

Autoimmunity in atherosclerosis: a protective response losing control?

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Atherosclerosis is a chronic inflammatory disease characterized by accumulation of oxidized lipoproteins, increased cell death and hypertrophic degeneration of the arterial intima. The disease process is associated with local formation of modified self antigens that are targeted by both innate and adaptive immune responses. Although it remains to be firmly established it is likely that these autoimmune responses initially have a beneficial effect facilitating the removal of potentially harmful rest products from oxidized LDL and dying cells. However, studies performed on hypercholesterolaemic mice deficient in different components of the immune system uniformly suggest that the net effect of immune activation is

pro-atherogenic and that atherosclerosis, at least to some extent, should be regarded as an autoimmune disease. These observations point to the possibility of developing new treatments for atherosclerosis based on modulation of immune responses against plaque antigens, an approach presently tested clinically for several other chronic inflammatory diseases with autoimmune components. Pilot studies in animals have provided promising results for both parental and oral vaccines based on oxidized LDL antigens. The time when this concept is ready for clinical testing is rapidly approaching but it will be important not to underestimate the difficulties that will be encountered in transferring the promising results from experimental animals into humans.

Keywords: atherosclerosis, immunity, lipoproteins, T cells, vaccines.

Introduction

A chronic inflammation of the arterial intima is the principal driving force behind the development of atherosclerosis [1, 2]. This inflammation is regulated by a complex interplay of innate and adaptive immune responses presumably aiming to clear the arterial wall of potentially harmful antigens [3]. Although some studies have implicated the involvement of infectious microorganisms in this process, there is clear evidence that the major targets for these immune responses are modified endogenous structures. There is also convincing evidence from hypercholesterolaemic mouse models deficient in specific components of innate and adaptive immunity that the net effect of both systems

is pro-atherogenic [3, 4]. Accordingly, there is considerable support for the concept that atherosclerosis, at least in part, should be considered as an autoimmune disease. This concept is well in line with recent genetic studies demonstrating associations between polymorphisms in genes regulating the expression of antigen-presenting molecules and cardiovascular disease [5]. It could also explain why other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis are associated with increased cardiovascular risk. However, to strictly define atherosclerosis as an autoimmune disease is most likely an oversimplification, because metabolic and haemodynamic factors provide initiating stimuli for the disease process and also as many of the autoimmune

responses in atherosclerosis seem to be protective by facilitating the removal of waste products such as modified lipoproteins and apoptotic fragments. There are several ways for the body to control the activity of these autoimmune responses, an important one being the regulatory T cells [6]. If these control systems fail the autoimmune response may accelerate into a destructive pro-inflammatory process causing plaque growth and destabilization. In this review we will discuss the balance between protective and disease-promoting autoimmunity in atherosclerosis and the possibility of targeting mechanisms regulating this balance to develop new treatments for cardiovascular disease.

Autoantigens in atherosclerosis

Atherosclerotic plaques express autoantigens that are targeted by both IgM and IgG (Fig. 1). The best characterized autoantigen in atherosclerosis is oxidized LDL. Some LDL particles entering the artery wall become trapped on proteoglycans in the extracellular matrix [7, 8]. Autopsy studies of children and young adults demonstrate that arterial lipoprotein retention precedes activation of local inflammation and preferentially occurs at sites where atherosclerotic lesions commonly develop later in life [9]. This retention is dependent on the interaction between negatively charged sulphate groups on proteoglycan sugars and certain positively charged domains of the LDL protein apo B-100 [7]. The pro-inflammatory properties of these retained LDL particles have been linked to modifications caused by oxidation and enzymatic attacks resulting in the release of pro-inflammatory phospholipids and lipid peroxides, which rapidly activate an inflammatory response in surrounding cells [10, 11].

The oxidation process is also associated with major structural modifications of LDL including fragmentation of apo B-100 and generation of various aldehyde and phospholipid-adducts to apo B-derived peptides. Following antigen presentation by dendritic cells and macrophages epitopes such as malondialdehyde-lysine give rise to clonal expansion of oxidized LDL-specific T cells and expression of oxidized specific autoantibodies to oxidatively generated autoantibodies [12,

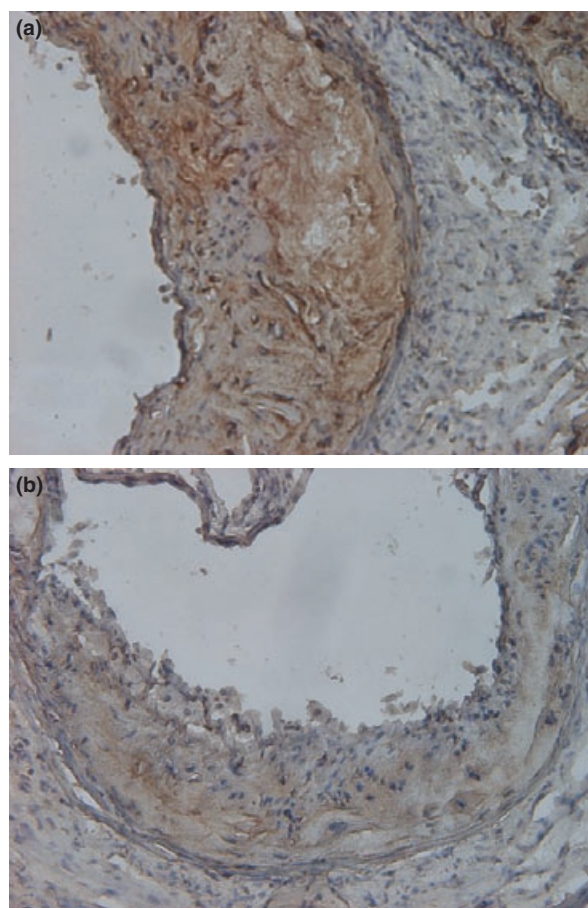


Fig. 1 Immunohistochemical staining (brown) for IgM (a) and IgG (b) in subvalvular atherosclerotic plaques for 25-week-old apoE^{-/-} mice. Magnification $\times 200$.

13]. About 10% of all T cells present in human atherosclerotic plaques are oxidized LDL-specific [14] and such T cells are also present in the circulation [15]. Autoantibodies to oxidized LDL are common in humans but their association with cardiovascular disease remains a matter of controversy possible because of difficulties in standardizing the antigens used for detection in analytical assays [16, 17].

A second category of autoantigens that have been implicated in atherosclerosis are the stress-induced heat shock proteins (HSP) [18]. HSP act as molecular chaperons facilitating refolding of denatured proteins in stressed cells. Interestingly, some of them have also been implicated in loading of immunogenic peptides

to major histocompatibility class (MHC) I and II molecules [19]. HSP60 is expressed by vascular cells in response to infections, fever, oxidant stress, cytokines and mechanical injury [18]. A possible explanation for the development of autoimmunity against endogenous HSP60 is molecular mimicry with similar HSP expressed by pathogens such as *Chlamydia pneumoniae* and *Helicobacter pylori* [20, 21]. Antibody levels against HSP-60/65 are increased in subjects with established cardiovascular disease and predict further development of the disease [22]. These antibodies specifically react with cells in atherosclerotic plaques and mediate lysis of stressed endothelial cells and macrophages *in vitro* [23].

Parental immunization of experimental animals with mycobacterial HSP-65 results in the development of atherosclerosis, and T cells isolated from these plaques respond specifically to HSP-65 *in vitro* [24, 25]. Whilst there seems to be a logical reason for the body to mount an immune response that could help remove potentially cytotoxic oxidized LDL particles, there appears less to be gained by activating autoimmunity against stressed cells trying to survive by expressing HSP. Therefore, autoimmunity to HSP60 may conceivably be due to molecular mimicry with pathogen antigens. This is also in line with the observation that immunization with oxidized LDL inhibits lesion formation in experimental animals [26] whereas parental immunization with HSP is pro-atherogenic.

A third category of antigens that may be involved in atherosclerosis are expressed by dying cells. Cell death in the atherosclerotic plaque may occur by apoptosis, a physiological and well-controlled process that allows damaged cells to be removed without injuring the surrounding tissue, or by necrosis [27]. The uptake of apoptotic cells by macrophages and some subsets of dendritic cells induce an anti-inflammatory response and play an important role in maintaining peripheral immune tolerance [28, 29]. It was recently demonstrated that regulatory B cells in the spleen contribute to apoptotic cell tolerance by stimulating T cells to produce IL-10 [30]. Conversely, uptake of necrotic cells or even a delayed uptake of apoptotic cells may result in immune activation and

risk for the development of autoimmunity [31]. Failure to effectively clear apoptotic cell fragments is believed to be involved in the pathogenesis of autoimmunity in SLE and be responsible for the expression of autoantibodies against nuclear antigens that characterize this disease [32]. Recent studies by Ait-Oufella *et al.* [33] showing that mice deficient in lactadherin, a protein that mediates binding of apoptotic cells to phagocytes, have accelerated atherosclerosis and impaired regulatory T-cell function suggest that similar processes may be of importance also in atherosclerosis. Interestingly, the T-15 type natural antibodies that are produced by B-1 cells in the spleen recognize the same phospholipid epitope on both oxidized LDL and apoptotic cells, linking the autoimmune responses against these structures [34, 35]. The role of oxidized LDL natural antibodies is discussed in more detail by Chou *et al.* in another paper of this issue of the journal.

Innate immune responses contribute to plaque development

The innate immune system provides a rapid and efficient defence against most microorganisms encountered in daily life. It is based on an array of inherited pattern-recognition receptors that bind broad classes of pathogens [36, 37]. They are widely expressed and trigger immediate responses including phagocytosis and inflammation. These receptors recognize highly conserved pathogen-associated molecular patterns such as bacterial CpG-containing DNA, double-stranded RNA and lipopolysaccharides (LPS) that allow the immune system to discriminate nonself pathogens from self. Accumulating evidence suggests that pattern-recognition receptors also interact with endogenous neo-antigens or self antigens through molecular mimicry and that these interactions play important roles in atherosclerosis [38, 39]. Again, a good example comes from oxidized LDL that is recognized by a group of endocytic pattern-recognition receptors called scavenger receptors [40]. These receptors rapidly mediate uptake of oxidized LDL in macrophages leading to the formation of foam cells, one of the most characteristic features of the atherosclerotic plaque.

A major ligand in oxidized LDL recognized by the scavenger receptor CD36 is oxidized phosphatidylcholine [41, 42]. Interestingly, these receptors also bind the same epitope on apoptotic cells, thus providing a link between the immune responses to oxidized LDL and dying cells [35]. The ability of the innate immune system to rapidly recognize molecular patterns expressed by damaged self structures, such as oxidized LDL and apoptotic cells, and to remove these potentially cytotoxic structures by endocytosis before they cause injury to the surrounding tissue can be said to represent a form of protective innate autoimmunity.

The uptake of oxidized LDL by macrophage scavenger receptors also represents a mechanism for making cholesterol that has accumulated in the vascular extracellular matrix available for reverse cholesterol transport through the ABC-A1–Apo AI pathway. However, the function of this protective response is far from perfect. Macrophage scavenger receptors lack a negative feedback mechanism which makes it possible for the cells to literally engulf themselves to death [43]. Once macrophages have turned into foam cells they also appear to have a limited ability to leave the vascular tissue [44]. The results of scavenger receptor gene knock-out studies have been inconsistent and it remains to be fully clarified whether scavenger receptors have a protective function in atherosclerosis or if they contribute to disease process [45–47].

The second major group of pattern-recognition receptors consists of the Toll-like receptors (TLRs). In contrast to the scavenger receptors that mainly mediate phagocytosis the TLRs are signalling receptors that rapidly activate an inflammatory response against invading pathogens [48, 49]. Some TLRs are expressed on the cell surface and recognize pathogen membrane structures such as lipopeptides (TLR1), peptidoglycan and lipoteichoic acid (TLR2) and LPS (TLR4) whilst others appear to function at intracellular locations (primarily the endoplasmic reticulum) where they interact with double-stranded viral RNA (TLR3), single-stranded viral RNA (TLR7 and TLR8) and CpG-containing bacterial DNA (TLR9). Most TLRs use a common signal pathway involving the

Toll/IL-1 resistance (TIR) domains and the adapter protein myeloid differentiation protein-88 (MyD88).

Endothelial and macrophage expression of TLR1, TLR2 and TLR4 have been identified in atherosclerotic lesions [50, 51]. Evidence that they have a functional role in the disease process has come from studies demonstrating that deletion of MyD88 in apo E^{-/-} mice results in decreased lesion formation and reduced number of inflammatory cells in the remaining plaques as well as from studies demonstrating that hypercholesterolaemic mice lacking TLR2 or TLR4 develop less atherosclerosis [52–54]. These findings also imply that hypercholesterolaemia results in the formation of endogenous TLR ligands. Several endogenous molecules promoting sterile inflammation have been proposed to signal through TLR including HSP60 and 70, fibrinogen, extra domain A of fibronectin, high mobility group box-1 protein (HMGB1) and soluble hyaluronan [55], but in the presence of hypercholesterolaemia it appears more likely that the relevant ligand is lipid or lipoprotein derived. Again it appears logical to focus attention on the possible role of factors generated by the oxidation of LDL. However, although minimally oxidized LDL as well as oxidized phospholipids stimulate cytokine expression in cultured cells, this response appears to be mediated by non-TLR pathways. Oxidized phospholipids have actually been found to inhibit LPS-induced activation of TLR4 [56]. An alternative mechanism was recently identified by Shi *et al.* [57] who demonstrated that exposure of cultured macrophages to saturated fatty acids such as palmitate and oleate stimulates the expression of IL-6 and tumour necrosis factor- α through a TLR-4-dependent signal pathway and that this mechanism explained the release of pro-inflammatory cytokines in adipose tissue.

Autoantigen presentation in atherosclerosis

Dendritic cells present antigens for discrimination of self from nonself

Pathogens that break the early defence lines made up by innate immunity will be attacked by an adaptive immune response. The power of adaptive immunity

resides in its high degree of specificity and its ability to create an immunological memory. Specificity depends on stochastic generation of a huge repertoire of T and B cell receptors through somatic rearrangement of TCR and Ig gene fragments. Therefore, the immune repertoire is carried by these two cell types alone.

Adaptive immune responses require uptake, processing and presentation of antigens by professional antigen-presenting cells (APC) that continuously scout all compartments of the body. The dendritic cells (DCs) are the most effective APC, although macrophages and B cells can also perform this function. Small numbers of DCs are usually present in the intima and adventitia of normal arteries with a preferential location to low shear stress segments that are predilection sites for atherosclerosis [58, 59]. They also become enriched in atherosclerotic lesions suggesting that plaque-specific antigens are involved in the disease.

The role of DCs is to continuously present molecules to antigen-detecting cells in order to permit the latter to discriminate self from nonself. At the site of an infection immature DCs will pick up pathogenic antigens that simultaneously activate the DC through stimulation of their TLRs. Activation leads to maturation of DC. Mature DCs will cease to sample antigens and instead up-regulate adhesion molecules and migrate to lymph nodes where they present peptide fragments of antigens on MHC molecules to T cells. If such a peptide–MHC complex ligates the T-cell receptor, that particular T cell will respond by activation, clonal expansion and cytokine secretion. The DCs use MHC class I molecules to present intracellular-derived peptide antigens for CD8⁺ T cells, MHC class II molecules to present extracellular-derived peptide antigens for CD4⁺ T cells and CD1d to present lipid antigens for NKT cells. By simultaneously expressing different co-stimulatory molecules (such as CD40, CD 80, CD 86 and LICOS) or by actively sustaining from expressing any co-stimulatory molecules when presenting the antigen the APC can modulate the subsequent T-cell response [60, 61]. For example, DCs that take up antigens in the absence of TLR stimulation (i.e. a noninfectious situation) usually

present antigens without expressing co-stimulatory molecules which in turn provides a suppressive signal to T cells. Theoretically, this should be the situation when a DC encounters an antigen formed under disturbed metabolic conditions such as hypercholesterolaemia. However, the ability of adaptive immunity to react with high specificity against an enormous variety of different nonself antigens, many of which are very similar to self antigens, creates a considerable challenge for the immune system. This challenge must be even greater when the immune system encounters modified self antigens such as oxidized LDL. Indeed, the immune system needs to retain a certain level of autoreactivity to be able to respond sufficiently well to pathogens but failure to control this autoreactivity may result in the development of autoimmune disease.

The presence of oxidized LDL-specific T cells and IgG in most humans clearly demonstrates that adaptive immune responses against oxidized LDL exists [12, 14]. It will become important to clarify whether this response reflects pathological autoimmunity, protective immunity or perhaps a mixture of both. As discussed below, experimental animal studies in which mice with specific genetic immune deficiencies have been cross-bred with atherosclerosis-prone mice, such as apo E^{-/-} and LDL receptor^{-/-} mice, are providing the answer these questions. However, as both apo E and LDL receptors are critically involved in the process of lipid antigen presentation [62] it is of great importance to keep the limitations of these models in mind.

Antigen presentation by DCs in atherosclerosis

Although it appears logical to assume that APC will encounter antigens of importance for atherosclerosis in the vascular wall, this is probably not always true. Studies by Angeli *et al.* [63] in apo E^{-/-} mice have demonstrated that hypercholesterolaemia is associated with increased accumulation and activation of DCs in the skin. Interestingly, it was also found to be associated with impaired migration of DCs to draining lymph nodes and suppression of immunological priming due to the formation of oxidized phospholipids

such as platelet-activating factor. A consequence of this process is induction of dermal inflammation suggesting that local sequestration of activated DCs in the periphery may favour aggravation of local inflammation. This may occur at the cost of a lost tolerogenic response in draining lymph nodes. It does not seem unlikely that similar disturbances of DC function may occur in the environment of the atherosclerotic plaques (Fig. 2). The observation that inability of APCs

to take up apoptotic cell antigens and convey tolerogenic immune signals results in increased atherosclerosis [33] is well in line with this concept. Interestingly, studies of arteritis suggest that adventitial DCs are critically involved in tolerization against vascular self antigens but that entrapment of activated DCs in the adventitia leads to loss of local tolerance and activation of arterial inflammation [64]. An even more complex situation may arise if DCs also are

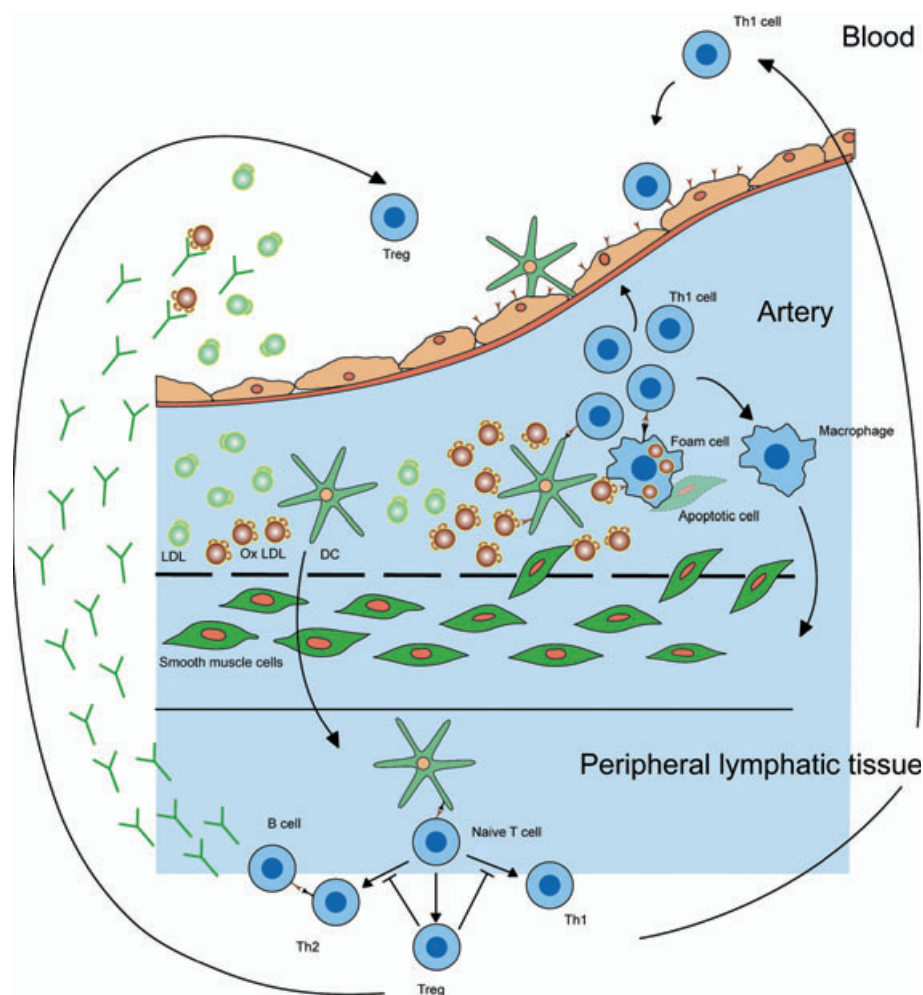


Fig. 2 Adaptive immune responses in the atherosclerotic plaque. LDL (green) accumulates in the arterial intima and is oxidized (oxLDL, red). oxLDL particles can be taken up by dendritic cells (DC), which migrate to lymph nodes and present oxLDL-derived antigens to naïve T cells. Upon antigen-specific activation, the latter differentiate into T effector cells (Th1, Th2, also Th17, which are not shown in the figure) or regulatory T cells (Treg). Circulating T effector and Treg cells can enter forming atherosclerotic lesions after adhesion to activated endothelial cells. When recognizing antigens presented to them by macrophages and DC, Th1 effector cells produce proinflammatory cytokines that promote vascular inflammation and the progression of atherosclerosis.

exposed to TLR ligands in the plaque because of invading microorganisms. Niessner *et al.* [65] reported that stimulation of plaque DCs with the TLR 9 ligand CpG results in activation of a T-cell attack on smooth muscle cells expressing markers of cellular stress providing one example as to how pathogens can induce pro-atherogenic autoimmunity. Indeed, considerable challenges appear to meet DCs entering atherosclerotic plaques. First they must be able to identify modified self antigens such as oxidized LDL and debris from degenerating cells as not being nonself. They also have to be able to discriminate almost identical HSP in stressed vascular cells from those expressed by microorganisms. Finally, they must resist signals provided by TLR activation and local entrapment to mount pro-inflammatory autoimmunity. Failing any of these tasks is likely to turn the DC into a potent pro-atherogenic cell.

Pro-inflammatory Th1 responses predominate in atherosclerosis

T cells are abundant in atherosclerotic lesions but the primary activation of T cells is more likely to occur in secondary lymphoid organs such as the peripheral lymph nodes to which DCs that have ingested atherosclerosis-relevant antigens migrate. In hypercholesterolaemia, periadventitial lymphoid infiltrates may actually form secondary lymphoid structures such as germinal centres [66, 67]. The primed T cell subsequently migrates to vascular tissues for a second activation by the same antigen and at this stage macrophages, B cells and endothelial cells may also act as APC. T-cell activation by APC is dependent on binding of the peptide/MHC complex to the T-cell receptor, an interaction that also requires binding of CD4 (for MHC class II) or CD8 (for MHC class I) to the MHC molecule. However, as discussed above, APC also use cell surface co-stimulatory molecules to regulate the mode of T-cell activation. Expression of the co-stimulatory signals CD80 and CD86 (also called B7.1. and B7.2) provides strong activating signals when interacting with CD28 on the T cell [68]. Other strong co-stimulatory signals are provided by CD40 interacting with the CD40 ligand on the T cell and the Ox40 ligand that interacts with Ox40 on the

T cell. Additional co-stimulatory pairs of molecules include CD137/CD137L and ICOS-L/ICOS. Binding to a T-cell receptor in the absence of co-stimulation not only fails to activate the T cell but also induces anergy so that the T cell will become refractory to subsequent stimulation by that specific antigen. As an alternative mechanism of control, certain ligands for co-stimulatory receptors confer inhibitory rather than stimulatory signals, such as CTLA-4 which competes with CD80 and CD86 for binding to CD28 on the T cell. Regulation of co-stimulatory signalling therefore represents an important mechanism to fine-tune immune responses and prevent autoimmunity. The APC may further modulate the T-cell response by secreting different types of cytokines [69]. Activated CD4⁺ T cells that are exposed to IL-12 and γ -interferon (INF- γ) develop into pro-inflammatory Th1 cells through activation of the transcription factor T-bet, whilst CD4⁺ T cells exposed to IL-4 develop into Th2 cells that function as inducers of B cells and eosinophils. There is solid evidence from hypercholesterolaemic animal models that activation of several of these pathways play important roles in the development of atherosclerosis. Transfer of CD4⁺ cells to apoE^{-/-}/SCID mice accelerates atherosclerosis [70], whilst hypercholesterolaemic mice deficient in IL-12, INF- γ , CD80/CD86, CD40 ligand, Ox40, CD4 and T-bet all develop less disease [71–80]. This clearly demonstrates the involvement of adaptive Th1 immunity in atherosclerosis.

Adaptive immune responses to lipid antigens similarly appear to promote plaque development as CD1d-deficient mice develop smaller lesions in response to both hypercholesterolaemia [81] and mechanical injury [82]. The role of Th2-type CD4⁺ T cells is less clear. Although IL-4 deficiency inhibits lesion development pointing to a pro-atherogenic role of Th2 immunity [71, 83], increased atherosclerosis has been observed in mice deficient in another Th2 cytokine IL-5 [84].

The role of CD8 cells in atherosclerosis remains to be fully characterized. Of note, these cells are more abundant in human lesions than in the arteries of hypercholesterolaemic mice on the C57BL/6 background. Treatment of hypercholesterolaemic mice with

a CD137 agonist leads to influx of CD8⁺ T cells into lesions in parallel with accelerated lesion development and plaque inflammation [85]. Furthermore, activation of CD8⁺ T cells to respond to an artificial transgenic antigen expressed by vascular smooth muscle cells causes dramatically increased atherosclerosis in hypercholesterolaemic mice [86].

In human atherosclerotic plaques, both CD4⁺ and CD8⁺ T cells are present, with a ratio of approximately 2 : 1 [87]. They are often located in the vicinity of MHC class II-expressing macrophages and DC, and the number of activated T is increased in culprit lesions from patients with acute coronary syndromes [88, 89]. T cells in human plaques predominantly express cytokines characteristic of Th1 activation [14, 90]. Although many T cells will be recruited unselectively to atherosclerotic lesions, almost 10% of T cells in human plaques are specific to oxidized LDL antigens [14].

Taken together, these observations suggest a scenario in which hypercholesterolaemia results in a situation where tolerance to oxidized LDL is broken due to disturbed APC function, which subsequently leads to induction of pro-inflammatory Th1 responses targeting arterial tissue (Fig. 2). If this concept is correct atherosclerosis should be considered as an autoimmune disease. However, it is also possible that the immune system normally maintains a low level of autoimmunity against oxidized LDL to facilitate the removal of these particles but loses control if arterial oxidized LDL accumulation becomes excessive.

Regulating tolerance

It is essential that effector T cells do not attack self antigens. Most autoreactive T cells are deleted in the thymus by negative selection but this process is far from complete because it would compromise the ability of recognizing pathogen antigens and some autoreactive T cells therefore escape [6]. It is possible that T cells that recognize altered self antigens, such as those expressed in oxidized LDL, also belong to the autoreactive T cells that escape negative selection.

The activity of autoreactive T cells is normally suppressed by regulatory T cells (Tregs) and loss of Treg function results in the development of autoimmune diseases [91]. Tregs also have an important function in limiting the injury made by nonself-specific effector T cells on host tissues. Interestingly, some pathogens have learned to use Tregs to down-regulate immunity resulting in chronic infections. Most Tregs require TCR recognition to be activated but their dependence on antigen specificity is much less studied than that of the effector cells. Several subtypes of Tregs have been identified. Natural CD4⁺ CD25⁺ Foxp3⁺ Tregs are developed in the thymus and then enter peripheral tissues where they constitute 5–10% of all T cells [6]. These natural Tregs have previously been believed to function by cell to cell contact inhibition but more recent studies suggest that they act by depleting effector cells of IL-2 [92]. In contrast to natural Tregs, the IL-10-producing T regulatory 1 (Tr1) and TGF- β -producing T helper 3 (Th3) cells are generated from naïve T cells in the periphery following antigen presentation and activation by DCs. Several lines of evidence have implicated dysregulation of Treg function in atherosclerosis [93]. Atherosclerotic plaques contain relatively few Foxp3-positive Tregs (1–5% of all T cells) compared to normal arterial tissue or inflammatory skin lesions where Tregs constitute about 25% of all T cells, suggesting that tolerance protection is impaired in atherosclerotic plaques [94]. There is also accumulating functional evidence for a protective role of Tregs in atherosclerosis. Depletion of Tregs through deletion of CD80/86, CD28 or ICOS as well as anti-CD25 antibody treatment significantly increases plaque formation [95, 96]. Similarly, inhibition of Th3 cells through deletion of the T-cell receptor for TGF- β markedly enhances the progression of the disease [97] whilst administration of a clone of ovalbumin-specific Tr1 cells together with its cognate antigen inhibited plaque development in apo E^{-/-} mice [98]. Our understanding of the role of regulatory T cells in atherosclerosis is still very incomplete but they represent a novel target for intervention of considerable interest. As discussed in this review it is clear that accumulation of LDL in the arterial wall and the subsequent development of atherosclerotic

lesions are associated with formation of several types of modified self antigens. As many of these modified self antigens are potentially toxic for surrounding cells it appears reasonable that they are recognized and removed by the immune system, i.e. a form of protective autoimmunity. However, it is also apparent that this autoimmunity must be tightly regulated to avoid unnecessary damage to the artery wall. It is reasonable to assume that regulatory T cells play an important role in this control and that plaque inflammation and cell death reflect a failure of the regulatory T cells to perform this function. If this line of reasoning is correct one could predict that stimulation of regulatory T cells should inhibit disease development, an approach that presently is investigated in many other autoimmune diseases. The drawback of this is that if regulatory T cells become too suppressive they will impair both CD8 T-cell-dependent tumour cell surveillance and the defence against infections. Accordingly, it is vital that any approach to design a Treg-mediated therapy is antigen specific and that the effect is restricted to atherosclerotic plaque tissue.

Therapeutic approaches targeting the immune system

As the importance of immunity in atherosclerosis has been revealed it has become increasingly interesting to determine whether this knowledge can be used to develop new treatments for cardiovascular disease. The first evidence that this could be possible came out from studies in which hypercholesterolaemic rabbits were immunized with oxidized LDL [99, 100]. The initial aim of these studies was to test whether activation of oxidized LDL immunity was associated with a more aggressive progression of disease but in contrast to expectations it was found that oxidized LDL-immunized animals developed a partial protection against atherosclerosis. This observation was subsequently confirmed in a number of different animal models of atherosclerosis [79, 101–105] and suggested the fascinating possibility that it could be possible to develop a vaccine for atherosclerosis based on antigens present in oxidized LDL. However, as oxidized LDL is a complex particle with an antigen composition that is difficult to

standardize and as it potentially may also contain harmful antigens it is in itself not an ideal vaccine component. Over the last few years considerable effort has therefore been put on characterizing the exact antigens in oxidized LDL that induce atheroprotective immunity.

The first antigens in oxidized LDL to be identified were the oxidized phospholipids. Palinski *et al.* [106] established a panel of B cell hybridomas from apo E^{-/-} mice and found that several clones produced antibodies specifically binding to modified phospholipids in oxidized LDL. The oxidized phospholipid antigens are not exclusively expressed by oxidized LDL but are also found on apoptotic cells and on some microorganisms such as *Streptococcus pneumoniae* [34]. These antigens are recognized by a subclass of IgM referred to as natural antibodies. They are usually defined as antibodies that are found in complete absence of exogenous antigenic stimulation and are produced primarily by B-1 cells in the peritoneal cavity and spleen [107]. They provide a first line of defence against invading microorganisms, but react also with self antigens associated with senescent cells and cellular debris. Binder *et al.* [108] were the first to study the functional role of anti-phospholipid antibodies in atherosclerosis by immunizing LDL receptor knockout mice with *S. pneumoniae*. This treatment was found to result in induction of high levels of oxidized LDL-specific IgM and a modest reduction in atherosclerosis. Fario-Neto *et al.* [109] subsequently showed that treatment with anti-phosphorylcholine IgM isolated from a T15 idiotype hybridoma reduced vein graft atherosclerosis in apo E^{-/-} mice. Finally, Caligiuri *et al.* [110] found that immunization with phosphorylcholine coupled to keyhole limpet hemocyanin (KLH) was associated both with a threefold increase in specific antibodies and a 40% reduction in atherosclerosis at the aortic root in the same type of mice. Taken together, these observations suggest that stimulation of the immune response against oxidized phospholipids represents one possible approach for the development of an immuno-modulatory therapy for atherosclerosis. The major challenges associated with this approach are: (i) the poor understanding of how the expression of natural antibodies is regulated;

(ii) effect of cross-reactivity with antigens expressed on other structures than oxidized LDL; (iii) relation to the anti-phospholipid antibodies associated with thrombotic disease.

The other major class of antigens in oxidized LDL are the peptide fragments generated as a result of proteolytic degradation and aldehyde-modification of apo B-100, the only protein permanently associated with LDL. These antigens have the advantage of being specific to oxidized LDL because they have a unique amino acid sequence. The antigens presented by MHC class II molecules are generally 13–17 amino acids long which make the chance for cross-reactivity with other peptide sequences minimal. Fredrikson *et al.* [111] used ELISAs based on a library of 20-amino acid-long polypeptides covering the complete apo B-100 sequence to identify a number of apo B-associated antigens recognized by autoantibodies present in human plasma. Immunization of apo E^{-/-} mice with several of these apo B-100 peptides was found to reduce atherosclerosis by up to 70 %, as well as to decrease macrophage and increase collagen contents of remaining plaques [112–114]. Immunizations resulted in a marked increase in specific IgG, but had only marginal effects on IgM levels. IgG expression also changed from IgG_{2a} to IgG₁ suggesting activation of a Th₂ response [113]. The best athero-protective response was found when immunizations were made with the apo B-100 peptide sequences between amino acids 16–35 (p2), amino acids 631–650 (p45) and amino acids 3136–3155 (p210) and native peptides were generally more effective than MDA-modified peptides. Interestingly, subsequent clinical studies have shown that high levels of IgG against native p45 and p210 are associated with a lower risk of cardiovascular disease [115] whilst high antibody levels against the corresponding MDA peptides appear to be markers of more severe disease [111, 116]. It remains to be fully understood how these observations should be interpreted but they may imply that immune responses against more mildly modified forms of LDL are more beneficial than immune responses against severely oxidized forms of LDL. The high specificity and the possibility of producing

standardized vaccine preparations are some advantages of apo B peptide-based vaccines and human vaccines are presently in preclinical development. The disadvantages with this approach include the need for human leucocyte antigen (HLA) genotyping of patients before treatment and the risk that vaccines need to be individualized depending on HLA type. However, as the protective effect of oxidized LDL immunization does not require functional CD4 cells, the role of HLA-dependent antigen presentation in this process needs to be studied further [79].

A limitation with both phosphorylcholine- and apo B peptide-based vaccines is that the mechanism of action is poorly understood. In mice immunized with oxidized LDL a more effective inhibition of atherosclerosis is seen in the mice with the highest antibody response suggesting that the protection may be mediated by antibodies [104]. Several other observations also favour this possibility. Treatment with polyclonal IgG significantly reduces the development of atherosclerosis in apo E^{-/-} mice and B cell reconstitution rescues apo E^{-/-} mice from the enhanced development of atherosclerosis caused by splenectomy [117, 118]. Schioppa *et al.* [119] produced human recombinant IgG against an aldehyde-modified form of the apo B p45 peptide and demonstrated that four treatments with this antibody reduced atherosclerosis by almost 50% over a 5-week period. An even more dramatic effect of p45 IgG treatment was observed in mice carrying the human gene for apo B-100 [120] and studies performed in apobec^{-/-}/LDL receptor^{-/-} mice demonstrated that antibody treatment markedly increased plaque regression caused by transferring the mice from a high-fat to a low-fat diet [121]. Although these studies collectively provide strong support for the notion that the protective effect of immunization depends on generation of specific antibodies, other mechanisms may also be involved. If autoimmune Th1 responses against oxidized LDL and other plaque-specific antigens are involved in the disease process, they should be susceptible to down-regulation by antigen-specific Tregs. One frequently used approach to induce tolerogenic responses is by mucosal (oral or intra-nasal) immunization [122]. Oral tolerance is an important physiological mechanism to

avoid development of delayed-type hypersensitivity and other allergic reactions to food proteins. van Puijvelde *et al.* [123] recently reported that oral administration of oxidized LDL is associated with suppression of atherosclerosis and that this is associated with an induction of antigen-specific Tregs in peripheral lymphoid tissues. Inhibition of atherosclerosis has also been observed following mucosal immunization with HSP 60/65 and β 2-glycoprotein I [124–126] further supporting the notion that induction of tolerance against atherosclerosis-associated antigens by mucosal immunization represents one promising approach for future atherosclerosis vaccine development. However, experience from other autoimmune disease points to difficulties in developing oral tolerance-based immunotherapy for humans. Although oral administration of disease-specific antigens effectively has prevented development of multiple sclerosis, type 1 diabetes and rheumatoid arthritis in animals, clinical trials to treat ongoing disease in humans has thus far been unsuccessful [127]. Whether activation of antigen-specific regulatory T cells is also involved in the effect of subcutaneously administered oxidized-LDL antigen vaccines remains to be elucidated but the finding that injection of high doses of oxidized LDL in neonatal apo E^{-/-} mice results in suppression of T-cell responses against oxidized LDL and inhibition of atherosclerosis provides some support for this possibility [128].

Future perspectives

The development of atherosclerosis involves a complex interplay of inflammation, autoimmunity and tissue-specific degeneration. The possibility of preventing and treating diseases with similar characteristics by targeting the immune system is presently being tested clinically for type 1 diabetes, rheumatoid arthritis, multiple sclerosis and Alzheimer's disease, whereas relatively little has been done so far to explore this possibility for the treatment of cardiovascular disease. Since the first evidence of involvement of immune responses in atherosclerosis was discovered more than 20 years ago, a large number of studies performed in various animal models have clearly established that it plays a pivotal role in the disease

process. Animal studies have also provided proof-of-principle support that it is possible to inhibit the development of atherosclerosis by modulating immune responses against plaque antigens by vaccines. It can be expected that a number of vaccines and other immunomodulatory treatments for atherosclerosis will enter into clinical testing within the next few years. This work will greatly benefit from learning the lessons made during early clinical testing of immunomodulatory treatments for other chronic diseases [129]. One important thing to be learned from this work is the need for internationally standardized and validated assays for immunological biomarkers. These will be critical both for identification of patients who will benefit from treatment as well as for monitoring the effect of treatment. We also need to expand our knowledge of the association between HLA type and cardiovascular disease. These associations are well characterized for many other chronic diseases with autoimmune components but they remain relatively unexplored when it comes to cardiovascular disease. The use of peptide-based vaccines will require that the patient has an HLA type that makes it possible for the peptide to be presented. It will also be important to carefully consider the side effects that may be associated with this type of treatments. Vaccines targeting autoimmunity are frequently designed to stimulate immunosuppressive Tregs. However, if this immunosuppression is not restricted to the affected tissue, there is a risk that the defence against infections as well as immune-dependent tumour surveillance will be compromised. When inducing an antibody response against a common endogenous antigen the risk of causing tissue injury by accumulation of immune complexes must also be considered. The immune system represents a novel and promising target for prevention and treatment of cardiovascular disease. The time when this concept is ready for clinical testing is rapidly approaching but it will be important not to underestimate the difficulties that will be encountered in transferring the promising results from experimental animals into humans.

Conflict of interest statement

No conflict of interest was declared.

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