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Vaccination to Modulate Atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease of the artery wall. Adaptive immunity plays a key role in the pathogenesis of atherosclerosis. Recently, modulation of the immune response against atherosclerotic plaque antigen(s) has attracted attention as a potentially preventive and therapeutic approach. Here we review a series of studies on immunization with various antigens targeting treatment and prevention of atherosclerosis. Atherosclerosis-related antigens include oxidized LDL, apolipoprotein B-100, and heat shock protein 60/65. Accumulating evidence supports the idea that immunization with these antigenic proteins or peptides may reduce atherosclerosis. In this review, we discuss the current status of immunization studies and possible associated mechanisms of atheroprotection.

Keywords

Atherosclerosis; immunization; apolipoprotein B-100; low density lipoprotein; heat shock protein 65

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by intimal deposition of lipoproteins in large and medium-sized arteries. It is accompanied by innate and adaptive immune responses (1–3). Atherosclerosis is thought to be caused by a chronic response to arterial injury. In the last two decades, the role of local arterial tissue inflammation has been hypothesized to be a major underlying mechanism in the development of atherosclerosis (4–6). Subsequent investigations into the pathogenesis of atherosclerosis have revealed that both innate (7–9) and adaptive (3, 10) immunity play a significant role in the development and progression of atherosclerosis (4, 11, 12). While several auto-antigens have been suggested to trigger adaptive immune responses, the top three candidates for activating T cell mediated immune responses are oxidized LDL (oxLDL) (13), apolipoprotein B-100 (ApoB-100) (14, 15) and heat shock protein (HSP) 60/65 (16).

The current management of atherosclerotic vascular disease includes statins, antiplatelet drugs and antihypertensive compounds (17). However, several randomized clinical trials of statins and other preventive treatments suggest that it is difficult to achieve relative risk reductions exceeding 40% with current strategies (15). Accordingly, modulation of immune responses against atherosclerotic plaque antigens has attracted attention as a novel therapeutic strategy. In this review, we discuss recent progress in our understanding of the role of adaptive immunity in atherosclerosis, which provides a rationale and blueprint for the development of vaccines for modulation of adaptive immunity and prevention of cardiovascular disease.

Adaptive and humoral immunity in atherosclerosis

Adaptive immunity's role in atherosclerosis was first hypothesized based on correlations between immunological biomarkers from blood samples of heart disease patients and examinations of atherosclerotic lesions (1). Serum titers of antibodies against HSP60 or HSP65 (18) and against oxLDL (19, 20) were found to be elevated in cardiovascular disease. Additional evidence suggests that T cells mediate proinflammatory and regulatory immune responses in atherosclerosis. In *Rag1^{-/-} Ldlr^{-/-}* mice, development of atherosclerotic lesions was significantly reduced compared to immunocompetent *Ldlr^{-/-}* mice (21), suggesting that lymphocytes play a role in atherosclerosis. Zhou et al showed that *Apoe^{-/-}* mice with severe combined immunodeficiency (SCID) developed less severe atherosclerosis compared to immunocompetent *Apoe^{-/-}* mice (22). Importantly, transfer of CD4⁺ T cells from *Apoe^{-/-}* to immunodeficient *Apoe^{-/-}* mice aggravated atherosclerosis (22), strongly suggesting involvement of CD4⁺ T cells.

Patients with autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis have a significantly enhanced risk of atherosclerotic cardiovascular disease (23), which also supports the notion that adaptive immune responses are involved in its pathogenesis.

Humoral immunity

As mentioned above, patients with clinical evidence of atherosclerosis develop high titers of IgG and IgM antibodies to (modified) LDL (19, 20). In some studies, antibody titers were found to be inversely related to atherosclerosis (24), but other studies found a positive correlation (25, 26); thus, the relation between antibody titers and atherosclerotic disease burden and progression remains unclear. While so-called “natural” IgM antibodies can be produced by B1 cells without T cell help (27), most IgG production is dependent on isotype switching and requires T cell help (28). Specifically, follicular helper CD4⁺ T cells (T_{FH}) are required for isotype switching (28).

B-1 cells secrete natural antibodies that are predominantly IgM and IgA (2). Several studies suggest that natural IgM antibodies have atheroprotective effects. Direct experimental evidence has shown that *Ldlr^{-/-}* mice deficient in serum IgM developed larger atherosclerotic lesions when compared with wild-type *Ldlr^{-/-}* mice (29). In humans, levels of plasma IgM reactive to oxLDL have an inverse relation to carotid atherosclerotic plaque size (24) and to coronary artery disease (20).

IgG antibodies are produced by B-2 cells in a T_{FH}-dependent manner (2). T-helper (Th)1 and Th2 cells specifically activate mature B cells to produce IgG2a and IgG1 subclasses, respectively (30). In human studies, controversial results have been obtained with regard to association between anti-oxLDL IgG antibody and coronary artery disease. Some studies showed a positive correlation (26, 31), whereas other study showed a negative relationship (32).

T cells

How T cells affect the development and progression of atherosclerosis has been an area of intense study. T cells express either $\gamma\delta$ or $\alpha\beta$ T cell receptors. In general, $\alpha\beta$ T cells express either CD4, a co-receptor for MHC-II, or CD8, a co-receptor for MHC-I. CD4⁺ T cells are further broadly categorized into functional subsets, such as into Th1, Th2, Th17, regulatory T cells (Treg) and T_{FH} cells. T_{FH} typically do not leave the lymph node, but rather stay in or near the germinal centers.

Th1 subsets are predominant in atherosclerotic plaque in humans and mice (33, 34), and cytokines associated with this subsets (such as IFN- γ) have been shown to play an important role in the pathogenesis of atherosclerosis based on several studies. Inhibition of Th1 polarization in *Apoe*^{-/-} mice results in significant reduction of atherosclerotic lesion (35). While administration of IFN- γ increases atherosclerotic plaque (36), reduction of atherosclerotic lesion have been observed in IFN- γ deficient mice (37, 38) and IFN- γ receptor knockout mice (39). Consistently, deficiency of T-bet, which is the key transcription factor determining Th1 lineage, results in significant decrease of atherosclerosis in *Ldlr*^{-/-} mice as well as shift of immune response toward Th2 (40). These observations support the evidence for the pathogenic role of Th1 cells in atherosclerosis.

Tregs are negative regulators of immune effector T cells. Natural Treg cells (nTreg cells) are produced in the thymus, and recognize auto-antigens. Naive T cells in the periphery can acquire Foxp3 expression and consequently Treg function, which are called induced Treg (iTreg). Although both nTregs and iTregs express CD4, CD25 (IL-2R), Foxp3, and CTLA-4 and have overlapping function, each subset differentiates distinctly and has different epigenetic status (41). The differentiation of nTreg cells in the thymus is involved with increased interactions with self-peptide-MHC complexes, while the differentiation of iTreg cells likely occurs in response to non-self antigens including allergens, food, and the microbiota (42). The balance between effector and regulatory T cells is believed to vary based on the antigen, the type of antigen-presenting cell (APC) and the co-stimulatory molecules. Besides Foxp3⁺ Tregs, it is thought that other types of Treg cells are induced from naive T cells in the periphery (43). CD4⁺ T cells secreting IL-10 and TGF- β , called Tr1 cells, are produced by antigenic stimulation of naive T cells in the presence of IL-10 (44, 45). Antigen-specific TGF- β -secreting T cells, called Th3 type Tregs, are TGF- β dependent and were discovered in the context of oral tolerance (46). Several studies have reported that Tregs may be protective in atherosclerosis. Adoptive transfer of Tr1 cells induced a significant decrease in Th1 responses revealed by reduced IFN- γ production, increased IL-10 production and significant reduction in atherosclerotic lesion size when compared

with control mice (47). Adoptive transfer of CD4⁺CD25⁺ nTregs into *Apoe*^{-/-}*Rag2*^{-/-} mice ameliorated atherosclerosis in the aortic root (48).

The possible involvement of Th2 cells to progression of atherosclerosis remains to be elucidated. Both *IL-4*^{-/-}*Apoe*^{-/-} mice and *IL-4*^{-/-}*Ldlr*^{-/-} mice have shown no significant difference in atherosclerotic lesion compared to *IL-4* wild-type control mice (49). In contrast, bone marrow transplantation from *IL-5*^{-/-} mice into *Ldlr*^{-/-} mice aggravated atherosclerosis, possibly due to decreased production of natural IgM antibody (50), suggesting IL-5 may be atheroprotective.

Th17 is a CD4⁺ T cell subset that produces IL-17A, IL-17F, and IL-22. The involvement of Th17 in atherosclerosis has been also investigated in recent years, but their role is still unclear (6). Treatment with mouse anti-mouse IL-17A antibody did not affect atherosclerosis development although the IL-17 signaling was abolished (51). Inconsistent results have been reported in several studies of genetic IL-17 deficiency in *Apoe*^{-/-} mice, which include no difference (52), aggravation (53), and reduction (54, 55) of atherosclerotic lesion.

Although CD8⁺ T cells were found in human atherosclerotic plaque, the role of cytotoxic CD8⁺ T cells is not well investigated (56). A potential role for this cell type is suggested by the finding that cytotoxic and proinflammatory CD8⁺ T lymphocytes promote the development of vulnerable atherosclerotic plaques by perforin- and granzyme B-mediated apoptosis, resulting in necrotic core formation and inflammation (57).

Complex Immunogens: Immunization with oxLDL and MDA-LDL

Oxidation of lipoproteins (and oxidative processes in general) are thought to play an important role in the initiation and progression of atherosclerotic lesions (13). The oxidation of LDL results in various structural modifications and the formation of a large number of neopeptides that may be recognized by antibodies (58). While the oxidation of LDL may be a trigger of the immune response, the mechanistic details are not known. Antibodies recognizing oxLDL-specific epitopes were detected in both human and rabbit atherosclerotic lesions (59). T cells reactive to oxLDL were also demonstrated from human plaque tissue two decades ago (60). Based on these findings, studies of protective immunity started with immunization with complex immunogens including native LDL, but also oxLDL, or malondialdehyde-modified LDL (MDA-LDL).

Palinski et al. reported that subcutaneous immunization with homologous MDA-LDL formulated in complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) reduces atherosclerosis in the thoracic aorta by 27% and in the abdominal aorta by 32% in *Ldlr*-deficient rabbits (61). These immunized rabbits had 10-fold higher MDA-LDL specific antibody titers (61). The ratio of antibody binding to MDA-LDL to antibody binding to native LDL was 10-fold, 2-fold, and equal in serum IgG, IgA, and IgM, respectively (61). Similarly, subcutaneous immunization with homologous LDL and copper-oxidized LDL reduced atherosclerotic lesions in high-cholesterol diet-fed rabbits in the proximal aorta by 74% and 48%, respectively (62).

Subsequently, several studies have supported the immunization concept in atherosclerotic mouse models. Freigang et al. reported that vaccination with both homologous native LDL and MDA-LDL reduced aortic atherosclerosis by 37% and 46%, respectively in *Ldlr*^{-/-} mice (63). Induction of atheroprotection and high titers of anti-MDA-LDL IgG antibody by homologous MDA-LDL immunization was also observed in *Apoe*^{-/-} mice (64, 65). It has been shown that administration of IgM with specificity for hypochlorite-oxLDL reduced atherosclerosis in *Ldlr*^{-/-} mice (66) in a model of passive immunization. Interestingly, serum IgG binding to MDA-LDL and oxidized cholesterol was increased in MDA-LDL immunized mice, but not the native LDL group. In the MDA-LDL group, the titer of both IgG1 and IgG2a binding to MDA-LDL increased to the same extent. Since atheroprotection was observed in the LDL group in the absence of increased antibody titers, these data suggest that cellular immune responses may be involved with LDL-induced atheroprotection.

CD4⁺ T cells as a whole likely induce atherosclerosis. *Apoe*^{-/-} mice deficient in CD4 developed less severe atherosclerotic lesion and low titers of IgG1, IgG2a, and IgM against MDA-LDL (67). When these mice were immunized with homologous MDA-LDL, a strong atheroprotective effect of CFA and IFA alone (adjuvant effect) with only weak additional atheroprotection by co-immunization with MDA-LDL was observed (67). These results suggest that CD4⁺ T cells as a whole play an important role in the progression of atherosclerosis and that atheroprotection induced by immunization may be mediated by CD4⁺ T cells.

However, several additional pieces of evidence suggest a potential dual role for CD4⁺ T cells. Adoptive transfer of oxLDL-pulsed dendritic cells (DCs) reduced atherosclerosis in *Ldlr*^{-/-} mice (68). In contrast, some studies suggest that administration of oxLDL induces atherosclerosis. Adoptive transfer of bone marrow-derived DCs treated with MDA-LDL in vitro exacerbated atherosclerosis in *Apoe*^{-/-} mice (69). In addition, transfer of CD4⁺ T cells isolated from oxLDL-immunized mice into *Apoe*^{-/-} *scid/scid* mice induced increased fatty streak lesions in aortic root (70). These transferred T cells presumably include Th1 cells known to be atherogenic. This finding suggests that immunization with oxLDL potentially activates proinflammatory Th1 cells rather than Tregs. This may depend on the route and frequency of immunization and the adjuvants used.

Immunization with ApoB-100 Peptides recognized by autoantibodies

Researchers have focused on humoral immunity at an early stage because elevation of anti-ApoB antibody titers was frequently observed in patients and experimental animals. Three injections of antibody against MDA-modified ApoB-100 peptide (P45) improved atherosclerosis in *Apoe*^{-/-} mice (71). The level of IgG against ApoB-100 peptides (P45 and P210) in serum from patients with cardiac disease was found to be lower than in healthy controls (32, 72). Taken together, these findings suggest that IgG reactive to ApoB-100 could be atheroprotective.

Because of the finding of naturally existing IgG and IgM to oxLDL, Fredrikson et al. tried to identify sequences from human ApoB-100 that are able to bind to IgG and IgM antibodies

isolated from patients with acute coronary heart events (14). In their studies, several of these peptides have been used to immunize mice, including P45 (ApoB-100_{661–680})(73), P143 (ApoB-100_{2131–2150}) (74), and P210 (ApoB-100_{3136–3155}) (74). Immunization with a mixture of P143 and P210 reduced atherosclerosis in the descending aorta of *ApoE*^{−/−} mice by about 60% but induced slightly larger lesions in the subvalvular area (74). There was no difference in infiltrated macrophage count in plaque. The titer of IgG antibody against MDA-modified ApoB-100 was elevated, but the IgM titer was not changed (74). Similarly, immunization with another MDA-modified human ApoB-100 peptide (P45) reduced atherosclerotic plaque by 48% (73). Surprisingly, expression of IFN- γ , which is a proinflammatory Th1 cytokine and an exacerbating factor in atherogenesis, was highly elevated in aorta of P45 immunized mice (73). The authors argued that immunization with ApoB-100 peptide induce a shift in immune response from Th1 to Th2 because IgG1 against MDA-modified P45 was strongly induced. However, induction of inflammatory cytokines (i.e. IFN- γ) did not support this notion. Immunization with human ApoB-100 peptides (P45 and P210) was also examined in human ApoB-100 transgenic *Ldlr*^{−/−} mice. Although injection of only native P45 significantly reduced aortic plaque by 66%, there was no detectable induction of peptide-specific antibodies and no difference in expression of Foxp3 and IL-10, both markers of Tregs (75). This is not too surprising, because P45 (and other peptides like P210) do not bind MHC-II and therefore cannot induce a CD4⁺ T cell response (6). In conclusion, the review of the data existing to date suggests that antibody responses are neither necessary nor sufficient to explain the disease modulation observed upon peptide immunizations.

Immunization with ApoB-100 Peptides and T cells responses

Hermansson et al. reported that T cells responsive to LDL express the T cell receptor cell receptor(76). Depleting these T cells with an antibody against TRVB31 reduced atherosclerosis, suggesting that ApoB-100 reactive T cells may be involved with pathogenesis of atherosclerosis (76). In contrast, other studies have suggested that immunization with ApoB-100 causes an increase in Treg cells, resulting in reduction of atherosclerosis. Wigren et al have reported that immunization with human ApoB-100 peptide (P210) reduced plaque in the descending aorta by 37% and induced an increase in CD4⁺CD25⁺Foxp3⁺ cells in the spleen compared to control mice treated with PBS lacking adjuvant (77). Administration of anti-CD25 antibody (depleting Tregs) abrogated atheroprotection, which suggests that atheroprotection induced by this peptide involves Tregs. However, it should be taken into consideration that adjuvant alone could modulate immune response (78) and that anti-CD25 antibody treatment could abolish immune cells other than Tregs such as activated T cells and B cells. In a separate study, continuous subcutaneous injection of MDA-modified ApoB peptides (P210 and p240) reduced atherosclerosis in *ApoE*^{−/−} mice and treatment with an anti-CD25 antibody again abrogated the atheroprotective effect (79). Elevated IL-10 production was also observed in plasma after immunization with human ApoB-100 peptides (80). In addition, adoptive transfer of DCs stimulated by ApoB-100 peptide P210 induced CD4⁺CD25⁺Foxp3⁺ cells in the spleen (80). Although these studies indicate Tregs might be involved with atheroprotection in immunized mice, further investigation is needed to clarify the role of Treg cell in immune

response to peptide vaccination. Hermansson et al has examined atheroprotective effects of tolerogenic DCs treated with ApoB-100 protein and IL-10 (81). Injection of these tolerogenic DCs induced reduction of atherosclerosis, possibly due to suppressing activation of ApoB-100 reactive T cells (81). A vaccine containing human ApoB-100 peptide (P210) was in preclinical development for human safety and efficacy studies (82), but no active clinical trial is currently under way.

As mentioned above, the function of Th2 cells in atherosclerosis is unclear. It has been reported that elevation of IL-4 production in spleen correlated with reduction of atherosclerotic lesions in ApoB-100 immunized mice (73). However, another study has shown that Th2 cytokines and Th2-associated IgG1 against LDL induced by human ApoB-100 does not reduce atherosclerosis in *ApoE*^{-/-} mice (83).

The role of CD8⁺ T cell on atherogenesis is also not well investigated. It was reported that adoptive transfer of CD8⁺ T cell from spleen of ApoB-100 peptide (P210) immunized mice attenuated atherosclerosis in *ApoE*^{-/-} mice, although adoptive transfer of CD4⁺CD25⁺ cells did not affect atherosclerosis (84). In this study, it should be noted that the transferred CD8⁺ cell count was 3.3-fold higher than the transferred CD4⁺CD25⁺ cell count.

Immunization with MHC class II binding ApoB-100 Peptides

Although the peptides derived from human ApoB-100 were identified based on the binding capacity to human plasma antibody, it is interesting that these peptides also appear to induce Treg cells (77, 79, 80). The mechanism of this effect is unknown. To directly affect CD4⁺ helper T cell activation, the peptide would have to first bind MHC-II on an APC and be subsequently presented to a specific T cell whose T cell receptor recognizes the peptide-MHC complex (6). As we have reported (6), we found that peptide P210 has very low affinity for I-A^b (MHC class II in C57BL/6 mice) compared to well-known I-A^b-restricted T-cell epitopes, such as ovalbumin₃₂₃₋₃₃₉ peptide (85) and myelin oligodendrocyte glycoprotein₃₈₋₅₁ peptide (86). Likewise, for a CD4⁺ T cell to recognize molecules of (ox)LDL, APCs must first internalize oxLDL and present antigenic peptides in a manner that the T cell can recognize (Figure 1). For CD4⁺ T cells, this requires presentation by MHC-II. The protein component of LDL, ApoB-100, is processed into peptides by APCs, some of which bind MHC class II molecules, and are displayed on the APC cell surface (87). Indeed, ApoB-100 peptides are frequently displayed by HLA-DR (human MHC-II) molecules in cultured human lymphoblastoid cells (88).

To effectively induce a CD4⁺ T cell response, any antigenic peptide must bind MHC-II (I-A^b). Because of this requirement, we screened ApoB-100 peptides for binding to I-A^b using a competitive peptide binding assay to recombinant I-A^b purified from cell line (89). In our first study, two ApoB-100 peptides (ApoB₃₅₀₁₋₃₅₁₆ [SQEYSGSVANEANVY] and ApoB₉₇₈₋₉₉₃ [TGAYSNASSTESASY]) with high affinity for MHC class II were identified and shown to induce proliferation of peptide-specific T cells in vitro. These same peptides were effective in reducing atherosclerosis by about 40% in the aorta of *ApoE*^{-/-} mice and one peptide also reduced lesions in the aortic roots (90). This study was aimed at prevention of atherosclerosis by immunizing around the time western diet was started. The mice were

immunized with peptide in CFA followed by four booster injections with peptide in IFA. Among all cytokine mRNAs measured, only IL-10 mRNA was significantly elevated in the aortas of immunized mice. Although immunization induced highly peptide-specific IgG1 and IgG2c antibodies, these antibodies did not cross-react with native or modified LDL (90), suggesting that the protective mechanism is unlikely to be humoral. In an unpublished study, three more I-A^b binding peptides, but not a non-binding peptide, were found to be atheroprotective. These studies suggest that presentation by MHC-II is required for ApoB-100 peptides to induce atheroprotection and points to a likely Treg mechanism.

Immunization with HSP60/65

HSP60/65 is also mentioned as a candidate which can trigger an atheroprotective immune response (91). Human HSP60 shows mimicry with mycobacterial HSP65 and chlamydial HSP65 (92, 93). The expression of HSP60 is increased when cells are exposed to elevated temperatures or other stressors, and is the only HSP for which direct atherogenic potential has been proven in experimental and clinical studies (16). The titer of anti-HSP60 antibody is increased in blood sample in patients with hypertension and coronary artery disease (94, 95). When the therapeutic potential of HSP65 immunization was tested, controversial results were obtained. In fact, early studies suggested that HSP65 immunization increases atherosclerosis. In normocholesterolemic rabbits, atherosclerotic lesions developed after immunization with mycobacterial HSP65 in CFA and IFA adjuvant, although the levels of cholesterol in serum remained normal (96). In wild-type C57BL/6J mice, immunization with mycobacterial HSP65 led to an earlier onset of atherosclerotic lesions (97). To examine the involvement of T cell mediated immune response, Metzler et al immunized T cell depleted rabbits with mycobacterial HSP65 (98). Although arteriosclerotic lesions were observed in the aortic arch of immunocompetent rabbits as expected, rabbits depleted of T cells with anti-CD3 antibody developed significantly milder atherosclerosis (98). It has been reported that subcutaneous immunization with recombinant HSP65 and IFA as adjuvant promotes early atherosclerosis in *Ldlr*^{-/-} mice (99). These immunized mice developed elevated titers of IgG antibody against HSP65, which suggests that this antibody alone is not atheroprotective. In addition, both adoptive transfer of HSP65-reactive lymph node cells and intraperitoneal administration of IgG derived from HSP65-immunized mice enhanced fatty-streak formation, suggesting the potential of HSP65 specific IgG to aggravate atherosclerosis (100). In addition, passive transfer of anti-HSP60 autoantibody from patients with coronary artery disease induced atherosclerosis in *ApoE*^{-/-} mice (101).

In contrast, subcutaneous immunization with mycobacterial HSP65 in alum adjuvant and anti-CD45RB monoclonal antibody reduced atherosclerotic lesions in *ApoE*^{-/-} mice (102). IgG1 antibody titer (a Th2 driven IgG) against HSP65 was also elevated (102). The discrepancy of these results with earlier studies is possibly due to the sort of adjuvant used or the stage of atherosclerosis. In fact, some studies indicate that adjuvant itself can modulate the immune response, usually leading to reduced atherosclerosis (78). Immunization with a combination ApoB peptide P45 (71) and HSP60₁₅₃₋₁₆₃ peptide (103) induced a significant reduction of atherosclerosis compared to P45 immunization alone (104), possibly due to reduced production of IFN- γ and increased production of IL-10.

Other immunization approaches

Several antigens other than ApoB-100 and HSP60/65 have been examined as potential immunogens. β 2-glycoprotein I (β 2GPI) has been thought to be the target of autoimmune anticardiolipin antibodies (105). In a study of subcutaneous immunization with human β 2GPI, atherosclerosis was worsened in *Ldlr*^{-/-} mice although the titer of anti- β 2GPI antibody was elevated (106). Another group reported that intravenous injection of human β 2GPI without adjuvant attenuated early atherosclerotic lesions in *Ldlr*^{-/-} mice (107).

Binder et al found that antibodies against oxLDL cross-react with *Streptococcus pneumonia* (pneumococcus). Interestingly, subcutaneous immunization with pneumococcal immunogen decreased the extent of atherosclerosis and induced circulating oxLDL-reactive IgM (108). This finding suggests that vaccine-induced anti-pneumococcus antibody cross-reactive with oxLDL may have an atheroprotective potential. In two unconfirmed studies, immunization with complement C5a receptor peptide with alum adjuvant was atheroprotective compared to KLH with alum (109). Immunization with rat MDA-modified fibronectin reduced atherosclerosis in descending aorta and subvalvular lesion (110).

Interestingly, some studies suggest that administration of adjuvant alone can be atheroprotective. Freund's adjuvant is commonly used, an emulsion of killed mycobacteria in mineral oil. CFA contains killed cells of *Mycobacterium tuberculosis* while IFA does not. Alum refers to adjuvants that comprise various aluminum salts. Alum is widely used for human vaccines. It has been reported that subcutaneous injection of CFA followed by intraperitoneal injection of IFA reduced atherosclerotic lesions in aortic roots of *ApoE*^{-/-} mice (78). Intraperitoneal injection of alum also induced atheroprotection (78). Similarly, subcutaneous injection of alum reduced atherosclerosis, possibly due to increased CD4⁺CD25⁺Foxp3⁺ T cells in the spleen (111).

Mucosal Immunization to Induce Tolerance

Mucosal immunization with antigens has been investigated in an effort to induce tolerance. Mucosal administration of antigen is known to generate tolerance and suppresses Th1 immune responses through production of IL-4, IL-10, and TGF- β , which induces unresponsiveness of immune system against a given antigen (112). Mucosal immunization has been explored using oxLDL and ApoB-100 peptides. Oral administration of human oxLDL and human MDA-LDL reduced atherosclerotic plaque in the aortic root and doubled the CD4⁺CD25⁺Foxp3⁺ cell count in spleen and mesenteric lymph node in *Ldlr*^{-/-} mice (113). Nasal immunization with human ApoB-100 peptide (P210) fused to the B subunit of cholera toxin resulted in reduction of atherosclerosis in *ApoE*^{-/-} mice (114). Although increased mRNA expression of Foxp3 was observed in the thoracic aorta of P210-immunized mice, there was no difference in the level of IL-10 expression between P210 and ovalbumin₃₂₃₋₃₃₉ peptide fused to cholera toxin.

While parenteral immunization with mycobacterial HSP65 is atherogenic, mucosal administration of HSP65 seems to induce atheroprotection. Nasal administration of mycobacterial HSP65 reduced atherosclerotic plaque as well as infiltration of CD4⁺ T cell and macrophages in *Ldlr*^{-/-} mice (115). The titer of IgG1 antibody against HSP65 was

increased in this study (115). Nasal administration of mycobacterial HSP65 reduced atherosclerosis also in high cholesterol diet-fed rabbits (116). In addition, oral administration of mycobacterial HSP65 reduced reactivity of lymphocytes to HSP65 and reduced atherosclerosis in *Ldlr*^{-/-} mice (117). Similarly, oral administration of both mycobacterial HSP60 and HSP60₂₅₃₋₂₆₈ peptide reduced atherosclerosis (103). In the latter study, although the number of CD4⁺CD25⁺Foxp3⁺ T cells and expression of IL-10 and TGF- β were increased, causality was not tested. Oral administration of mycobacterial HSP65 delivered by *Lactococcus lactis* reduced atherosclerotic plaque in aortas of *Ldlr*^{-/-} mice, accompanied by elevation of IL-10 production (118).

Recently, it has been reported that oral administration of a combination of human ApoB-100 peptide (P45) and HSP60₁₅₃₋₁₆₃ peptide induced enhanced reduction of atherosclerotic lesion compared to ApoB-100 peptide alone or HSP60 peptide alone in *Ldlr*^{-/-} mice which express ApoB-100 but not ApoB-48 (119). The reason for this apparent synergistic effect is not known.

Conclusion

Over the past quarter century, immunization with the goal of preventing or reducing atherosclerosis has been explored in rabbits and mice. Immunization with complex antigens like oxLDL or MDA-LDL appears to be effective. Later, specific peptides derived from ApoB-100 were identified by reactivity with autoantibodies found in cardiovascular patients. These peptides, as well as several HSP60/65 peptides, were reported to be atheroprotective, although their ability to be presented by MHC-II was not tested. One of these peptides (P210) in fact does not bind mouse MHC-II, so it is surprising that these studies have suggested that the atheroprotective mechanism is primarily via increase in Foxp3⁺ Treg cells. Adjuvants commonly used in vaccination approaches can have strong atheroprotective effects independent of the antigen used. Oral tolerization has been reported to be successful in several studies, but the specificity of the tolerization was not assessed. Our report is the only one so far showing that MHC-II binding ApoB-100 peptides induce a CD4⁺ T cell response that is involved with the suppression of inflammation. Further work is needed to fully understand the mechanism of protective autoimmunity in atherosclerosis and to better understand the effects of immunization with self peptides in general.

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Figure 1. Hypothetical adaptive immune response to atheroprotective immunization

After self peptide (purple) is processed in CD103⁺ dendritic cells (DCs, green), antigen-MHC class II complex is presented to antigen-specific CD4⁺ T cells (red). Adjuvant stimulates toll-like receptors (TLR) and enables co-stimulation (not shown). Antigen presentation by CD103⁺ tolerogenic DCs likely induces the clonal expansion and the differentiation of antigen-specific CD4⁺ T cells to Treg cells, resulting in the suppression of inflammation and the reduction of atherosclerosis. Memory Tregs may form, persist and recirculate, resulting in durable atheroprotection.