or BSA) via Schiff base formation with the reactive sn-2 aldehyde. Such covalently bound OxPL explain the ability of apoB of OxLDL to bind to CD36, as observed in experiments showing that POVPc linked to BSA also bound to...
CD36 strongly support the interpretation that the PC moiety of OxPL was a sufficient ligand to mediate binding to CD36. This was confirmed by demonstrating specific binding of a labeled POVPc peptide to CD36-transfected COS-7 cells, and that cleavage of the PC moiety abrogated this binding.37 We also showed that cells undergoing apoptosis contain an increased content of such OxPL, and that their binding and uptake by macrophages could be inhibited entirely both by E06, a monoclonal NAb that specifically recognizes the PC moiety of OxPL, as well as by POVPc-BSA.6,7 Thus, the exposed PC head group of OxPL in OxLDL and apoptotic cells is a DAMP recognized by macrophage scavenger receptors.

A second motif on OxPL was demonstrated in an elegant series of studies by Hazen, Podrez, and their colleagues, who demonstrated that various oxidized moieties on the sn-2 side chain of OxPL (in both PC- and phosphoserine-containing PL) can also serve as ligands for CD36.55-57 These have the common motif of oxidized and truncated sn-2 fatty acids that terminate in γ-hydroxy (or oxo)-α,β-unsaturated carbonyl groups. Irrespective of the head group, the presence of these motifs on OxPL are also sufficient ligands to mediate binding of both OxLDL and apoptotic cells to CD36.43-45 However, this motif could not account for the ability of modified apoB of OxLDL to bind to CD36. Indeed, we speculate that the binding of both the PC and the oxidized side chain moieties of OxPL (in both the lipid and apoB moieties) to different sites on CD36 would lead to cooperative high-affinity binding of OxLDL to CD36. These data illustrate what appears to be the common “rule” that such innate PRRs have the capacity to bind to multiple DAMPs. Another example illustrating this rule is the observation that both OxLDL and acetyl LDL bind to SR-A, but at different molecular sites.46 Because these PRRs are germline encoded, and thus limited in number, it is likely that most such conserved receptors have the capacity to bind multiple related epitopes, such as oxidation-specific epitopes, rather than one specific ligand.

Toll-Like Receptors

TLRs have been traditionally considered PRRs that sense the presence of microbial PAMPs, which initiate complex signal transduction pathways and often excessively strong inflammatory responses. TLRs often depend on binding coreceptors to mediate ligand binding and presentation. Thus, CD14 on the cell surface first binds LPS, then mediates LPS binding to TLR4/MD-2, the receptor complex that dimerizes with a different TLR4/MD-2 pair on binding of two LPS molecules. Among these LPS-binding receptors, only TLR4 has a transmembrane domain and is capable of initiating signaling.47,48 CD14 also aids TLR2 signaling, and CD36 has been shown to associate with TLR2 and with TLR4 to provide ligands for their activation.49-51 These examples show a multifaceted, ligand-dependent architecture of PRR complexes that determines the variety of cellular responses.

Work from several laboratories have now developed the idea that TLRs also bind numerous host-derived DAMPs.52 Work from our laboratory has demonstrated that mmLDL (made by exposure of LDL to 15LO-expressing cells) binds to CD14 and activates macrophages via TLR4/MD-2.53 (Importantly, all mmLDL preparations used in these studies were shown to have negligibly low endotoxin levels.) We found that 15LO-CE was responsible for many (but not all) TLR4-dependent macrophage responses to mmLDL. The presence of a hydroperoxide group in 15LO-CE is critical because a hydroperoxide-reducing agent, ebselen, abrogated the TLR4-mediated biological activity of both mmLDL and 15LO-CE.5,26

In addition to oxidized CE, OxPLs have been suggested to activate TLR as well. For example, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) (a collection of PC-containing OxPLs) has been shown to bind membrane CD14 and an yet unidentified GPI-anchored receptor on endothelial cells (ECs), leading to activation of ECs via TLR4.10,54 In contrast, OxPAPC and other OxPLs binding to soluble CD14 and LPS-binding protein (LBP) prevent recognition of LPS by these proteins and impair LPS activation of TLR4.54-56

Because CD36 was shown to bind OxLDL and OxPL,36,37,45 as well as to present diacylglycerides to the TLR2/TLR6 signaling complex,50,57 several laboratories sought experimental evidence for CD36-mediated TLR2 activation by OxLDL. However, recent results suggest that CD36 mediates OxPL activation of the TLR4/TLR6 heterodimer, but not of TLR2. Moreover, the OxLDL-induced TLR4/TLR6 activation occurred not on the cell surface, but in the endosomal compartment.51

Most of the studies to date have used mixtures of OxPL generated by air oxidation of PAPC, and it is likely that future studies with specific OxPLs will help to more clearly identify the exact interactions between the various OxPLs and TLRs and their coreceptors. What these studies reveal is that TLR4, TLR2, and associated binding proteins represent important PRRs for a variety of oxidized lipids, including various OxPLs and OxCEs, which constitute endogenous DAMPs.

Soluble PRRs

Many PRRs expressed on the cell surface also exist in a soluble form, including soluble CD14, MD-2, LOX-1, and CD36. Their functions have been postulated to be two-fold. Similarly to their membrane-bound form, some soluble PRRs can activate their signaling counterparts. However, at higher concentrations and when bound to different ligands, they are inhibitory, as, for example, was suggested for soluble CD14 and OxPL.54

Other circulating PRRs include LBP, various lectins, the complement family of proteins, and the pentraxins. The short pentraxin C-reactive protein (CRP), an acute-phase protein, is a valuable biomarker of inflammation and cardiovascular disease. However, this protein was originally noted for its ability to bind to the PC adduct covalently bound to the cell wall polysaccharide of Streptococcus pneumoniae and for its ability to mediate enhanced clearance of this and other pathogens. We have shown that CRP specifically binds to the PC moiety of OxPLs, whether present on OxLDL or on apoptotic cells, but does not bind to the PC of nonoxidized phospholipids.58 Furthermore, CRP is found in atherosclerotic lesions and is colocalized with PC-containing OxPL, presumably on OxLDL and apoptotic cells.58 We hypothesize that in future studies many other highly conserved soluble
PRRs will be identified with the capacity to bind oxidation-specific epitopes.

Natural Antibodies

The NAb response is an important arc of innate immunity. NAbs can be considered immunoglobulin PRRs, having in common with cellular and soluble PRRs a limited repertoire and yet a wide range of pattern recognition. In mice, B-2 cells of adaptive immunity secrete predominantly IgG antibodies, which have undergone affinity maturation, and represent delayed, but highly specific humoral immune responses. In contrast, a special subset of innate-like B-cells termed B-1 cells, secrete NAbs, which are predominantly IgM and IgA (and some IgG3) and represent a rapid and first-line defense against pathogens. In contrast to B-2 cells of adaptive immunity, which are negatively selected in fetal life producing anergy, B-1 cells are positively selected, leading to the presence of NAbs at birth or shortly thereafter. Indeed, consistent with the ability to secrete NAbs in the complete absence of external antigenic stimulation, a full repertoire of such NAbs can be found in gnotobiotic mice.59 In uninfected mice, most, if not all, IgM in plasma are of B-1 cell origin and are predominantly secreted from the spleen even in the absence of antigen. However, established B-1 cell clones can be expanded later in life by antigen exposure, leading to increased IgM levels in plasma. B-1 cells have restricted use of VH genes that are minimally edited; thus, the IgM they generate are reflective of germline usage, with minimal to no mutations. Because they are conserved by natural selection, the presumption is that, fundamentally, NAbs provide advantageous properties maintaining homeostasis, such as their crucial role in immediate host defenses against PAMPs and a wide range of pattern recognition. In mice, B-2 cells, secrete NAbs, which are predominantly IgM and IgA (and some IgG3) and represent a rapid and first-line defense against pathogens. In contrast to B-2 cells of adaptive immunity, which are negatively selected in fetal life producing anergy, B-1 cells are positively selected, leading to the presence of NAbs at birth or shortly thereafter. Indeed, consistent with the ability to secrete NAbs in the complete absence of external antigenic stimulation, a full repertoire of such NAbs can be found in gnotobiotic mice.59 In uninfected mice, most, if not all, IgM in plasma are of B-1 cell origin and are predominantly secreted from the spleen even in the absence of antigen. However, established B-1 cell clones can be expanded later in life by antigen exposure, leading to increased IgM levels in plasma. B-1 cells have restricted use of VH genes that are minimally edited; thus, the IgM they generate are reflective of germline usage, with minimal to no mutations. Because they are conserved by natural selection, the presumption is that, fundamentally, NAbs provide advantageous properties maintaining homeostasis, such as their crucial role in immediate host defenses against PAMPs and DAMPs60,61; the latter include oxidation-specific epitopes.

A good example is the prototypic E06 NAb that we cloned from a panel of hybridomas from cholesterol-fed apoE-deficient mice, which had high IgM titers to OxPL.60 E06 bound to both the lipid and apoB moiety of OxLDL and to apoptotic cells.62,63 We subsequently showed, by direct sequencing of the VH/VL chains and use of antiidiotypic antibodies, that E06 was 100% homologous to the classic germline-encoded NAb T15, secreted by a well-characterized antibody-deficient mice, which had high IgM titers to OxLDL.62 E06 from a panel of hybridomas from cholesterol-fed apoE-deficient mice, most, if not all, IgM in plasma are of B-1 cell origin and are predominantly secreted from the spleen even in the absence of antigen. However, established B-1 cell clones can be expanded later in life by antigen exposure, leading to increased IgM levels in plasma. B-1 cells have restricted use of VH genes that are minimally edited; thus, the IgM they generate are reflective of germline usage, with minimal to no mutations. Because they are conserved by natural selection, the presumption is that, fundamentally, NAbs provide advantageous properties maintaining homeostasis, such as their crucial role in immediate host defenses against PAMPs and DAMPs60,61; the latter include oxidation-specific epitopes. Among these, we found a high prevalence of IgM to MDA and to the complex structural adducts that occur when MDA is added to proteins. We previously documented a high titer of IgM to MDA-LDL even in wild-type C57BL/6 mice as well as in healthy adult humans.60,61 Remarkably, in recent studies of newborn human umbilical cord blood, we found a similar high titer of IgM to MDA epitopes, and similar to such NAbs in mice, they bind apoptotic cells and atherosclerotic tissues.59 Because IgM do not cross the placenta, such IgM are of fetal origin and considered to represent the human equivalent of innate NAbs. Thus, both in mice and humans, oxidation-specific epitopes are an important target of innate NAbs.

These data demonstrate that oxidation-specific epitopes are an important target of innate immune PRRs. Thus, PRR-Mediated Biological Responses to Oxidation-Specific Epitopes

Cytoskeletal Rearrangements and Cell Migration

The actin cytoskeleton dynamics regulates cell morphology, motility, and endocytosis. During the process of phagocytosis, the macrophage extends actin-driven filopodia to surround bacteria (recognized via PAMPs) or apoptotic cells (recognized via DAMPs). Macropinocytosis is similar to phagocytosis in which extensive membrane ruffling caused by actin cytoskeletal rearrangements results in internalization of large volumes of extracellular liquid. Several studies have suggested that oxidation-specific epitopes regulate cytoskeletal function of macrophages.

In the filopodia of macrophages phagocytosing apoptotic cells, we observed polymerized F-actin colocalized with 12/15LO, and addition of mmLDL to macrophages resulted in robust actin polymerization, membrane ruffling and cell spreading.59,70 As discussed earlier, mmLDL is in fact a carrier of the products of 12/15LO-catalyzed oxidation of lipids, and addition of mmLDL to macrophages resulted in robust actin polymerization, membrane ruffling and cell spreading.59,70 Such spreading would contribute to reduced migratory capacity and in turn to macrophage localization to sites of enhanced content of oxidized CE, such as in atherosclerotic lesions. Although mmLDL-treated macrophages had enhanced uptake of modified LDL (see below), the polydirectional actin...
polimerization, in turn, interfered with the macrophage phagocytosis of apoptotic cells, a mechanism that may contribute to defective clearance of apoptotic cells observed in atherosclerotic lesions.

Active components in mmLDL responsible for macrophage cytoskeletal rearrangements were found to be polyoxygenated CE hydroperoxides, such as 15LO-CE discussed earlier, which activate a TLR4-dependent signaling cascade. This mmLDL/OxCE-mediated TLR4-dependent signaling was MyD88-independent, and mediated by spleen tyrosine kinase (Syk) recruitment to a TLR4 signaling complex. This led to TLR4 and Syk phosphorylation, downstream activation of small GTPases Rac, Cdc42, and Rho GTPases, and the formation of a N-WASP/Arp2 complex, leading to dramatic, actin-dependent membrane ruffling (Figure 1). In addition, mmLDL induced TLR4-independent activation of PI3K, which also contributes to cytoskeletal rearrangements.

Steinberg and colleagues originally showed that components of OxLDL were not only chemotactic for monocytes but also led to inhibition of migration of macrophages, which would both enhance the local accumulation of macrophages within sites of inflammation and oxidative events. In addition to the OxCE noted above, different oxidation-specific epitopes, formed by LDL oxidation with myeloperoxidase/hydrogen peroxide/nitrite, specifically bind to CD36 and also induce macrophage spreading and reduce macrophage migration. The CD36-mediated actin polymerization and cell spreading involves Src-dependent activation (phosphorylation) of focal adhesion kinase (FAK) and NADPH oxidase; the latter produces ROS, which inactivate SHP-2 protein tyrosine phosphatase, contributing to reduced dephosphorylation of FAK.

Lipoprotein Uptake and Foam Cell Formation

Lipoprotein uptake by macrophages within the intima and formation of foam cells is a major atherogenic process connecting lipid deposition in the vessel wall with vascular inflammation. Unregulated uptake by macrophages of Ox-LDL and other modified LDLS bound to the intimal matrix, combined with downregulated efflux of lipids, stimulates expression of proinflammatory cytokines, antigen presentation, secretion of matrix-degrading enzymes, and often results in cell death, thereby promoting further lesion development and its eventual rupture. The mechanisms of uptake of modified LDL by macrophages are diverse and, in part, reflect the multitude of oxidative modifications of LDL, as well as other modifications. Depending on the prevalence of different oxidation-specific epitopes, macrophages could use different combinations of binding receptors and different uptake mechanisms to remove these DAMPs from the tissue, but at the expense of generating foam cells.

In vitro experiments in which macrophages were exposed to OxLDL and acetylated LDL only, showed that CD36 and SR-A were responsible for 75% to 90% of uptake of these lipoproteins. It has been demonstrated that the CD36-mediated OxLDL uptake depends on the activation of Src and c-Jun N-terminal kinase (JNK) kinases (Figure 1). Similarly to mouse macrophages, a comparison of monocyte/macrophage from patients with a total deficiency of CD36 with normal monocyte/macrophages suggests that ≈50% of the in vitro uptake of OxLDL is attributable to this receptor, under the conditions studied. However, epidemiological data of CD36-deficient subjects with respect to cardiovascular disease are not yet available, and murine model studies produced mixed results. Using hypercholesterolemic CD36−/− and SR-A−/− mice, several groups suggested that SR-A and CD36 play quantitatively important roles in mediating uptake of OxLDL and promoting the development of atherosclerosis in apoE−/− mice. In contrast, a different group demonstrated that both SR-A−/−apoE−/− and CD36−/−/apoE−/− double knockout mice, although having significant reductions in peritoneal macrophage lipid accumulation in vivo, had increased atherosclerosis or no change in the lesion size. A follow-up study by this group confirmed that even the combined CD36/SR-A deficiency in apoE−/− mice had no effect on foam cells in atherosclerotic lesions or the lesion size, but revealed reduced complexity and size of necrotic

Figure 1. Oxidized lipid moieties induce lipoprotein accumulation in macrophages. Macrophage lipoprotein uptake mechanisms can be separated into: (1) macropinocytosis, when actin polymerization and extensive membrane ruffling result in the ruffles closing into large endosomes and capture of large volumes of extracellular material, including all classes of native and oxidized LDL present in the vicinity of the cell; and (2) micropinocytosis, when ligand–receptor binding leads to membrane invagination and nearly stoichiometric internalization of the lipid or the lipoprotein carrying this ligand. mmLDL and polyoxygenated OxCEs induce Syk recruitment to TLR4, Syk, and TLR4 phosphorylation and subsequent ERK1/2-dependent activation of small GTPases Rac, Cdc42, and Rho and phosphorylation of paxillin, leading to actin reorganization and membrane ruffling. Resulting macropinocytosis promotes foam cell formation. Binding of OxLDL or OxPL to CD36 initiates Lyn-dependent phosphorylation of JNK, which is essential for CD36-mediated OxLDL uptake, although the control of the degradation of these proteins is not understood.

Lipoprotein uptake mechanisms to remove these DAMPs from the tissue...
areas in the lesions, suggesting a role for CD36 and SR-A in cell death.84 The discrepancy between the results obtained in different laboratories underscores the complexity of the mechanisms of macrophage lipid accumulation and atherogenesis.32,76

As noted earlier, mmLDL does not bind to CD36; it binds to CD14 and induces TLR4/MD-2-dependent cytoskeletal rearrangements. Remarkably, mmLDL-induced formation of membrane ruffles results in the ruffles closing into large endosomal vesicles (macropinosomes). This mmLDL (and 15LO-CE)-induced process of macropinocytosis captures lipoproteins from the cellular microenvironment and results in macrophage uptake of mmLDL itself, as well as other lipoproteins in the vicinity, such as native LDL and even OxLDL (Figure 1). Knockdown of TLR4 or Syk abolishes macrophage lipoprotein accumulation stimulated by either mmLDL or 15LO-CE.9 Mouse experimental studies support a role for TLR4 in the development of diet-induced atherosclerosis. TLR4 is expressed on endothelial cells and macrophages within human and mouse atherosclerotic lesions.83,86 A deficiency in TLR4 attenuates the development of atherosclerosis in hyperlipidemic apoE−/− mice.87 Overexpression of both TLR2 and TLR4 in the intima of carotid arteries of hyperlipidemic rabbits significantly augmented atherosclerosis, although transfection of only TLR4 or only TLR2 resulted in little change in atherosclerosis.88 In human epidemiological studies, the common Asp299Gly TLR4 polymorphism (loss-of-function) was associated with a decreased risk of carotid artery and femoral artery atherosclerosis and cardiovascular cause of death90; however, subsequent studies from different laboratories that evaluated different clinical manifestations of atherosclerosis reported inconsistent results.90–95

Unlike the defined models of OxLDL or mmLDL that we use in the laboratory, OxLDL in atherosclerotic lesions is likely more complex, containing not only oxidation-specific epitopes found in both OxLDL and mmLDL but yet unidentified epitopes as well. How can one measure in vivo the quantitative importance of each individual PRR-mediated uptake mechanism? We have recently suggested to tackle this problem by comparing the kinetics of lipid accumulation in vivo by wild-type macrophages versus that by various PRR-deficient macrophages, eg, TLR4-deficient macrophages.96 For this purpose, we used optically transparent zebrafish larvae as a model for monitoring vascular lipid accumulation.30,97 An advantage of the optically transparent zebrafish model is that unlike cell culture models and wild-type mouse macrophages were injected into zebrafish larvae in which a high-cholesterol diet has induced the formation of fatty streaks. Because zebrafish larvae are transparent and because we used fluorescently labeled cells and fluorescent dietary lipids, we were able to quantify macrophage lipid uptake in vivo, directly in the environment of a fatty streak. Using this model, we found that the rate of in vivo lipid uptake by TLR4-deficient macrophages was significantly lower compared to the uptake by wild-type macrophages, supporting results of our in vitro experiments.98–99 Similar measurements with macrophages lacking other receptors and signaling molecules could be used in the future to measure a set of rate constants to compare different mechanisms of foam cell formation and specifically, the quantitative role of different PRRs.

Proinflammatory Gene Expression

The first report of proinflammatory properties of OxPL was that they induced monocyte binding to vascular ECs,98,99 which is a critical early step in the formation of vascular lesions. OxPAPC has been widely used as a collection of biologically active OxPL. ECs stimulated with OxPAPC secrete chemoattractants MCP-1 and interleukin (IL)-8 and express adhesion molecules connecting segment 1 (CS1) fibronectin and P-selectin on cell surface, which lead to monocyte recruitment and binding to the ECs.98 The expression of CS-1 fibronectin is mediated by cAMP-induced R-Ras and PI3K activity.100 PEIPC (1-palmitoyl-2-(5,6)-epoxyisoprostane E2-α)-glycerol-3-phosphocholine) was identified as a highly active component of OxPAPC, and exerted its activity via binding to prostanoglandin E2 receptor.101 In addition to MCP-1 and IL-8, OxPAPC induced expression of IL-6, CCL3, CCL4, and vascular endothelial growth factor (VEGF).102,103 Although TLR4 seems to be involved in OxPAPC-induced IL-8 secretion by ECs,10 the nuclear factor (NF)-κB pathway was not activated, and the EC response to OxPAPC was mediated by the c-Src/JAK2/STAT3, SREBP and unfolded protein response pathways.103–106 VEGF receptor (VEGFR)2 mediates OxPAPC-induced recruitment of Rac1 to the NADPH oxidase-4 complex, resulting in strong ROS generation by ECs. The ROS generation was, in turn, responsible for expression of inflammatory and sterol metabolism genes, but not the genes of unfolded protein response and antioxidative responses (Figure 2).107 It should be noted that the PC-containing OxPL present on apoptotic cells and their apoptotic bodies can also mediate such biological effects. Thus apoptotic cells and blebs can induce IL-8 release from endothelial cells, effects blocked by antibody E06,7,108 mmLDL signals via TLR4/MD-2, but unlike LPS, the bacterial ligand for TLR4, mmLDL induces only modest levels of expression of proinflammatory cytokines.72 This is likely attributable to TLR4 clustering with different cellular receptors,51,109 as well as to the different set of adaptors recruited to the cytoplasmic domain of TLR4 in response to mmLDL as compared to LPS. mmLDL induces some MyD88-dependent effects, but a major signaling pathway for mmLDL seems to be the recruitment of Syk to TLR4, and Syk-dependent activation of ERK1/2 and phospholipase Cγ.9,110 The latter activates protein kinase C and NADPH...
oxidase-2, resulting in generation of ROS. ROS has been suggested to play a major role in regulation of many intracellular signaling pathways, including NF-κB. We have demonstrated Nox-2 dependent expression of RANTES, IL-1β, and IL-6 in macrophages stimulated with mmLDL. In turn, RANTES induced migration of vascular smooth muscle cells (Figure 2).110

Because of the signaling differences in the LPS- and mmLDL-induced activation of TLR4, we hypothesized that costimulation of macrophages by mmLDL and by low levels of bacterial LPS would result in cooperative effects, leading to greater activation than achieved by either stimulus alone. Indeed, we demonstrated that mmLDL and low levels of LPS cooperatively upregulated expression of a number of proinflammatory genes, including chemokines Cxcl2, Ccl3 and Ccl4, both in vitro and in vivo.111 A de novo motif analysis of promoters of cooperatively activated genes suggested involvement of activator protein (AP)-1 transcription factors, a finding that agrees with an ERK1/2-dependent character of mmLDL activation. In addition, mmLDL induced phosphorylation of c-Jun and the release of NCoR (nuclear receptor corepressor) from the promoter regions of Cxcl2 and Ccl3 (Figure 3).111 These molecular mechanisms of cooperative macrophage activation with mmLDL and low levels of LPS may be relevant to the increased risk of acute cardiovascular events in patients with atherosclerosis complicated by chronic infections, obesity and type 2 diabetes and other conditions associated with subclinical endotoxemia.

Although initial evidence placed pattern recognition on the cell surface, new data suggest that signaling by intracellular PRRs is as important as the ones originating from the plasma membrane. We will review two examples, both published in 2010. One demonstrates that recognition of OxLDL (and amyloid-β peptide) by CD36 triggers assembly of a heterotrimeric complex composed of CD36, TLR4 and TLR6.54 The signaling likely originates on the cell surface, where OxLDL induces CD36-mediated activation of a Src kinase Lyn, which, in turn, initiates TLR4/TLR6 dimerization. The assembly of the CD36/TLR4/TLR6 complex is completed in the endosomal compartment, where both the MyD88 and

OxPAPC activation of VEGFR2.107 ROS in endothelial cells mediate secretion of MCP-1 and IL-8 and thereby promote monocyte migration.

Figure 2. Oxidized LDL and OxPL induce ROS generation in vascular cells. In macrophages, OxLDL binding to CD36 induces recruitment of Lyn, a Src kinase, its activation, and phosphorylation of Vav, which in turn activates Rac.24 Unlike OxLDL, mmLDL induces recruitment of Syk to TLR4 and Syk-dependent activation of Vav and Rac.9 In addition, mmLDL induces TLR4- and Syk-dependent activation of phospholipase C (PLC-γ) and protein kinase C (PKC), which induces recruitment of p47phox, and p67phox (not shown) to the NADPH oxidase-2 (Nox2) enzyme complex, and activated (GTP-bound) Rac completes the complex, leading to ROS production.9,110 High levels of extracellular ROS may exacerbate the oxidative damage, whereas intracellular ROS are important signaling molecules, mediating cytokine secretion. One of mmLDL-induced, Nox2-dependent chemokines, RANTES, induces vascular smooth muscle cell migration.110 Nox4 is the predominant NADPH oxidase in endothelial cells. It is activated by OxPAPC via VEGFR2-dependent Rac recruitment.107 However, the mechanism of the OxPAPC activation is unclear. Although OxPAPC is capable of inducing VEGF production by endothelial cells,103 anti-VEGF antibodies do not block high levels of extracellular ROS. ROS in endothelial cells mediate secretion of MCP-1 and IL-8 and thereby promote monocyte migration.
TRIF adaptors propagated the signal, leading to activation of NF-κB, generation of ROS, and expression of Cxcl1, Cxcl2, Cc19, Ccl5, and IL-1β.114

The second example involves not an oxidized lipid, but cholesterol crystals. However, lipid peroxidation is known to promote formation of crystalline structures of free cholesterol.112 Recent studies using laser reflection and fluorescent microscopy suggested that cholesterol crystals emerge at the earliest time points of diet-induced atherogenesis, together with the appearance of immune cells in the subendothelial space.113 Cholesterol crystals localized both extracellularly and inside macrophages in atherosclerotic lesions. Microcrystals in general and cholesterol crystals in particular have been shown to activate the intracellular PRR, NLRP3 (cryopyrin), the critical component in the caspase-1–mediated production of active IL-1.114 Indeed, cholesterol crystals induced IL-1β secretion by wild-type but not NLRP3−/− macrophages, and transplantation of NLR3−/− or IL-1α/β−/− bone marrow into LDLR−/− mice significantly reduced diet-induced atherosclerosis.115

Expansion of Oxidation-Specific NAbs and Atherosclerosis

Activation of cellular PRR by oxidation-specific epitopes results in many proinflammatory and proatherogenic effects, as summarized above. In contrast, NAbs seem to protect against atherosclerosis. In vitro, the E06 NAb, which binds the same PC-containing OxPL as does CD36, prevented OxLDL and apoptotic cell uptake by macrophages.6,63 Because immunization of mice with heat-inactivated PC-containing S. pneumoniae was known to robustly expand T15/E06 B-1 cell clones, we immunized cholesterol-fed LDLR−/− mice with heat killed pneumococci to see whether this would reduce atherosclerosis. Indeed, immunization induced high titers of E06/T15 IgM and significantly reduced atherosclerosis.116 Thetheroprotective property of anti-PC IgM was corroborated in a vein graft atherosclerosis model in which T15 IgM was infused intravenously,116 and by the demonstration that immunizing apoE−/− mice with PC-KLH was atheroprotective.117 Furthermore, we have shown that E06 can bind to and block proinflammatory effects of PC-containing OxPL,7,11,108 and this property undoubtedly also contributes importantly to the anti-inflammatory and antiatherogenic properties of these oxidation-specific IgM. The overall importance of IgM to atherosclerosis was demonstrated by the observation that when secretory IgM knockout mice were crossed with LDLR−/− mice, atherosclerosis was dramatically enhanced.118 Because we have shown that oxidation-specific epitopes are a major target of NAbs in mice (and humans), it is very likely that IgM to such epitopes play a major role in this protective effect. We predict that similar to E06, NAbs to other oxidation-specific epitopes, such as MDA, 4-hydroxy-2-nonenal, and oxidized cardioli pin, will be antiinflammatory and atheroprotective as well.

Because NAbs have been conserved by natural selection, it is likely that on balance they must have been beneficial to the host, aside from their role in atherogenesis. For example, we and others have shown that by binding to oxidation-specific epitopes on the surface of apoptotic cells, they facilitate complement-dependent enhanced clearance in vivo.59,119,120 Because we have shown in principle that increasing the titer of such antibodies can be antiatherogenic, identifying the epitopes to which such NAbs bind should identify antigens that could be used to develop an atheroprotective vaccine. In addition, enhancing such NAb titers, possibly even passively, could be used to limit the proinflammatory effects of oxidized lipids in acute situations, such as in acute coronary syndromes,121 or in viral induced respiratory distress syndromes, a situation in which the oxidized lipids to which they bind are proinflammatory.11,122 Antibodies to these epitopes could be used to image not only atherosclerotic lesions123 but a variety of inflammatory settings in which such epitopes are ubiquitously expressed, as well as to target therapeutic molecules to active sites of inflammation. It can be envisioned that a better understanding of this important compartment of innate immunity will ultimately identify novel therapeutic targets that can be exploited to interfere with atherogenesis and inflammatory states in general.

Conclusions

In this article, we have summarized evidence that PRR recognition of oxidation-specific epitopes initiates many important innate immune processes, having both proatherogenic and antiatherogenic consequences. To put these findings into perspective, because PRRs are germline-encoded, the observation that oxidation-specific epitopes are targeted by multiple innate PRRs strongly implies that these epitopes represent important endogenous “danger signals” against which multiple defenses are selected to provide homeostasis. Moreover, endogenous oxidation-specific epitopes likely exert positive selection pressure for NAb-producing B-1 cells, which are selected in fetal life, or shortly thereafter. NAbs are present even in germ-free mice, never exposed to microbial PAMPs. The observation that similar epitopes on infectious pathogens are also recognized by NAbs suggests that such pathogens are likely to have also exerted selection pressure later in life. A similar argument may be offered that oxidation-specific epitopes are among the primary selecting antigens for other innate immune receptors as well.

We further suggest that the fundamentally important altered-self antigens leading to selection of oxidation-specific innate PRRs are apoptotic cells and the apoptotic blebs and cellular debris resulting from such programmed cell death (Figure 4). Cells undergoing apoptosis develop mitochondrial disruption, leading to generalized enhancement of oxidative events. In turn, a wide variety of lipids are oxidized, including cardiolipin, phosphatidylserine, and phosphatidylcholine.124 (Future studies will determine whether OxCEs are also found on dying cells.) Such epitopes are greatly enriched in the apoptotic blebs that bud off from such cells6,108 and are present in the circulation as microparticles. Scavenger receptors participate in apoptotic cell recognition and clearance by phagocytes. We have shown that syngeneic apoptotic cells are highly immunogenic and proinflammatory if not promptly cleared,7 and because apoptosis is a universal biological event from the earliest stages of development, we suggest that this provides a strong evolutionary pressure for innate mechanisms to efficiently clear such dying cells. Indeed, the fact that there are apparently multiple and redundant mechanisms to effect clearance of apoptotic cells indicates the biological
importance of this process. Remarkably, nearly all of the different oxidation-specific monoclonal antibodies we have cloned to date, both from murine or human libraries, bind to apoptotic cells and apoptotic bodies, presumably to effect their removal, as noted above. Furthermore, these same oxidation-specific epitopes are ligands mediating apoptotic cell recognition by various scavenger receptors. Failure to adequately clear the daily burden of apoptotic cells would likely lead to inflammation, immune activation, and pathology, such as, for example, promotion of atherogenesis.

In contrast to atheroprotective oxidation-specific NAbs, cellular PRR recognition of oxidation-specific epitopes have predominantly proatherogenic effects, at least under conditions of marked hypercholesterolemia generated in the Western diet fed murine models studied. However, such marked hypercholesterolemia is not likely to have been influential in evolutionary selection. Rather, PRRs likely evolved as protective mechanisms against the proinflammatory effects of oxidation-specific epitopes, such as those found in apoptotic cells, or even the oxidized lipids found extracellularly, such as on apoptotic blebs or lipoproteins. Because many of the same oxidation-specific PRR also recognize similar molecular patterns on pathogens, we speculate that the ability to protect against infectious pathogens provided a strong secondary selecting pressure as well. It is also plausible that these cellular PRRs developed accompanying stronger cellular responses to such infectious pathogens than those resulting from host-derived activation. This implies that the intracellular signaling pathways linking a PRR response to an endogenous DAMP may be different from those involved in the same PRRs response to an infectious PAMP. Indeed, examples of differential responses of TLR4 to mmLDL (and its OxCE) and to LPS are discussed above. Thus, to provide a strong host response to an acute invading pathogen, TLRs initiate an “overreactive” cellular response. In contrast, the cellular response mediated by the same PRR to an endogenous DAMP, such as OxCE, is more muted, because the OxCE is ubiquitously present and the cellular response is aimed at maintaining homeostasis. Nevertheless, chronic exposure to increased levels of DAMPs, such as oxidation-specific epitopes induced by hypercholesterolemia, induces low-grade but sustained activation of PRRs and, thus, contributes to the inflammatory state characteristic of atherogenesis.

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Miller et al. Oxidation-Specific DAMPs of Innate Immunity


Oxidation-Specific Epitopes Are Danger-Associated Molecular Patterns Recognized by Pattern Recognition Receptors of Innate Immunity

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