Innate sensing of oxidation-specific epitopes in health and disease

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Abstract | Ageing, infections and inflammation result in oxidative stress that can irreversibly damage cellular structures. The oxidative damage of lipids in membranes or lipoproteins is one of these deleterious consequences that not only alters lipid function but also leads to the formation of neo-self epitopes — oxidation-specific epitopes (OSEs) — which are present on dying cells and damaged proteins. OSEs represent endogenous damage-associated molecular patterns that are recognized by pattern recognition receptors and the proteins of the innate immune system, and thereby enable the host to sense and remove dangerous biological waste and to maintain homeostasis. If this system is dysfunctional or overwhelmed, the accumulation of OSEs can trigger chronic inflammation and the development of diseases, such as atherosclerosis and age-related macular degeneration. Understanding the molecular components and mechanisms that are involved in this process will help to identify individuals with an increased risk of developing chronic inflammation, and will also help to indicate novel modes of therapeutic intervention.

In the course of evolution, most organisms have developed mechanisms to utilize oxygen for various biological processes, such as the synthesis of biomolecules, energy production and phagocytosis. Cellular usage of oxygen leads to the constant generation of reactive oxygen species (ROS), which are mainly generated by the mitochondria. In addition, ROS are generated in peroxisomes, the endoplasmic reticulum and the plasma membrane. Within eukaryotic organisms, superoxide, peroxide and hydroxyl radicals represent the main types of ROS that have high reactivity and low stability and that actively drive many cellular reactions. The biological effects of ROS greatly depend on the amounts of ROS present, which supports the idea that cellular ROS generation has characteristics of hormesis\textsuperscript{1}. Indeed, at low concentrations, ROS participate in physiological processes, such as cell renewal, cellular metabolism, proliferation and differentiation\textsuperscript{1-4}. However, high ROS production during an oxidative burst is crucial for host defence to ensure microbial killing\textsuperscript{5} and the formation of neutrophil extracellular traps (NETs)\textsuperscript{6}. These cellular responses and the modulation of immune responses by ROS have been recently reviewed\textsuperscript{3,4,7}. However, both exogenous triggers, such as ultraviolet (UV) irradiation, chemical toxins and hyperthermia, and endogenous conditions, including senescence, metabolic changes and inflammation, can impair the balance between the generation and the elimination of intracellular ROS, which can lead to ROS accumulation and oxidative stress. The harmful effects that are caused by oxidative stress are typically limited by antioxidant responses, which include the induction of enzymes and proteins that eliminate ROS. However, if unresolved, oxidative stress generates cellular and extracellular damage by irreversibly modifying DNA, RNA, proteins and lipids, which ultimately leads to molecular modifications that disrupt normal functions and often results in cell death. Oxidative modifications of biomolecules also lead to the generation of secondary free radicals, which further propagate oxidative damage\textsuperscript{3,4,7}.

Lipids, particularly phospholipids, are prime targets for oxidation owing to their abundance as important building blocks of cells, extracellular vesicles and lipoproteins. The oxidation of lipids not only alters their biological function but also results in the generation of various degradation products through a process that is known as lipid peroxidation\textsuperscript{8} (BOX 1). The newly generated products propagate the effect of oxidative stress by directly modulating cellular functions and responses such as metabolism and inflammation\textsuperscript{8,9}. Furthermore, as the degradation products of oxidized lipids are often highly reactive, they modify self-molecules and thereby generate structural neo-epitopes that are recognized by receptors of the immune system. These neo-epitopes have been termed oxidation-specific epitopes (OSEs) and represent a common set of epitopes present on various oxidatively modified self-proteins and lipids\textsuperscript{14,19}. OSEs, including oxidized phospholipids (OxPLs) and malondialdehyde...
Box 1 | Lipid peroxidation

Lipid peroxidation is the oxidative damage of lipids that is initiated by the removal of a hydrogen atom from the C=H group within the double bonds of polyunsaturated fatty acids (PUFAs). The subsequent addition of oxygen radicals and the formation of lipid-peroxy radicals, which are transformed into lipid-hydroperoxide molecules can further propagate this reaction. Both enzymatic and non-enzymatic mechanisms can lead to lipid peroxidation. The enzymatic mechanisms involve lipoxygenases, cyclooxygenases and cytochrome P450, and result in the generation of products with high stereo-specificity. Depending on the PUFAs substrate, major products of lipoxygenase oxidation are hydroxyperoxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins, hydroxyoctadecanoid acids (HODEs), hepatoxins and resolvins. Oxygenation by cyclooxygenases results in the generation of prostaglandins, prostacyclin and thromboxanes, whereas cytochrome P450 generates epoxyspecies and eicosanoids (EOEs), 20-HETE, thromboxanes and prostacyclins. Non-enzymatic mechanisms are mediated by free radicals that are generated by NADPH oxidases and nitric oxide synthases in the presence of transition metal ions (Fe²⁺ and Cu²⁺) and result in a mixture of nonspecific stereoisomers.

Moreover, the enzyme myeloperoxidase can also initiate non-enzymatic lipid peroxidation through the generation of reactive oxygen species (ROS), such as HOCl, H₂O₂ and NO. In contrast to products of enzymatic lipid oxidation, the products of non-enzymatic processes are considered to be more toxic and damaging to the host. Continuous degradation of lipid-hydroperoxides by cyclization and fragmentation in the presence of reducing metal ions (Fe²⁺ and Cu²⁺) results in the generation of highly reactive terminal degradation products such as reactive aldehydes 4-hydroxynonenal (4-HNE), malondialdehyde (MDA) and 2-(ω-carboxyethyl)pyrrole (CEP). Peroxidation products that are generated by both mechanisms, modulate a multitude of physiological processes such as cell signalling, wound healing, immune tolerance, skin barrier function, coagulation, vasodilation, modulation and the resolution of inflammation. They are rapidly removed in healthy tissues but accumulate in many pathological conditions in which they can cause adverse effects.

(MDA)-modified amino groups, have been documented on the surface of apoptotic cells, microvesicles and damaged structures such as oxidized low-density lipoproteins (OxLDLs) and oxidized phosphatidylcholine (OxPCs). Oxidized phosphatidylcholine (OxPCs) and oxidized phosphatidylethanolamine (OxPEs) have been studied as prototypical markers of oxidative stress and can be measured by the frequently used 2-thiobarbituric acid reaction (TBAR) method. As a vast diversity of OxPLs can be generated, many more OSEs derived from these may exist. Moreover, the oxidation of other lipids, such as cholesterol and cholesterol esters, can lead to structural changes and altered biological activities, but their ability to be recognized by innate immune receptors is still being characterized. It will be important to elucidate the exact structures of the relevant OSEs to better understand their recognition by the immune system. Owing to their biological activities, these products of lipid peroxidation have been implicated in a wide variety of physiological and pathological processes, and immune responses that target these products can modulate many of these effects.

Oxidation-specific epitopes (OSEs): Novel epitopes that form as a result of lipid peroxidation in response to various pathological and pathological situations. These epitopes are enriched on oxidized low-density lipoprotein and on membranes of microvesicles and apoptotic cells. They are recognized by humoral and cellular immune responses.

Microvesicles
Small (0.1–1 μm) membrane vesicles shed from activated or dying cells. They expose phosphatidylserine and carry surface markers of their parental cells. They mediate the transfer of proteins, nucleic acids and lipids between cells. Elevated levels of circulating microvesicles have been reported in acute and chronic inflammatory diseases.

Immune recognition of OSEs
OSEs have important roles in physiological processes, as they mark oxidatively modified endogenous molecules, such as proteins and/or lipoproteins and apoptotic cells, as being damaged by increased oxidative stress. This facilitates their removal by the housekeeping functions of the immune system to maintain homeostasis. In pathological situations — in which oxidative events are greatly increased and the homeostatic functions of innate immunity are overwhelmed and/or impaired — the accumulation of OSEs leads to sterile inflammation, which is a mechanism that should ultimately restore normal tissue integrity. If this fails as a result of persistent tissue damage and/or impaired resolution, the
Box 2 | Sterile inflammation

Sterile inflammation is an inflammatory response that is elicited in response to damage-associated molecular patterns (DAMPs), which are released locally in response to tissue damage21. DAMPs are intracellular and extracellular host-derived molecules that are not usually sensed by the immune system but that are released or become modified into altered self-molecules upon tissue damage21. In analogy to pathogen-induced inflammation, sterile inflammation is triggered by the activation of the innate immune response through the recognition of DAMPs by pattern recognition receptors (PRRs), resulting in the enhanced secretion of cytokines and chemokines. Membrane-bound PRRs, such as Toll-like receptors (TLRs), and intracellular PRRs, such as the inflammasome, are key mediators of sterile inflammation. Cytokines belonging to the interleukin-1 (IL-1) family have been proposed to be important drivers of sterile inflammation34. Increased cytokine and chemokine secretion at the site of initial damage ultimately results in an enhanced recruitment of immune cells, such as neutrophils and macrophages. The resolution of sterile inflammation should lead to tissue repair and the re-establishment of homeostasis. Unresolved sterile inflammation is implicated in the development of several medical conditions, such as gout, Alzheimer disease, rheumatoid arthritis and atherosclerosis.

Oxidized low-density lipoproteins (OxLDLs). Low-density lipoprotein (LDL) particles (20–25 nm) are the major transporters of cholesterol in the circulation. LDL particles consist of 45% cholesterol, 20% phospholipids, 10% triglycerides and 25% protein. LDL can be oxidatively modified by enzymatic and non-enzymatic mechanisms that result in many different changes to the lipid and protein moiety of LDL. Unlike LDL, OxLDL is immunogenic and pro-inflammatory, and can be recognized by macrophage scavenger receptors.

Damage-associated molecular patterns (DAMPs). Intracellular and extracellular components that are typically hidden from recognition by the immune system until cellular damage occurs. Once released into the extracellular environment they trigger sterile inflammation. Prototypical examples include high mobility group box 1 protein (HMGB1), heat shock proteins (HSPs), uric acid, mitochondrial proteins, DNA, RNA and cholesterol crystals.

2-thiobarbituric acid reaction (TBAR). The TBAR method is used to measure lipid peroxidation products, such as malondialdehyde (MDA) or other reactive aldehydes, as these products generate thiobarbituric acid-reactive substances that can be detected by colorimetric or fluorometric measurements. Accumulation of OSEs can trigger chronic inflammation. Therefore, OSEs have important roles in both the regulation of tissue homeostasis and inflammatory responses (FIG. 2). The detailed characterization of OSEs and the immune responses against these OSEs have shown that they are recognized by both cellular and soluble pattern recognition receptors (PRRs), including natural IgM antibodies44,51 (TABLE 1).

OSE sensing by cellular PRRs. OSEs are recognized by a wide variety of cell surface receptors (TABLE 1), most of which are expressed by macrophages. Macrophages mediate the uptake and phagocytosis of oxidatively altered molecules and cells, and macrophages also sense the accumulation of OSEs in the local microenvironment, which triggers signalling pathways that induce the secretion of inflammatory chemokines and cytokines44,51,53. Scavenger receptors represent the prototypical class of innate receptors for OSEs57. They are multi-ligand receptors that bind both modified self-structures and non-self-molecules. Different types of scavenger receptors have been identified, including CD36, SRA1, SRA2, SRB1, CD68 and lectin-like oxidized LDL receptor 1 (LOX1; also known as OLR1)57. Among these, SRA1, SRA2 and CD36 are considered to be responsible for most of the OxLDL uptake by macrophages in in vitro assays, because macrophages that are deficient in CD36, SRA1 and SRA2 show 75–90% decreased binding and degradation of OxLDL58. Detailed structural studies have shown that OxPL is a high-affinity ligand that mediates OxLDL binding to CD36 (REF 39), and, specifically, that this is mediated by the phosphocholine (PC) headgroup of OxPL (PC-OxPL) but the PC of unoxidized phospholipids does not serve as a ligand49. Binding can also be mediated by the truncated sn-2 acyl chain of OxPLs51,52, which indicates that other classes of oxidized phospholipids such as OxPS can bind to CD36 (REFS 4, 3, 44). Similarly, PC-OxPL also mediates the uptake of OxLDL by SRB1 on macrophages41. Examples of other OSEs that are recognized by scavenger receptors include CEP-modified proteins, which are recognized by CD36 (REF 46) and MDA-modified proteins, which are taken up by SRA1 and SRA25,7. A recent study showed that the endothelial scavenger receptor LOX1 mediates MDA-induced nitric oxide (NO) production49 and this receptor also binds 4-HNE50.

Toll-like receptors (TLRs) also recognize and respond to OSEs. Macrophages stimulated with oxidized 1-palmitoyl-2-archidionyl-sn-glycero-3-phosphocholine (OxPAPC) secrete interleukin-6 (IL-6), whereas IL-6 production is absent in macrophages that lack TLR4 or TIR-domain-containing adaptor protein inducing interferon-β (TRIF; also known as TICAM1)52. Sensing of PC-OxPL seems to be important for the TLR4-mediated effects, as the PC-specific IgM T15/E06 inhibited IL-6 secretion induced by OxPAPC but did not inhibit IL-6 secretion induced by lipopolysaccharide (LPS)51. OxPLs have also been reported to stimulate macrophages via a TLR2 pathway51. Moreover, chemokine secretion by macrophages stimulated with OxLDL has been shown to require the cooperation of CD36 with a TLR4–TLR6 heterodimer51. Conversely, OxPLs induce apoptosis in macrophages via TLR2–TLR6 in cooperation with a CD36 ligand48. As most of these studies have used mixtures of different OxPL products, individual OxPL moieties might use TLR receptors in varying combinations to generate different signalling pathways. The common role of TLRs as sensors of OSEs is further supported by the findings that CEP adducts are bound by TLR2, but not by TLR4, on both endothelial cells55 and macrophages48. Furthermore, CEP selectively augments TLR2–TLR1 signalling in macrophages48 and has been shown to activate platelets via TLR2–TLR1 (REF 56). TLRs have not yet been directly implicated in chemokine secretion that is induced by MDA or 4-HNE, but this process is probably also mediated by the cooperation of scavenger receptors with other signalling PRRs57. Oxidized cholesteryl esters (OxCEs) trigger pro-inflammatory macrophage responses via TLR4 and these involve non-classical signalling via spleen tyrosine kinase (SYK)53,54. Thus, some OSEs represent endogenous TLR ligands that have the capacity to trigger inflammatory responses. Notably, in endotoxin-induced inflammation, soluble OxPLs impair complex formation of TLR4 with CD14, MD2 and LPS-binding protein and thereby dampen LPS-induced responses23,59. Recently, epoxycyclopentenones that are present in OxPAPC were identified as active components responsible for the inhibitory effects of OxPAPC on the activation of specific TLRs by synthetic ligands50. An interesting property of the inflammatory responses to OxPL is the tight cooperation with scavenger receptors, such as CD36, which mediate recognition of OxPLs and probably also facilitate sensing and signalling by TLRs. Although OxLDL uptake via CD36 seems to be necessary for some macrophage signalling pathways, it is unknown to what extent scavenger receptor-mediated uptake of other OSEs is required for their cellular responses.

OSE sensing by soluble PRRs. Soluble PRRs include secreted versions of cellular PRR, pentraxins and proteins of the complement system41. Whereas some of
these proteins interact with cellular PRRs to mediate the signals of bond ligands, others participate in the housekeeping functions of the host. Soluble PRRs distinguish between healthy and damaged tissues by sensing metabolic byproducts and OSEs presented on dying cells (TABLE 1).

C-reactive protein (CRP) was originally identified as the C-reactive component of plasma that could bind the PC present on the capsular polysaccharide of Streptococcus pneumoniae.

Figure 1 | The generation of OSEs. Tissue damage, cellular stress and cell death result in increased oxidative stress, which promotes lipid peroxidation. Lipid peroxidation can occur through non-enzymatic mechanisms, such as reactive oxygen species (ROS), and through enzymatic mechanisms, including myeloperoxidases, 12/15-lipoxygenases and cytochrome P450. The oxidation of sn-2 polysaturated fatty acids (PUFAs) of membrane phospholipids leads to fragmentation and the generation of highly reactive breakdown products, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE).

In addition, different types of oxidized phospholipids (OxPLs) can be generated from otherwise modified low-density lipoproteins, including oxidized phosphatidylcholine, oxidized phosphatidylethanolamine (OxPE), oxidized phosphatidylserine (OxPS) and oxidized cardiolipin (OxCL). The newly generated breakdown products and the oxidized and truncated residual domain OxPLs can in turn react with free amino groups of protein side chains or lipids that are localized in their vicinity, forming stable covalent adducts and creating oxidation-specific epitopes (OSEs). Because phosphocholine (PC) as an OSE is only presented as an epitope in the context of OxPL, these epitopes are termed PC-OxPLs for clarity. The PC moiety can also be a component of the capsular polysaccharide of bacteria, where it is not part of a phospholipid backbone and is constitutively presented as an epitope. In addition, an adduct between an oxidative fragment of docosahexaenoic acid, (E)-4-hydroxy-7-oxohept-5-enoic acid and lysines of proteins (or aminophospholipids) can lead to the formation of 2-(ω-carboxyethyl)pyrrole (CEP). OSE-modified proteins or lipids are sensed by innate immune responses and represent a unique class of damage-associated molecular patterns (DAMPs).
Role of innate immune responses targeting OSEs. Oxidized low-density lipoproteins (OxLDL), apoptotic cells, microvesicles and cellular debris display various types of oxidation-specific epitopes (OSEs)\(^{17,20,21,22,23}\). Components of cellular and humoral immune responses sense OSEs and mediate sterile inflammation or clearance and neutralization depending on the availability and context. Macrophages sense OSEs through various pattern recognition receptors (PRRs) that are expressed on their surface. Scavenger receptor CD36 preferentially binds and mediates the uptake of the phosphocholine (PC) of OxPL (PC-OxPL), oxidized phosphatidylethanolamine (OxPS) and 2-γ-carboxyethylpyrrole (CEP). Scavenger receptor SRB1 recognizes PC-OxPL; SRA1 and lectin-like oxidized LDL receptor 1 (LOX1) bind malondialdehyde (MDA); and LOX1 recognizes 4-hydroxynonenal (4-HNE)\(^{24,25}\). Toll-like receptors (TLRs) mediate pro-inflammatory signals. PC-OxPL transmit inflammatory signals through a heterotrimer of TLR4–TLR6–CD36, as well as through TLR2, and CEP signals through TLR2 in cooperation with CD36 (REFS 35, 46). Humoral immune responses to OSEs include natural IgM antibodies, C-reactive protein (CRP), members of complement and other soluble PRRs that are involved in the clearance of apoptotic cells. Soluble PRRs inhibit the recognition of OSEs by cellular PRRs and mediate uptake via alternative pathways. Natural IgM bound to PC-OxPL, MDA and 4-HNE epitopes are taken up by macrophages by C1q–calreticulin–CD91-dependent or mannose-binding lectin (MBL)– and MBL receptor-dependent mechanisms. CRP bound to PC-OxPL is taken up by C1q–calreticulin–CD91-dependent mechanisms\(^{18,21,22,23}\). Alternative recognition of C1q is mediated by CD93 (not shown). Complement factor H (CFH) bound to MDA provides cofactor activity for the cleavage of C3b into iC3b opsonins\(^{26}\) that mediate anti-inflammatory clearance via complement receptor 3 (CR3). Milk fat globule-epidermal growth factor 8 (MFG-E8) bound to OxPS or OxPE mediate clearance via \(\alpha v\beta 3\) integrins\(^{27,28}\).

OSEs are major targets of natural IgM antibodies. OSEs have been identified as prominent antigens of natural IgM antibodies (TABLE 1). IgM antibodies in human umbilical cord blood, which represent naive natural antibodies of fetal origin, have specificity for OSEs\(^{29}\). In fact, titres of IgM with specificity for OxLDL and MDA-LDL were higher in cord blood samples compared with matched maternal blood samples. The specific binding properties of natural IgM antibodies have been investigated by characterizing IgM antibodies derived both from \textit{in vitro} stimulated B-1 cells and from the plasma of mice lacking recombination-activating gene 1 (RAG1) that were selectively reconstituted with B-1 cells\(^{30}\). These studies revealed that several OSEs, including PC-OxPL, MDA and 4-HNE adducts are bound by up to 30% of all natural IgM found in the plasma of both wild-type and gnotobiotic mice, and by a similar percentage of IgM-secreting cells from the spleens of wild-type mice\(^{31}\). Furthermore, several monoclonal natural IgM antibodies with specificity for different OSEs have been characterized. For example, the well-studied PC-binding T15/E06 IgM — which contains a variable region that is composed of a canonical rearrangement of germline genes in both the \(\nu_a\) and \(\nu_b\) chains — was originally cloned from the spleens of atherosclerosis-prone apolipoprotein E (ApoE\(^{−/−}\)) mice as an antibody with specificity for OxLDL, which was later found to be specific for PC-OxPL\(^{32,33}\). The same antibody idiotype, as an IgA, was originally described due to its ability to bind PC present in the capsular polysaccharide of \textit{S. pneumoniae}\(^{34,35,36}\). In addition, several other IgMs that bind different OSEs have been identified, such as the OxCL-specific LR01 and a number of others with specificity for MDA-type adducts, including E014, NA17 and LR04. Similar to T15/E06, all of the OSE-specific natural IgM antibodies that have been described exhibit germline or near-germline usage in their complementary-determining region 3 (CDR3), identifying them as natural IgM\(^{31,33,34,35,36}\). Thus, natural IgM antibodies prominently participate in the response to OSEs.

Role of OSEs in the maintenance of homeostasis

Physiological carriers of OSEs. Tissue homeostasis depends on the efficient removal of dying cells, which is achieved by molecular tags that mark cellular debris at different stages of cellular death. The quiescent removal of apoptotic cells is mainly mediated by phagocytes that sense several ‘find-me’ and ‘eat-me’ signals on apoptotic cells\(^{37}\). In addition, several other IgMs that bind different OSEs have been identified, such as the OxCL-specific LR01 and a number of others with specificity for MDA-type adducts, including E014, NA17 and LR04. Similar to T15/E06, all of the OSE-specific natural IgM antibodies that have been described exhibit germline or near-germline usage in their complementary-determining region 3 (CDR3), identifying them as natural IgM\(^{31,33,34,35,36}\). Thus, natural IgM antibodies prominently participate in the response to OSEs.
Enhanced efferocytosis

Nitric oxide production by the

OxCL

Unknown

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MDA

Uptake

MDA

4-HNE

Uptake

Inflammation

OxCL

Neutralization

Inflammation

TLRs

TLR4–TLR6

PC-OxPL

Inflammation

TLR2 and 

TLR2–TLR6

•

CEP

•

OxPL

Inflammation

TLR2–TLR6

Inflammation

TLR2–TLR6

Angiogenesis

ER stress

Apoptosis (iC3b)

Neutralization

Modulation of efferocytosis

Enhanced efferocytosis

PC-OxPL

Enhanced efferocytosis

PC-OxPL

Modulation of efferocytosis

OxPS

OxPE

Enhanced efferocytosis

E014

MDA

Neutralization

(?)

4-HNE

Unknown

4-HNE, 4-hydroxynonenal; CEP, 2-(ω-carboxyethyl)pyrrole; CFH, complement factor H; CRP, C-reactive protein; ER, endoplasmic reticulum; LOX1, lectin-like oxidized LDL receptor 1; MDA, malondialdehyde; MFG-E8, milk fat globule-epidermal growth factor 8; OSE, oxidation-sensitive epitope; OxCL, oxidized cardiolipin; OxPE, oxidized phosphatidylethanolamine; OxPL, oxidized phospholipid; OxPS, oxidized phosphatidylserine; PC-OxPL, phosphocholine (PC)-containing OxPL; PRR, pattern recognition receptor; SR, scavenger receptor; TLR, Toll-like receptor.

Physiological role of OSEs. In situations that are associated with increased oxidative stress, which favour OSE generation, a large amount of cellular waste is generated. For example, exposure to high light intensities in an oxygen-rich environment results in enhanced lipid peroxidation. Consequently, the retina is one of the tissues with the highest cell turnover rate, and accordingly requires high phagocytic activity for the clearance of aged photoreceptors, which are enriched in long-chain PUFAs. Both retinal pigment epithelium (RPE) and microvascular endothelial cells express several scavenger receptors that mediate the clearance of apoptotic cells, cellular debris and oxidized lipids\(^8\). Indeed, rats with a deletion variant of CD36 exhibit impaired phagocytosis of photoreceptor rod outer segments\(^8\). Whereas anionic phospholipids on apoptotic cells have been suggested to mediate this binding\(^8\), mass spectrometry studies have shown that the exposure of rat retinal cells to light results in elevated levels of OxPLs\(^9\). Importantly, OxPL inhibited the uptake of rod outer segments by wild-type cells but not by Cd36-/– RPE cells. OSEs may also be involved in efferocytosis via MFG-E8, which binds OxPS exposed on the surface of apoptotic cells\(^8\). MFG-E8 facilitates engulfment by phagocytes via the αvβ3 and αvβ5 integrin receptors, and has been shown to have an important role in the phagocytosis of photoreceptors of the outer segment\(^9\). Recent work has identified the 12/15-lipoxygenase product OxPE as a ligand for MFG-E8 with unique functional consequences in the context of apoptotic cell clearance during peritonitis\(^9\). In this study, OxPE that is exposed on the surface of alternatively activated macrophages led to the sequestration of MFG-E8, which favours the engulfment of apoptotic cells via phosphatidylserine receptors, such as T cell immunoglobulin and mucin domain 4 (TIM4), but blocks MFG-E8-dependent uptake of apoptotic cells by pro-inflammatory LY6C\(^9\) monocytes. Thus, in environments with high oxidative stress, the OSE-dependent clearance of cellular debris represents a physiological housekeeping function that directs the clearance of dying cells to specific pathways.

Accumulating evidence indicates that the recognition of OSEs may also be crucial for complement-mediated clearance mechanisms, particularly of late apoptotic and several triggers, were enriched in different OxPLs\(^7\). Similarly, microvesicles generated from monocytes that were loaded with unesterified cholesterol, as well as t-BuOOH-oxidized microvesicles that were derived from endothelial cells, carried both MDA and PC-OxPL epitopes in their membranes\(^8,9,10\).

More recently, we also identified a subset of circulating microvesicles as carriers of various OSEs\(^8\). Microvesicles have been implicated in several diseases owing to their pro-inflammatory and pro-coagulant activities\(^8\). Interestingly, MDA adducts consisting of simple MDA-lysine adducts, as well as more complex adducts made up of a lysine residue and two or more MDA moieties, were the most prominent OSE found on ~50% of circulating microvesicles, whereas PC-OxPLs were found on fewer microvesicles but were found to a greater extent on the surface of the same microvesicles. The presence of OSEs was generally independent of cellular origin, supporting their universal presence\(^8\). Moreover, a portion of the microvesicles also carried IgM bound on their surface, which was primarily directed against MDA, consistent with their prominent presence. Interestingly, a subset of circulating microvesicles also carry CRP on their surface, which is consistent with the presence of PC-OxPLs\(^8\).

Thus, different mechanisms of cell death and generation of microvesicles result in the surface presentation of various types of OSEs. An interesting area for future investigations includes understanding whether the presence of OSEs is associated with other types of cell death such as pyroptosis, necroptosis and ferroptosis\(^9,50\).
nescotic cells, including the activity of several complement proteins and receptors, such as CRP and natural IgM, as well as pentraxin [33,38,39]. Natural IgM antibodies are important in apoptotic cell clearance, as mice deficient in secreted IgM are more prone to developing autoimmunity when crossed with the lupus-prone MRL/lpr background [46,100]. To date, all characterized OSE-specific natural IgM antibodies have also been found to bind apoptotic cells, and the PC-specific T15/E06 IgM and the MDA-specific NA17 antibodies enhance the uptake of apoptotic cells in mice in vivo [75,101]. Interestingly, MDA and 4-HNE accumulate in the serum of aged MRL/lpr lupus-prone mice [102]. MDA and PC-OxPL have also been suggested to be dominant drivers in IgM-C1q-mediated efferocytosis by dendritic cells [87]. Moreover, the administration of T15/E06 protected arthritis-prone DBA1 mice from collagen-induced arthritis [48], suggesting an anti-inflammatory potential of neutralizing PC-OxPL. As dying cells display different OSEs at the same time, IgM with different OSE specificity may have functionally additive effects. Similarly, CRP, which also binds apoptotic cells via PC-OxPL, enhances the clearance of dying cells in a complement-dependent manner [103]. Although CRP and IgM are potent activators of complement, their binding on apoptotic cell surfaces does not lead to terminal complement activation because this is limited by the recruitment of the soluble regulators CFH and C4 binding protein (C4BP) [104]. Importantly, recruitment of CFH to apoptotic blebs and to necrotic RPE cells can be mediated by MDA [49] and other previously described ligands [105]. This might allow CFH to provide cofactor activity for factor I on dying cells to inactivate C3b into iC3b. The phagocytosis of dying cells that have been opsonized with iC3b can inhibit nuclear factor-κB (NF-κB) transcription, and can thereby promote anti-inflammatory clearance by phagocytes [106]. Notably, CFH mediates cofactor activity on MDA-decorated surfaces, and MDA binding does not interfere with this process. Moreover, owing to its reduced MDA-binding capacity, the H402 variant of CFH displayed significantly impaired cofactor activity compared with the Y402 variant, which may result in the less efficient clearance of MDA-decorated structures and increased inflammation [107,108]. Interestingly, CFH was reported to slow down efferocytosis in vitro [104,108], which is beneficial in circumstances that require decreased release of ROS, nitrogen intermediates and lysosomal enzymes by phagocytes [98,109]. During this process, additional complement-independent neutralizing functions of CFH and OSE-specific IgM may also be crucial to prevent potential pro-inflammatory effects of the OSEs that have not yet been cleared. For example, CFH blocks MDA-induced CXCL-chemokine ligand 8 (CXCL8) and CXCL1 production in vitro and in vivo, respectively [107]. Similarly, the MDA-specific IgM LR04 and the PC-specific IgM T15/E06 inhibited microvesicle-induced CXCL8 secretion in human monocytes and the activation of endothelial cells, respectively [80,88]. These findings provide insights into the important physiological function of OSE-specific immune responses in the recognition and neutralization of cellular debris in the circulation.

In summary, OSEs mark dying cells and their cellular debris and distinguish them from viable and healthy tissues. This enables specific humoral immune responses such as natural IgM antibodies to coordinate the safe disposal of dying cells with a regulated activation of the early steps of the complement cascade. In addition, scavenger receptors that bind to OSEs mediate efferocytosis. However, if the enhanced generation of dying cells exceeds the clearance capacity of available phagocytes, the OSEs present on the accumulating apoptotic cells mediate pro-inflammatory signals and may promote autoimmunity.

Innate sensing of OSEs in disease

During acute inflammatory conditions OSEs can trigger sterile inflammation to ultimately restore tissue integrity. However, under certain conditions, the balance between the generation and the clearance of OSEs by innate immune functions is lost owing to the increased generation of OSEs and/or dysfunctional innate immune responses, which leads to increased or chronic inflammation.

OSEs have been documented in diseased tissues of patients with, for example, infectious and sterile acute lung injury, atherosclerosis, hepatitis, age-related macular degeneration (AMD), multiple sclerosis and Alzheimer disease [11]. The investigation of these diseases has provided important insights into the functional role of OSE-specific immunity in both acute and chronic inflammatory models.

Role of OSEs in acute inflammation

The OSE CEP transiently accumulates during acute inflammation in a mouse model of wound healing, and induces angiogenesis in endothelial cells in a TLR2–MYD88-dependent manner to stimulate wound healing and to protect against hindlimb ischaemia [31,55]. Administration of OxPL promoted acute lung injury in mice in a TLR4- and TRIF-dependent manner. In this context, F4/80+ and alternatively activated macrophages scavenge and degrade accumulated CEP in a process that requires binding of both TLR2 and CD36 [46]. Notably, CEP accumulates in ageing tissues, which may lead to adverse consequences.

OSEs also accumulate in a wide variety of acute situations [33,38,110]. They accumulate in the lungs of mice following acute lung injury induced by sterile acid aspiration, as well as following infection with the avian influenza virus H5N1 [51]. The pulmonary lavage fluid of injured mice contains OxPL that stimulates the production of IL-6 in macrophages, which can be ameliorated by the natural IgM antibody T15/E06. Administration of OxPL promoted acute lung injury in mice in a TLR4- and TRIF-dependent manner. In addition to PC-OxPL, MDA adducts also formed in the lungs of these mice, and intranasal administration of MDA-modified bovine serum albumin (BSA) induced CXCL1 secretion and neutrophil recruitment into the lungs [111]. This is consistent with the capacity of MDA-adducts to trigger chemokine secretion in macrophages, and further demonstrates the pro-inflammatory potential of OSEs in vivo. Notably, both PC-OxPL and MDA have been documented in the lung tissues.
Role of OSEs in atherosclerosis. Atherosclerosis is the prototypical chronic inflammatory disease in which the innate immune responses to OSEs are involved (Fig. 3). It is characterized by the deposition of LDL in the intima of large and medium-sized arteries, and the LDL subsequently undergoes oxidation to generate OxLDL. OSEs on OxLDL not only mediate uptake by macrophages to generate foam cells but also mediate many of the chronic inflammatory events that lead to the development of plaques. If plaques rupture, they can trigger thrombus formation, resulting in severe clinical manifestations such as myocardial infarction and stroke.12

PC-OxPL, MDA, 4-HNE, OxCE and CEP have all been documented in atherosclerotic lesions in humans and in animal models of disease.18,46,76,113–115. Endothelial cells and macrophages—which express different sets of scavenger receptors and TLRs—are major cellular sensors of OSEs in atherosclerosis. OxPLs induce the expression of chemoattractants, such as CC-chemokine ligand 2 (CCL2; also known as MCP1), fibronectin containing connecting segment 1, CXCL8 and P-selectin, and also trigger monocyte binding to endothelial cells15, which is partly mediated by TLR4 (Ref. 116). 4-HNE also induces ERK1/2 and NF-kB activation in endothelial cells via LOX1 (Ref. 50). LOX1 deficiency results in decreased lesion formation in Ldlr−/− mice17. CEP may also trigger a pro-atherogenic endothelial cell response via TLR2 (Ref. 53). The expression of TLR2 is increased in endothelial cells at sites of disturbed blood flow, and TLR2 deficiency in non-haematopoietic tissues reduces atherosclerosis in Ldlr−/− mice18. Thus, OSEs by endothelial cells is a key response in the development of atherosclerosis. Moreover, a recent study demonstrated that CEP accelerates platelet activation and thrombus formation in hyperlipidaemic Apoe−/− mice in a TLR2-dependent manner16, indicating a role for OSEs in triggering the clinical events that result from atherosclerosis.

A hallmark of atherosclerotic lesions is the formation of lipid-laden macrophages, known as foam cells, which occurs because of the enhanced uptake of OxLDL mediated by OSE binding to scavenger receptors, such as CD36 and SRA1 (Ref. 119). The enhanced uptake of cholesterol-rich LDL and subsequent inefficient removal of intracellular cholesterol can lead to a situation of supersaturation of cholesterol, leading to the formation of cholesterol crystals that damage lysosomes and that activate the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, resulting in IL-1β secretion19. Indeed, cholesterol loading of macrophages with OxLDL induces IL-1β secretion.19. However, cholesterol loading of macrophages with OxLDL induces IL-1β secretion, and Ldlr−/− mice reconstituted with NLRP3-deficient bone marrow develop less atherosclerosis.19. Moreover, deficiency of CD36 and/or SRA1 in Apoe−/− and Ldlr−/− mice, respectively, reduces the size and/or complexity of lesions compared with control mice.19. In addition, binding of OxLDL to CD36 induces chemokine expression and inflammasome priming through NF-κB activation, which is dependent on heterotrimer formation with TLR4–TLR6 (Refs 53,121). TLR4 signalling is important, as Tlr4−/−Apoe−/− mice exhibit significantly reduced lesion size12, and clinical studies have found an SNP in TLR4, which results in impaired signalling, to be associated with reduced plaque burden and acute coronary events12,124. These studies demonstrate that sensing of OSEs, and in particular PC-OxPL, by CD36 is central to several pro-atherogenic responses in macrophages. OxLDL, as well as excessive foam cell formation, induces macrophage apoptosis, which is observed in advanced atherosclerotic lesions.125. As a result of inefficient efferocytosis, apoptotic macrophages accumulate, which results in increased plaque necrosis and long-term atherosclerotic burden. Several studies demonstrate that deficiencies in certain proteins or receptors that mediate efferocytosis enhance lesion formation in atherosclerosis-prone mice.125. Furthermore, the accumulation of apoptotic macrophages within lesions, and their released microvesicles, which all bear OSEs, may compete with both OxLDL and apoptotic cells for clearance by macrophages and thereby further impair efferocytosis.

Humoral immune responses are increasingly being recognized as important modulators of atherogenesis. Although the ability of CRP to bind OSEs and to mediate apoptotic cell clearance suggests a role in atherosclerosis, there is no real support for an active role of CRP itself in the pathogenesis of the disease from animal or human studies.127,128. However, CRP is an excellent biomarker of inflammation, and increased levels of CRP independently predict manifestations of atherosclerosis.14. Contrast, natural IgM antibodies clearly modulate atherosclerotic lesion formation, as deficiency in secreted IgM promotes atherosclerosis in Ldlr−/− mice.128. As many natural IgM antibodies bind OSEs, their atheroprotective function may be mostly mediated by OSE-specific IgM, which includes housekeeping mechanisms and other protective effects. For example, T15/E06 IgM neutralizes the pro-inflammatory effects on endothelial cells and macrophages of late apoptotic cells and/or blebs carrying PC-OxPL. T15/E06 IgM antibodies have also been shown to block the binding and uptake of OxLDL in macrophages, preventing foam cell formation.27. Importantly, raising plasma levels of T15/E06 IgM by immunization with PC-containing pneumococcal extracts or passive infusion decreased atherosclerosis in Ldlr−/− mice and vein graft atherosclerosis in Apoe−/− mice, respectively.129,130. In analogy to PC-OxPL, MDA-epitopes also trigger tumour necrosis factor (TNF) and CXCL8 in monocytes and macrophages.17. In addition, the numbers of MDA-positive microvesicles substantially increased at the culprit lesion sites of patients suffering from an acute coronary event compared with the number found in peripheral circulation, which could further propagate inflammation.20. Thus, OSE-specific IgM can protect by neutralizing the pro-inflammatory effects of OSEs in the context of OxLDL, microvesicles and apoptotic plaques.
cells, and can promote the anti-inflammatory clearance of cellular debris. In line with this, several clinical studies have shown that lower levels of IgM antibodies directed against MDA-LDL and OxLDL are linked to an increased risk of cardiovascular disease (CVD)\textsuperscript{126}. Our recent identification of CFH as a major MDA-binding protein in plasma also suggests its potential involvement in atherosclerosis\textsuperscript{67}. CFH is present in human atherosclerotic lesions, where it localizes to areas rich in MDA-epitopes\textsuperscript{67,131}. Although several studies have
reported an association of the 402H allele — which decreases binding of CFH to MDA — with an increased risk of heart disease and stroke, a meta-analysis of eight different study populations failed to find a significant association of this gene variant with CVD\(^2\). However, to fully understand this function of CFH in CVD, the net contribution of all SNPs that potentially affect MDA binding needs to be explored.

These data support an important role for immune responses that target OSEs in atherogenesis. OxLDL and cellular debris contribute to an excessive accumulation of OSEs, which cannot be adequately targeted by beneficial clearance mechanisms and, consequently, pro-inflammatory responses in macrophages and endothelial cells are sustained. Similarly, OSEs may promote non-alcoholic steatohepatitis\(^3\), which is increasingly being recognized as a risk factor for CVD. Studies in cholesterol-fed \(\text{Ldlr}^{-/-}\) mice indicate that the expression of CD36 and SRA1 in Kupffer cells mediates hepatic inflammation\(^4\), whereas inducing plasma levels of the T15/E06 IgM is protective\(^5\). Consistent with these data, we recently showed that deficiency of sialic acid-binding immunoglobulin-type lectin G (Siglec-G), a negative regulator of B-1 cell function, results in increased levels of OSE-specific IgM and reduced atherosclerosis and hepatic inflammation in cholesterol-fed \(\text{Ldlr}^{-/-}\) mice\(^6\).

**OSEs in AMD.** OSEs have also been implicated in AMD\(^7\), which is characterized by the accumulation of cellular waste (drusen) in the retina. Several OSEs, including PC-OxPLs, and MDA- and CEP-modified proteins, have been identified as drusen components and have been shown to trigger sterile inflammation\(^8\). For example, CEP-modified proteins induce pro-inflammatory gene expression and prime the NLRP3 inflammasome in monocytes via TLR2 (REFS 55, 159). Drusen extracts isolated from AMD lesions activate the NLRP3 inflammasome, resulting in the secretion of IL-1β and IL-18 (REF 159). Similarly, MDA-modified proteins induce the expression of CXCL8 and CXCL1 in RPE cells in \textit{in vitro} and \textit{in vivo} models, respectively\(^9\). Disregulation of complement represents another major pathological driver of AMD, and genome-wide association studies have identified SNPs in genes encoding complement proteins as genetic risk factors of AMD\(^10\). Among these, the SNP rs1061170 in \textit{CFH} predisposes to increased risk for AMD\(^11,12\). Necrotic RPE cells and blebs thereof display MDA-epitopes that are recognized by CFH, which in turn facilitates the generation of iC3b for opsonization and anti-inflammatory removal. Compared with the Y402 CFV variant, the cofactor activity on MDA-decorated surfaces is severely impaired when the 402H CFH variant is used, demonstrating a functional consequence. As a result, MDA may accumulate and promote inflammation\(^13\). Indeed, transgenic mice that express the human 402H variant have increased susceptibility to oxidative stress-mediated injury in the retina as a result of MDA accumulation and increased inflammation\(^14\).

**Conclusions and future directions**

The recognition of OSEs by cellular and humoral immune responses enables the immune system to mediate important housekeeping functions, such as the removal of dying cells, cellular debris and damaged molecules. In situations of increased oxidative stress, the generation and burden of OSEs is greatly increased. As a consequence, OSEs are sensed by cellular signalling receptors, leading to the secretion of chemokines and pro-inflammatory cytokines. Thus, OSEs represent a unique class of DAMPs, which both mediate the recognition of cellular debris for housekeeping functions and, under certain circumstances, act as pro-inflammatory danger signals themselves. When this fine balance between the presence of OSEs and the capacities of the immune system to survey and respond to them is lost owing to the excessive generation of OSEs and/or their impaired removal, the development and propagation of chronic inflammatory diseases become manifest.

Although there have been a few attempts to treat patients with antioxidants to reduce the progression of CVD, these have not been successful\(^15\). These very limited studies have been criticized as premature as we currently lack sufficient knowledge to adequately design an effective therapeutic trial to test the role of oxidation in atherogenesis\(^16\). As discussed in this Review, oxidative events and the associated immune responses have beneficial effects as well as adverse consequences and we currently lack insight into the relative importance of these \textit{in vivo}, which could affect the proper design of antioxidant trials.

A better understanding of the role of OSEs and OSE-reactive immune responses in the maintenance of homeostasis and in controlling inflammation are important areas of future investigations. Moreover, it is important to elucidate how different components of OSE-specific immune responses cooperate at a functional level. The identification of the genetic variants that affect some of these responses, and mechanistic studies characterizing these, may provide exciting insights into different individual predispositions to responding to oxidative stress and to developing chronic inflammation.

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Competing interests statement
The authors declare competing interests: see Web version for details.