Keywords
Atherothrombosis, oxidized LDL, innate immunity, natural IgM antibodies

Summary
Atherosclerosis is a chronic inflammatory disease of the vascular wall that results from disturbed lipoprotein metabolism and increased oxidative stress. A major consequence of this is lipid peroxidation, which generates a number of breakdown products of membrane lipids that form so called oxidation-specific epitopes (OSE). OSE have been documented in oxidized lipoproteins and on the surface of dying cells and circulating microparticles, and their ability to trigger robust pro-inflammatory and pro-thrombotic responses has been demonstrated extensively. Recent studies have identified specific OSE as major targets of both cellular and soluble pattern recognition receptors of the innate immune system, including innate natural IgM antibodies. This allows the immune system to identify metabolic waste and mediate important physiological housekeeping functions, e.g. by promoting the removal of cellular debris and by neutralizing oxidized molecules. Indeed, innate B1 cells and B1 cell derived natural IgM with specificity for OSE have been shown to protect mice from the development of atherosclerotic lesions. Moreover, OSE-specific natural IgM antibodies bind and neutralize the pro-inflammatory and pro-thrombotic effects of OSE, and low levels of OSE-specific IgM are associated with an increased risk for myocardial infarction. Conclusion: Understanding the molecular components and mechanisms involved in this process, will help identify individuals with increased risk for atherothrombosis and indicate novel points for therapeutic intervention.

Schlüsselwörter
Atherothrombose, Oxidiertes LDL, angeborene Immunität, natürliche IgM Antikörper

Zusammenfassung

Atherosclerosis and oxidized LDL
Atherosclerosis is characterized by a chronic inflammation of the vessel wall of large and medium sized arteries, and its onset is tightly associated with pathological lipid accumulation. Initially being described as the mere retention and accumulation of different lipoproteins within the vascular wall, it is now clear that atherosclerosis is an active process in which several cells and soluble molecules and even tissues influence each other eventually leading to severe consequences, such as organ ischemia, stroke and myocardial infarction. These clinical events constitute the major causes of death globally (1) and

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result from atherosclerosis-associated thrombosis (2, 3) often consequent to plaque rupture and the exposure of plaque material to the blood flow. It is now increasingly recognized that also the mechanisms leading to atherothrombosis are critically influenced by a dynamic interplay of altered lipid metabolism, inflammation and hemostasis (4–6).

A number of risk factors for the development of atherosclerosis have been identified, but high plasma levels of low density lipoprotein (LDL) cholesterol and concomitant low levels of high density lipoprotein (HDL) remain key initiating factors of the disease. The early stages of atherosclerosis are characterized by the accumulation and retention of LDL from the circulation within the artery wall through interactions with extracellular matrix proteoglycans (7). Once LDL is trapped to the intimal layer, it becomes susceptible to modifications such as
• oxidation (8),
• glycation (9) and
• aggregation (10).

Oxidation, which may occur via enzymatic or non-enzymatic mechanisms, is by far the most investigated modification of LDL. A large body of literature is available focusing on the generation and the different types of oxidative modifications of both the lipid and protein moieties (11) as well as the pro-inflammatory and pathogenic roles of the oxidized LDL (OxLDL) (8, 12). OxLDL promotes atherogenesis in many ways. It has been shown to induce the activation of endothelial cells and promote the recruitment of monocytes from the circulation. Such recruited monocytes differentiate into macrophages which in turn start to take up the excess of OxLDL, leading to the generation of cholesterol-laden foam cells (13, 14). This uptake of OxLDL does not occur through the conventional LDL receptor which is downregulated by intracellular increased cholesterol levels, but instead involves different scavenger receptors most prominently CD36 and SR-A1 (13, 15). The constant accumulation of cholesterol esters within macrophages together with an ineffective cholesterol efflux mechanism, ultimately leads to their death by apoptosis (16). However, when the increased demand for clearance of apoptotic cells is not efficiently fulfilled, apoptotic cells and cellular debris start to accumulate and further propagate pro-inflammatory and pro-thrombotic effects.

### Oxidation-specific epitopes are targets of innate immunity

Because many of the modifications of LDL induced by lipid peroxidation result in the generation of immunogenic neo-epitopes on OxLDL, these modifications have been termed oxidation-specific epitopes (OSE) (11). Notably, OSE do not only occur on OxLDL but also on other autologous lipids and/or proteins e.g. in the membrane of dying cells. Such modified structures can be generated by extensive oxidative stress in virtually all cells and tissues, and they have been documented in many inflammatory conditions like atherosclerotic tissues, acute lung injury, age-related macular degeneration, multiple sclerosis and Alzheimer’s disease (17–20). Many OSE have been found to trigger pro-inflammatory responses such as chemokine and cytokine production in vitro and in vivo (11). Thus, OSE represent a new class of “danger signals” or damage-associated molecular patterns (DAMP) of innate immunity (12). The most prominent examples include
• phosphocholine (PC) of oxidized phospholipids,
• malondialdehyde (MDA),
• 4-hydroxynonenal (HNE),
• oxidized phosphatidylserine (oxPS),
• carboxyethylpyrrole (CEP) and
• oxidized cardiolipin (oxCL).

Consistent with their role as DAMP a number of innate immune responses specifically targeting OSE have been identified in the past years. These include cellular pattern recognition receptors (PRR) as well as humoral pattern recognition proteins and so called natural antibodies. PRR are broadly expressed on the surface of macrophages, with the function of recognizing non-self but also modified self to mount a quick immune response. Toll-like receptors (TLR) (21) and scavenger receptors (SR) are prominent examples of PRR. SR mediate the uptake of OxLDL and they represent the prototypic PRR for OSE, as deficiency of CD36 and SR-A1 has been found to reduce OxLDL-induced foam cell formation (13). Importantly, CD36 cooperates with a heterodimer of TLR4 and TLR6 to mediate pro-inflammatory signals of OxLDL (22). This is also important as through the engagement of this pathway components of the intracellular PRR, the nucleotide binding domain, leucine rich repeat containing domain protein 3 (NLRC3) became primed for subsequent activation by cholesterol crystals that are generated during foam cell formation (23). As a result of this, OxLDL contributes to the release of the proinflammatory cytokines IL-1 and IL-18 (13). Of note, the ongoing CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) trial is currently evaluating whether antibody-mediated blockade of IL-1 reduces the incidence of a second cardiovascular event in patients who already suffered a first heart attack (www.thecantos.org/cantos-summary.html).

In addition to the central role of macrophages in the innate immune response to OSE, humoral immune responses have been described as well. For example, C-reactive protein (CRP) specifically binds PC of oxidized phospholipids (24) and the major regulator of the alternative complement pathway factor H (CFH) has been shown to bind MDA-epitopes (20). The latter may have an important role in atherosclerotic lesion development as CFH was shown to neutralize the ability of MDA-epitopes to stimulate the production of IL-8 (20), which promotes lesion formation (25). Finally, a large part of natural IgM antibodies has been shown to bind various OSE (26). Natural antibodies represent a class of antibodies that do not require any exposure to pathogens or exogenous antigen to be generated. They are already present at birth and therefore constitute a first line of defense against invading pathogens in all individuals. These antibodies mainly belong to the IgM isotype and the variable regions of the heavy and light chains are encoded by germline genes with no or very little nucleotide insertions (27). The IgM repertoire is
thought to be the result of natural selection and it is restricted to highly conserved epitopes found in virtually all macromolecules (nucleic acids, protein, phospholipids and carbohydrate residues) of both pathogens and self-structures. Important insights for the understanding of the function of IgM antibodies came from the finding that a large part of these antibodies are able to recognize and bind OSE present on apoptotic cells, but not on viable cells – highlighting their housekeeping function in physiological conditions (26). IgM act therefore also as natural tool in the protection against endogenously generated waste, promoting the removal of cellular debris and the neutralization of excess accumulation of oxidized molecules, thereby preventing both inflammatory and autoimmune reaction. Evidence for the role of natural IgM in tissue homeostasis came from the observation that apolipoprotein E (ApoE) deficient mice displayed increased levels of autoantibodies recognizing different epitopes of OxLDL like MDA, 4-HNE and OxPC. This allowed the isolation of a panel of monoclonal antibodies with specificity for epitopes of OxLDL (19). Subsequently we showed that 30% of total plasma IgM has specificity for different OSE (26). Natural antibodies are secreted by a specific subset of self-replenishing B lymphocytes named B1 cells (28). B1 cells can be distinguished from B2 cells by their anatomical localization, activation state and specific surface marker expression. B1 cells were initially described in mice, but – although still controversial – a corresponding human B cell population has been identified. This human cell population is abundant in the umbilical cord, but is also found in peripheral blood and it displays typical properties that characterize B1 cells in mice, i.e. IgM secretion, ability to stimulate T-cells and potency of intracellular signaling (e.g. Syk and PLC-2 phosphorylation) (29).

Thus, several arcs of innate immunity have been described that specifically target different OSE that are present in OxLDL as well as the surface of dying cells. By binding OSE, these immune responses can actively modulate the biological activities of OxLDL and other molecules displaying OSE.

**OxLDL triggers pro-thrombotic responses**

The pro-atherogenic effects of OxLDL do not only depend on its pro-inflammatory potential, but also on its ability to affect pro-thrombotic responses for example by modulating the activation state of endothelial cells. In healthy conditions, the endothelial barrier has limited permeability to selected molecules and endothelial cells express a series of molecules with anti-thrombotic properties to stabilize the closed configuration of the endothelial junctions. Nevertheless, exposure of endothelial cells to OxLDL has been reported to induce a decreased endothelial barrier function and to up-regulate adhesion molecules such as VCAM-1, as well as modulate components of the coagulation cascade like enhanced pro-thrombinase and protein C inhibitor activity, up-regulation of tissue factor (TF) expression and activity, down-regulation of thrombomodulin and inactivation of TF pathway inhibitor thus resulting in an overall increased thrombogenic state of endothelial cells (30, 31).

TF is the most prominent factor that regulates thrombus formation. It acts as the initiator of blood coagulation in this context and its concentration and localization have been associated with the onset of cardiovascular events. For example, high levels of TF were found in the atheroma of patients with unstable coronary syndromes (32) supporting the hypothesis that local increase in the concentration of TF in the lesion is associated with increased thrombotic events. Upon plaque rupture sudden exposure of TF to the circulation triggers platelet activation and aggregation and the induction of the coagulation cascade, which can lead to thrombus formation and vessel occlusion. TF is mainly expressed on the cells of the vascular wall that are not in contact with blood, like pericytes and fibroblasts of the adventitia (33, 34). Nevertheless, under non-physiological conditions, for instance in sepsis, TF is induced also in activated macrophages, neutrophils, monocytes, platelets and endothelial cells. Expression of TF on monocytes was shown to be induced by stimulation with lipopolysaccharide (LPS) both in vitro and in vivo and deletion of the TF gene in mouse myeloid cells is sufficient for reduction of coagulation induced by LPS treatment (34). Similarly, in dyslipidemia TF is induced in cells that normally do not express it. Circulating monocytes of patients with hyperlipidemia do present higher TF levels compared to healthy controls (35). The involvement of aberrant TF expression in the thrombotic complications of atherosclerosis is supported by immunohistochemical evidences, as macrophages in human plaques express TF (36). TF is also present in plasma and blood-borne TF has been found in higher levels in association with acute myocardial infarction (37). OxLDL in particular has been described to trigger TF expression in monocyte-derived macrophages (36) and on circulating microparticles (MP). Notably, TLR4 has been suggested to be involved in the activity of TF-carrying MP because a deficiency of this receptor has been shown to drastically diminish the TF+ MP activity and to reduce coagulation in hypercholesterolemic mice (38).

OxLDL induces also pro-thrombotic effects by modulating platelet function, but the mechanisms that links increased concentrations of OxLDL to dysregulated platelet activation remain still elusive. This interaction may be of particular importance during plaque rupture, where the release of OxLDL has been documented (39), though lipoproteins carrying biologically active oxidized phospholipids are also present in the circulation (40). Several lines of evidence are available demonstrating that platelets from hyperlipidemic animals and humans show increased ability for aggregation in vitro. Notably, platelets highly express scavenger receptor CD36, which was shown to be responsible for binding OxLDL and to induce platelet activation in the context of hyperlipidemia. Mice deficient for ApoE that were fed a high fat diet have a higher platelet aggregation rate compared to wild type littermates. Importantly, platelet aggregation was reduced in ApoE/-/- mice also deficient in CD36, highlighting the important role of SR in platelet function. To observe such differences, mice needed to be in a hyperlipidemic state, as no effect was observed when platelet activity in ApoE/-/- and ApoE/-/-CD36/-/- mice on chow diet were compared (41).
In addition to platelets, MP are increasingly recognized as important players in hemostasis and thrombosis. MP are small vesicles between 0.1 and 1 µm in size that are released from the membrane of activated, apoptotic or necrotic cells (5, 42). Although MP are found in the circulation of healthy individuals, their concentration increases with pathological conditions including cardiovascular diseases. MP derived from different cellular origin, including platelets, erythrocytes, monocytes and endothelial cells have been described. The stimulus that triggers their shedding and the microenvironment surrounding their release determine their composition. Importantly, MP are not mere inactive membrane fragments but they mediate cell communication by transferring information from one cell to another. For example, they can transport mRNA and microRNA, and the presence of a variety of receptors and specific ligands on their surface allows MP to deliver and/or exchange genetic information and modulate cellular responses. Several reports indicate that MP released by different cells (platelets, leukocytes, endothelial or smooth muscle cells) are the main carrier of TF in the circulation and monocyte derived MP were found in high concentration in atherosclerotic plaques (43). In analogy to apoptotic cells, MP display phosphatidylserine (PS) on their outer leaflet, which is normally found in the inner leaflet of eukaryotic cells (42). This exposure of PS allows the deposition and activation of clotting factors, which initiates the clotting cascade similarly to the surface of activated platelets. Thus, circulating MP possess potent pro-coagulant activities that are in part due to the presence of bioactive lipids (44). Several studies have demonstrated the presence of OSE on in vitro generated MP and it has been shown that oxidized phospholipids contribute to monocyte adhesion to endothelial cells induced by MP and dying cells (45, 46). Moreover, we recently could show that a subset of circulating MP isolated from the plasma of healthy donors carries OSE in their membranes (47). In particular, nearly half of all circulating MP population displayed MDA-epitopes, while a lower percentage of MP also carried PC-epitopes. We also found that the levels of MDA-carrying MP were significantly increased in the coronary circulation at the culprit lesion site compared to the peripheral circulation of patients with acute myocardial infarction. Importantly, the presence of MDA-epitopes is partially responsible for the biological activity of MP, as the ability of platelet-derived MP to induce interleukin-8 (IL-8) secretion by primary human monocytes was inhibited by ~90% upon co-incubation with an IgM antibody that specifically recognize MDA-epitopes. Of note, a part of circulating MP was also found to carry endogenous MDA-specific IgM antibodies. Thus, circulating MP are novel carriers of OSE. Because the same OSE have been described in OxLDL, it is plausible to speculate that MP mediate similar pro-inflammatory and pro-thrombotic responses as described for OxLDL above. Immune responses targeting OSE may directly interfere with pro-thrombotic properties of MP by neutralizing their activities or simply promoting their removal from the circulation.

Thus, OxLDL and OSE also have the capacity to act as direct mediators of prothrombotic responses in the vessel wall and the circulation, and interfering with this specific activity may provide an interesting point of therapeutic intervention.

### OxLDL-specific immune response modulate atherothrombosis

The concept that atherosclerosis is actually an immunity regulated disease is unequivocally accepted (3, 48, 49). Both innate and adaptive immune cells take part to the initiation and progression of atherosclerosis, as it is shown by the presence of macrophages, dendritic cells, T cells, natural killer T cells within the plaque. Immune responses with specificity for OxLDL have been documented in mice and humans plaques. For example, T cells that were isolated from human carotid atherosclerotic lesions were found to specifically proliferate in response to OxLDL in an MHC class II restricted manner (50). In this regard, IFN-secreting Th1 cells with specificity for OxLDL have the capacity to modulate the inflammatory milieu within atherosclerotic lesions, which could also indirectly propagate thrombogenic responses. In turn, T regulatory cells with anti-atherogenic capacity dampen such activities (49).

Besides these responses, B cells and humoral immunity have gained much attention in the past years (51). Different B cell subsets contribute differently to the progression of the disease, which is in part due to their ability to preferential secrete IgM and IgG antibodies, respectively. B2 cells have been suggested to promote atherogenesis as B2 cell depletion in Ldlr⁻/⁻ and Apoe⁻/⁻ mice results in reduced lesion formation. They primarily secrete antibodies of the IgG isotype in a T cell-dependent manner, and although IgG antibodies to epitopes of OxLDL have been de-

### Table 1

Epidemiological studies correlating IgM and IgG titers and cardiovascular events

<table>
<thead>
<tr>
<th>antigen</th>
<th>antibody isotype</th>
<th>effect</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OxLDL</td>
<td>IgG</td>
<td>direct correlation with myocardial infarction</td>
<td>(57)</td>
</tr>
<tr>
<td>CuOx-LDL and MDA-LDL</td>
<td>IgM, IgG and IgA</td>
<td>no association of circulation antibodies levels and the risk of stroke</td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>inverse correlation with coronary artery disease and events</td>
<td>(59)</td>
</tr>
<tr>
<td>phosphatidylcholine</td>
<td>IgM</td>
<td>inverse association with ischemic stroke in men</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with development of myocardial infarction</td>
<td>(61)</td>
</tr>
<tr>
<td>MDA-LDL and CuOx-LDL</td>
<td>IgG and IgM</td>
<td>positive association of IgG and inverse association of IgM with risk of ischemic stroke, myocardial infarction, new-onset unstable angina, acute coronary interventions, and vascular death</td>
<td>(62)</td>
</tr>
</tbody>
</table>
scribed, their functional role in atherosclerosis is still unknown. OxLDL-IgG immune complexes have been suggested to promote inflammatory responses in macrophages via Fc receptors, but protective capacities of IgG may also exist.

In contrast to B2 cells, B1 cells mediate atheroprotection and natural IgM antibodies are largely responsible for this effect. Indeed, Ldlr<sup>−/−</sup> mice that are unable to secrete IgM display increased atherosclerosis. Moreover, it could be shown that the pro-atherogenic effect of splenectomy in atherosclerosis-prone mice can be reversed by adoptive transfer of B1 but not B2 cells. This effect could not be observed when B1 cells from mice deficient in secretory IgM were transferred. Different mechanisms have been proposed to explain the protective role exercised by natural IgM, which to a large extent may depend on the fact that a large part of natural IgM have specificity for OSE. For example, OSE-specific IgM inhibit binding of OxLDL by SR like CD36 and SR-1B on macrophages (52, 53), thereby limiting OxLDL uptake resulting in reduced formation of foam cells. In addition, by binding of OSE on apoptotic cells IgM facilitate their clearance via complement complex C1q by macrophages (54) preventing the accumulation of apoptotic bodies in growing lesions. Finally, IgM mediated neutralization of dangerous pro-inflammatory OSE exposed on OxLDL and cellular debris limits inflammatory and potentially pro-thrombotic responses. For example, the prototypic natural anti-OxLDL IgM T15/E06 that binds PC of oxidized phospholipids has been shown to block OxLDL binding to macrophages, promote the clearance of apoptotic cells, and inhibit cytokine secretion by macrophages and activation of endothelial cells by OSE (18, 45, 46, 53). Importantly, raising T15/E06 IgM levels in atherosclerosis-prone mice by active and passive immunization limits atherosclerotic lesion formation (55, 56).

Consistent with the protective role for OSE-specific IgM, a growing number of epidemiological studies in humans suggest a protective role of OxLDL-specific IgM. For example titers of IgM antibodies to models of OxLDL have been found to inversely correlate with the extent of carotid intima-media thickness, the incidence of cardiovascular diseases in general, but also the risk for clinical events such as heart attacks and strokes. On the other hand, IgG titers to OxLDL have been reported to show no or even a positive association with occurrence of cardiovascular disease (49). Further investigation is needed to fully define the role of OSE-specific IgG antibodies in the modulation of atherosclerosis and their mechanisms of action. A summary of studies in which IgM and IgG antibodies to OxLDL were determined in association with cardiovascular events is provided (Tab. 1).

Conclusions and outlook

The generation of OSE on OxLDL and on “stressed” cellular structures triggers multiple responses by innate immunity that contribute to the development of atherothrombosis. Newly identified physiological carriers of OSE, such as MP, may be a critical link between inflammation and thrombosis. Only a detailed investigation of the generation and biological activities of OSE and the capacity of specific immune responses (e.g. natural IgM) to block their effects may help identify new interventional strategies in the treatment of atherothrombosis.

Conflict of interest

The authors declare no conflict of interest.

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