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Apolipoprotein B100 autoimmunity and atherosclerosis – disease mechanisms and therapeutic potential

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Abstract

Purpose of review

Adaptive immune responses have been shown to play an important role in the atherosclerotic disease process and both pathogenic and protective immunity has been identified. Apolipoprotein (apo) B100 appears to be a key antigen and novel therapies modulating immune responses against apo B100 have shown promising results in experimental models. This review will discuss recent developments in the mechanistic understanding of apo B100 autoimmunity and approaches taken to use this knowledge for development of novel therapies.

Recent findings

It has recently been shown that not only apo B100 modified by oxidation but also non-modified apo B100 is targeted by autoimmune responses. This implies that a corresponding set of regulatory T cells with the same antigen specificity must exist and that these cells under normal circumstances are able to prevent autoimmunity against LDL. Recent studies also suggest that the athero-protective effect of apo B100 peptide immunization acts by re-enforcing the activity of such cells.

Summary

These novel findings suggest that aggravation of plaque inflammation may occur as result of a local loss of tolerance against LDL in the plaque due to insufficient activity of regulatory T cells. Restoration of lost tolerance represents an interesting novel approach for treatment of cardiovascular disease.

Key words: Autoimmunity, atherosclerosis, LDL, vaccine

Introduction

Atherosclerosis is characterized by a chronic inflammation affecting large- and mid-sized arteries and it has been established that this inflammation primarily is caused by an accumulation and oxidative modification of low density lipoprotein (LDL) in the artery wall [1]. The pro-inflammatory properties of oxidized LDL have been attributed to the generation of lysophospholipids and lipid peroxides as well as to general cytotoxicity. More recently it has also been found that this inflammation is modulated by immune responses against oxidized LDL antigens suggesting the involvement of autoimmunity in the atherosclerotic disease process [2, 3]. Interesting novel observations suggest that these autoimmune responses may not only target modified self-antigens in oxidized LDL but also un-modified apolipoprotein B (apoB)100 [4]**. There are several possible reasons for the existence of autoimmune responses against apo B100 and other antigens in oxidized LDL. The immune system is forced to allow a certain presence of autoreactivity in order to avoid limiting the immunological diversity required to mount effective immune attacks against all different forms of foreign antigens. Many bacterial proteins are for example very similar to proteins present in human cells and strictly deleting immune responses against such human proteins would compromise the defense against infections. The autoreactive T effector cells that escape deletion in the thymus are normally controlled by immune-suppressive regulatory T cells (Tregs) with corresponding self-antigen specificity preserving immunological tolerance. However, presentation of self-antigens in an inflammatory environment, such as an atherosclerotic plaque, favors a shift towards generation of pro-inflammatory Th1 cells and may result in loss of tolerance. It is also possible that the modifications that take place in response to oxidation are so significant that some antigens in the LDL particle are no longer recognized as self by the immune system. Interestingly, there is also evidence that some of the autoimmune responses against LDL antigens are protective and may facilitate oxidized LDL

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removal as well as to limit oxidized LDL-induced inflammation in a site-specific manner [2]. This has generated interest in the possibility of developing immune-modulatory therapies specifically targeting and enforcing athero-protective immune responses [5]. This review will focus on immune responses against apo B100 and how these can be targeted by novel intervention strategies.

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Oxidized LDL and atherosclerotic plaque inflammation

LDL particles that accumulate in the artery wall may become oxidized within a few hours resulting in activation of the transcription factor NF-κB that subsequently initiates the expression of pro-inflammatory cytokines and adhesion molecules [6]. Several mechanisms through which oxidized LDL can activate inflammation have been identified. Accumulating evidence indicate a role for the toll-like receptors (TLR) 2 and 4, that are key regulators of innate immune responses, in recognition of oxidized LDL [7, 8]. TLR4 has also been shown to mediate uptake of LDL in macrophages [9, 10]. Consistently, deletion of TLR2, TLR4 or MyD88, a key signal transduction protein of TLR 2 and 4, markedly reduces lesion development in hypercholesterolemic mice suggesting that activation of this pathway has an important role in atherosclerosis [11]. Lysophosphatidylcholine (lysoPC) and its metabolite lysophosphatidic acid (LPA) have been proposed to mediate the inflammatory effect of oxidized LDL. LysoPC is formed when oxidized phospholipids in LDL are hydrolysed by lipoprotein-phospholipase A2 (Lp-PLA2). Both lysoPC and LPA activate inflammatory responses in cultured vascular cells and recent studies have shown strong associations between the human atherosclerotic plaque contents of lysoPC, LPA and pro-inflammatory cytokines [12]. The importance of these lipid mediators is further supported by observations that treatment with an inhibitor of Lp-PLA2 reduces the development of coronary

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atherosclerosis in experimental animals [13] and halts the progression of plaque necrotic core expansion in man [14]. Ketelhuth and co-workers recently identified a sequence in apo B100 that when presented as a fragment to human mononuclear leukocytes activated the expression of IL-6, IL-8 and CCL2 [15]**. This apo B100 fragment was referred to as the apo B100 danger associated signal 1 (ApoBDS-1). It was shown to be present in a biologically active form in human atherosclerotic plaques and to function through activation of calcium signalling and mitogen-activated protein kinase indicating involvement of a specific receptor.

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Adaptive immune responses against oxidized LDL antigens

The first indication of the existence of autoimmune response against oxidized LDL came from studies identifying the presence of oxidized LDL-specific antibodies in human serum [16]. This notion of oxidized LDL autoimmunity was subsequently supported by findings of oxidized LDL-specific autoantibodies and T cells in atherosclerotic plaques [17, 18]. Early studies aiming to characterize the functional role of these immune responses unexpectedly showed that immunization with oxidized LDL resulted in a partial protection against atherosclerosis development rather than the anticipated aggravation of disease [19, 20]. This outcome was initially difficult to explain as other studies carried out at the same time appeared to confirm the original hypothesis that adaptive immune responses activated by hypercholesterolemia were pro-atherogenic [21]. However, as these studies continued to evolve it became evident that the role of the immune system in atherosclerosis was much more complex than originally anticipated. Deletion of cytokines and transcription factors required for $CD4^+$ Th1 effector cell function was consistently found to be associated with reduced development of atherosclerosis whereas studies on Tregs uniformly pointed to a protective role of the latter cells [3]. The role of $CD4^+$ Th2 effector T cells appears to be more

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multifaceted. There is evidence of a protective role of the Th2 cytokine IL-5 through stimulation of B1 cells to secrete so called natural IgM antibodies, some of which are binding to phospholipids on oxidized LDL and the plasma membrane of apoptotic cells. IL-33, a potent inducer of Th2 T cell responses, has also been shown to reduce atherosclerosis [22]. In contrast, there is experimental data suggesting a pro-atherogenic role of the Th2 signature cytokine IL-4 [3].

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Targeting cellular and humoral immune responses against apo B and oxidized LDL to inhibit atherosclerosis

The observation of an athero-protective effect of oxidized LDL immunization taken together with the increased understanding of the role of different T cell subsets in atherosclerosis has stimulated efforts to develop novel immune-modulatory therapies for treatment and prevention of cardiovascular disease (figure). We were able to identify several native and malondialdehyde (MDA)-modified peptide sequences in apo B100 that were recognized by autoantibodies in human plasma and showed that several of these peptides could inhibit the development of atherosclerosis when used to immunize *Apoe*$^{-/-}$ mice [23, 24]. The athero-protective effect of a prototype vaccine based on the MDA-modified apo B100 peptide p45 (amino acids 661-680) was shown to be associated with generation of MDA-p45 specific IgG [25]. To investigate the role of these antibodies recombinant human IgG with the same specificity were generated. Treatment of *Apoe* $^{-/-}$ mice with these antibodies was found to inhibit the development of atherosclerosis, reduce inflammation of remaining plaques and to facilitate plaque regression when applied in combination with lipid lowering [26, 27]. The mechanisms involved in the athero-protective effect of these antibodies remains to be fully characterized but appear to involve facilitation of oxidized LDL removal as well as

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neutralization of the pro-inflammatory effects of oxidized LDL. The possible athero-protective effect of these antibodies in humans is presently being investigated in the GLACIER trial (Goal of oxidised Ldl and ACtivated macrophage Inhibition by Exposure to a Recombinant antibody, www.clinicaltrials.gov).

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Although the studies described above point to an important role of antibodies in mediating the effect of immunization with apo B100 peptides other studies have shown that protection can occur also in the absence of an antibody response [28]. Recent studies by Wigren et al [29, 30]* have suggested that this protection involves activation of Tregs and that it may be enhanced by the use of aluminum-containing adjuvants. The possibility that the protective effect of apo B100 peptide immunization is mediated by activation of Tregs is in line with the documented athero-protective effect of these cells [31] as well as with studies demonstrating that mucosal delivery of oxidized LDL is associated with inhibition of atherosclerosis [32]. Mucosal delivery of antigens is a well-established approach to induce tolerance and studies by van Puijvelde et al [32] have shown that oral administration of oxidized LDL results in generation of oxidized LDL-specific Tregs. Taking advantage of this knowledge Klingenberg and coworkers [33] developed an intranasal vaccine based on recombinant fusion protein containing the native apo B sequence 3136-3155 (p210) and the B-subunit of cholera toxin (CTB). CTB promotes uptake of antigens via the nasal and oral mucosa and CTB-antigen conjugates have evolved as a promising therapeutic strategy to induce tolerance in a variety of autoimmune diseases. Immunization with p210-CTB for 12 weeks caused a reduction in aortic lesion size of *Apoe* $^{-/-}$ mice. The reduction of lesion size was accompanied by induction of regulatory T cells that markedly suppressed effector T cells re-challenged with apo B100 and increased numbers of interleukin (IL)-10^{+}CD4^{+} T cells. Another approach to activate apo B100 tolerance was applied by Herbin *et al.* [34]** that used subcutaneously implanted

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osmotic mini-pumps to continuously administer low doses of apo B100 peptides in absence of adjuvant during a 2-week period. This treatment lead to a reduction of lesion development in young *Apoe* $^{-/-}$ mice and abrogated atherosclerosis progression in older *Apoe* $^{-/-}$ mice. The protection conferred by apo B100 peptide administration was associated with activation of an apo B100-specific Treg response and could be cancelled by depletion of Tregs.

It is interesting to note that the different types of apo B100 peptide vaccines that have been shown to be athero-protective almost exclusively contain native peptides rather than peptides modified by aldehydes or other oxidation products suggesting that the target antigen could be present in un-modified rather than in oxidized LDL. A possible explanation to this apparent paradox was recently provided by Hermansson and co-workers [4]** that identified the existence of apo B100 reactive $CD4^+$ T cells. This observation came out of studies in which human apo B100 transgenic mice were immunized with oxidized LDL and T cell hybridomas subsequently generated by fusing cells from draining lymph nodes with thymoma cells. Unexpectedly, it was found that several of the generated hybridomas were specific for apo B100, whereas none reacted to oxidized LDL. The apo B-reactive T cell hybridomas were all characterized by expression of the T cell receptor variable β chain TRB31. Immunization of the mice with a TRB31-derived peptide blocked T cell recognition of apo B100 and inhibited the development of atherosclerosis. It remains to be fully clarified if these apo B100 autoreactive T cells target completely normal LDL particles or only particles with minor modifications. These observations still have considerable implications as they suggest that the existence of apo B100-reactive T cells may not be associated to atherosclerosis pathology in itself. If this notion is correct atherosclerosis would instead develop when Tregs with the corresponding antigen-specificity lose control over these cells. It is an interesting possibility

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that apo B100 peptide vaccines work by targeting the balance between apo B100-reactive T effector and Tregs.

Most studies have focused on $CD4^+$ cells as being the most important T cells in atherosclerosis. However, Chyu et al [35]* recently reported that adoptive transfer of $CD8^+$, but not $CD4^+$ T cells from mice immunized with apo B100 p210 vaccine reduced atherosclerosis in recipient of *Apoe*$^{-/-}$ mice. They also presented evidence that this effect was mediated through $CD8^+$ T cell cytolysis of dendritic cells. Studies carried out in mice lacking the ability to present antigens to $CD8^+$ T cells through MHC class I did not demonstrate any effect on atherosclerosis [36], but this does not exclude that both pro- and anti-atherogenic sub-sets of $CD8^+$ T cells may exist. Accordingly, it is possible that both $CD4^+$ and $CD8^+$ T cells are involved in mediating the protective effect of apo B100 peptide immunization.

There has also been attempts to further enhance the efficacy of apo B peptide vaccines by including other antigens in the vaccine and Lu *et al.* [37] have reported that immunization with the apo B100 p45 peptide in combination with a peptide derived from HSP60 is more effective than immunization with the apo B100 p45 peptide alone.

Dendritic cell therapy

Dendritic and other antigen presenting cells play a key role in determining the nature of T cell activation. Dendritic cells that express co-stimulatory molecules, such as CD80 and CD86, and secrete IL-12 activates differentiation and clonal expansion of pro-inflammatory effector T cells, while dendritic cells that interacts with co-inhibitory molecules, such as CTLA4 and PD1 on T cells, in presence of IL-10 induce Tregs [38]. The expression of co-stimulatory

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molecules and IL-12 is typically induced by activation of TLR receptors. Hjerpe *et al.* [39] reported that dendritic cells that have been isolated, pulsed with MDA-LDL in presence of the TLR4 activator LPS and subsequently transferred into apo $E^{-/-}$ mice aggravate atherosclerosis. However, using an almost identical design Habets et al [40] found that injection of dendritic cells pulsed with oxidized LDL inhibited the development of atherosclerosis in *Ldlr*$^{-/-}$ mice. The reason for these contradictory observations remains to be clarified but both findings underline the possibility of using *ex vivo* modulation of dendritic cell function as a therapeutic approach for prevention and treatment of atherosclerosis. This possibility was further investigated in a study by Hermansson *et al.* [41]** who showed that injection of human apo B100 transgenic mice with dendritic cells pulsed with apo B100 in the presence of IL-10 inhibits the development of atherosclerosis as compared with injection of dendritic cells pulsed with Apo B100 alone.

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Biomarkers of apo B100 and oxidized LDL autoimmunity

The most commonly used approach to study presence of autoimmune responses against oxidized LDL has been to determine circulating levels of autoantibodies against copper-oxidized or MDA-modified LDL. The presence of different organ-specific autoantibodies is a diagnostic criterion in many autoimmune diseases. This is not the case for oxidized LDL autoantibodies and cardiovascular disease, suggesting that atherosclerosis is not an autoimmune disease in the traditional sense. Instead autoantibodies against oxidized LDL can be detected in almost all individuals and the levels are often highest at an early age before the onset of clinical disease [42]. A large number of studies have been carried out to determine the association between autoantibodies against oxidized LDL and cardiovascular disease. However, the results of these studies have not been consistent with some reports

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demonstrating inverse associations between the level of circulating autoantibodies against oxidized LDL and the severity of atherosclerosis while others have shown the opposite association [43]. The reason for these inconsistencies remains to be fully clarified but may involve difficulties in standardizing the LDL antigens used in the antibody assays as LDL is extremely sensitive to modifications *ex vivo*. Notably, many of these studies also determined antibody levels against native LDL but only used these measures for background subtraction purposes. One approach to overcome the standardization problem of oxidized LDL antibody assays has been to determine autoantibodies against specific native or MDA-modified apo B100 peptide sequences. These studies have primarily used the p45 (amino acids 661-680) and p210 (amino acids 3136-3155) apo B100 peptides used in the development of immune-modulatory therapies discussed above. Inverse associations have been found between the level of autoantibodies against these apo B100 peptides and the severity of atherosclerosis and subjects that later suffered from acute myocardial infarction has been reported to have lower apo B peptide autoantibodies levels than controls matched for age and gender [44-47].

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Conclusions

It has become well established that immune responses against atherosclerotic plaque antigens play an important role in the disease process and that both pathogenic and protective immune responses exist. It was originally assumed that modified self-antigens, such as oxidized LDL, were the main targets for these immune responses but recent research implies that immune responses against native apo B100 may be of equal importance. The identification and cloning of the T cell receptor of apo B100 autoreactive $CD4^+$ cells represents a particularly important breakthrough in this respect and suggest that there exists a naturally occurring autoimmunity against LDL. As a consequence, this implies that a corresponding set of Tregs with the same

antigen specificity must exist and that these cells under normal circumstances are able to prevent autoimmunity against LDL. Recent studies also suggest that the athero-protective effect of apo B100 peptide immunization acts by re-enforcing the activity of such cells. Taken together, these novel findings suggest that aggravation of plaque inflammation and disease progression may occur as result of a local loss of tolerance against LDL in the plaque due to insufficient recruitment or activation of Tregs. If this is true, immune-modulatory therapy enhancing LDL tolerance represents an interesting novel approach for prevention and treatment of cardiovascular disease.

Key points

- Immune responses against atherosclerotic plaque antigens play an important role in the disease process and both pathogenic and protective immunity has been identified.
- It was originally assumed that modified self-antigens generated as a result of LDL oxidation were the most important auto-antigens in atherosclerosis but recent findings demonstrate that T cells that react with un-modified apolipoprotein B100 also exist.
- These apo B100 auto-reactive T cells should normally be controlled by regulatory T cells with corresponding antigen specificity, but this control appears to be lost in atherosclerotic lesions.
- Novel therapies enforcing apo B100 tolerance have shown promising results in experimental models.

Acknowledgements

Disclosures

Jan Nilsson is signed as co-inventor on patents describing the use of immune-modulatory therapy for atherosclerosis and has assigned patent-rights to Forskarpatent, Sweden. Forskarpatent has licensed patent-rights to Cardiovax, US and Bioinvent, Sweden. Jan Nilsson has received research support from Cardiovax and Bioinvent.

Figure legend

Apo B100 autoimmunity and novel therapeutic strategies modulating apo B100 autoimmune responses. Immunization with apo B100 peptides inhibits the development of atherosclerosis in mice. Modified and unmodified apo B100 peptides are recognized by autoantibodies in human plasma and treatment of mice with these antibodies can inhibit the development of atherosclerosis. Adoptive transfer of dendritic cells pulsed with apo B100 in the presence of IL-10 inhibits the development of atherosclerosis in mice. Also, adoptive transfer of $CD8^+$ T cells from mice immunized with apo B100 p210 vaccine reduced atherosclerosis in recipient mice. The existence of apo B100 reactive $CD4^+$ T cells raises the possibility that Tregs with the corresponding antigen-specificity exist to control these apo B-reactive T cells. When the balance between apo B100-reactive T effector and Tregs is shifted in favour of the effector T cells, a local loss of tolerance against LDL in the plaque could aggravate atherosclerosis. Restoration of lost tolerance represents an interesting novel approach for treatment of cardiovascular disease. ApoBDS-1 = apo B100 danger associated signal 1.

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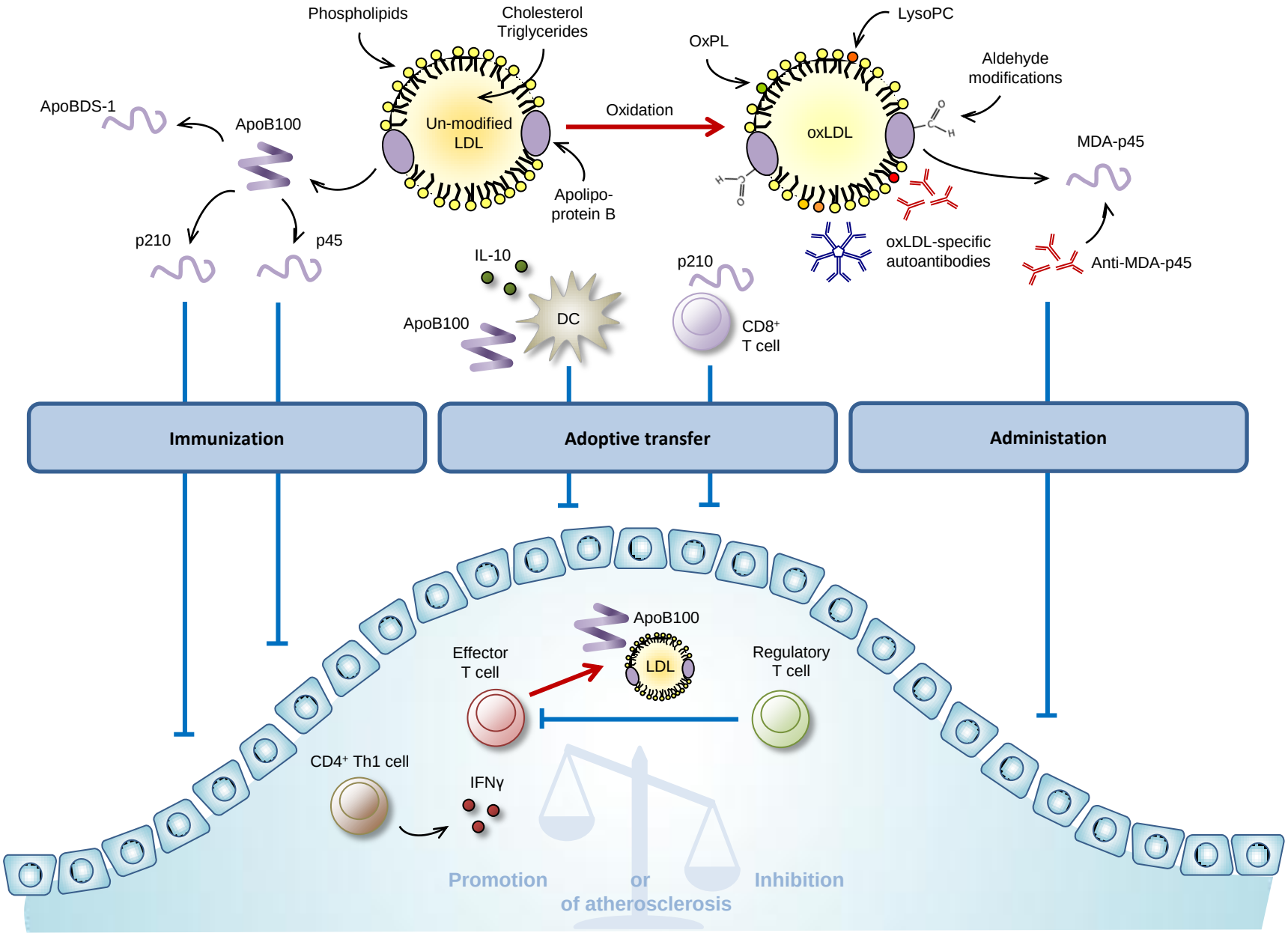
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LDL
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OxPL
LysoPC
Aldehyde
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oxLDL
ApoBDS-1
ApoB100
Apolipo-
protein B
MDA-p45
p210
p45
oxLDL-specific
autoantibodies
Anti-MDA-p45
IL-10
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DC
p210
CD8⁺
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