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Vaccination against atherosclerosis

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Atherosclerosis is a chronic inflammatory disease that causes most heart attacks and strokes, making it the biggest killer in the world. Although cholesterol-lowering drugs have dramatically reduced these major adverse cardiovascular events, there remains a high residual risk called inflammatory risk. Atherosclerosis has an autoimmune component that can be manipulated by immunologic approaches including vaccination. Vaccination is attractive, because it is antigen-specific, does not impair host defense, and provides long-term protection. Several candidate antigens for atherosclerosis vaccine development have been identified and have been shown to reduce atherosclerosis in animal models. In this review, we focus on two different types of atherosclerosis vaccines: antibody-inducing and regulatory T cell-inducing.

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Introduction

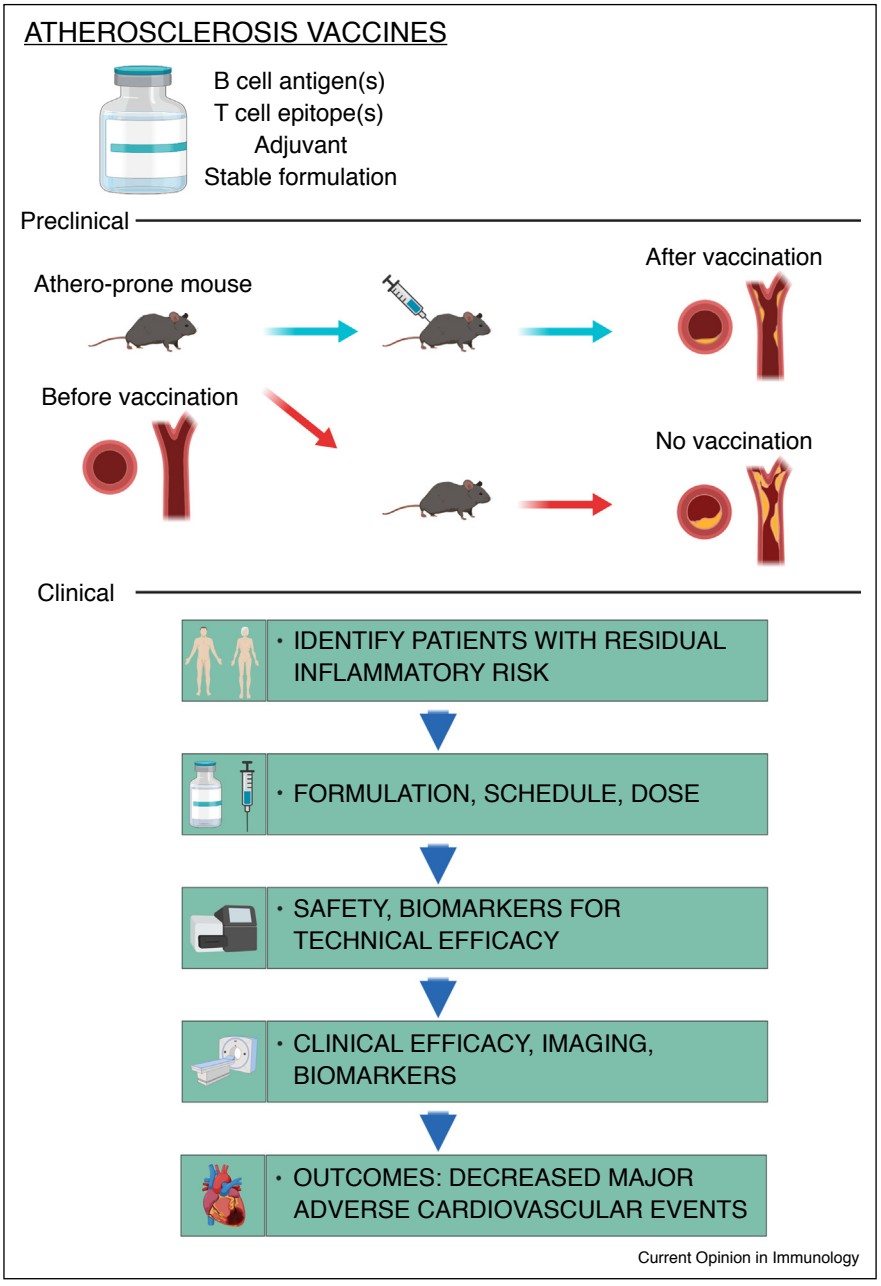
Atherosclerosis is a chronic inflammatory disease. It starts with high plasma levels of low-density lipoprotein (LDL) cholesterol in blood. LDL accumulates in the arterial wall and is modified by enzymatic and non-enzymatic processes including oxidation [1]. Oxidized LDL (oxLDL) binds Toll-like receptor 4 (TLR4), CD36 and other scavenger receptors [2,3], leading to pro-inflammatory signaling cascades. Macrophages and smooth muscle cells proliferate, immune cells are recruited from blood, and an atherosclerotic plaque forms. Unstable plaque causes coronary artery disease (angina pectoris) and leg ischemia (peripheral artery disease). Plaque rupture or erosion causes myocardial infarctions and strokes [4]. These atherosclerosis-dependent diseases are called cardiovascular diseases (CVD). The traditional risk factors for CVD include high plasma LDL cholesterol, smoking, hypertension, obesity, and diabetes mellitus. Two major LDL cholesterol-lowering medications are statins (HMG-CoA reductase inhibitors) and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors. Both drug types significantly reduce the incidence of CVD [5–7]; however, CVD is still the number 1 killer in the world. About 17.9 million (representing 31% of all global deaths) people died from CVD in 2016 (from WHO site URL: http://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)).

When LDL cholesterol-lowering drugs are administered at optimal doses, the 'cholesterol risk' is considered to be controlled. Under these conditions, major adverse cardiovascular events (MACE) are reduced by 30–40%. However, compliance (the patient actually taking the drug as prescribed) is incomplete so the reduction of events is less. An atherosclerosis vaccine could address this compliance gap, because it would be administered at a doctor's office and would not require daily pills. If the cholesterol risk is optimally managed, the remaining risk is considered inflammatory in nature. Recently, the IL-1β blocking antibody Canakinumab was successfully tested in a large clinical trial (CANTOS: Canakinumab Anti-inflammatory Thrombosis Outcome Study). Canakinumab significantly reduced MACE [8•], especially in people in which it reduced the inflammatory biomarker C-reactive protein (CRP). However, significantly increased rates of infections were observed, reflecting impaired host defense. Currently, Canakinumab is not FDA-approved for use in CVD treatment. Vaccination is an alternative strategy that is expected to spare host defense, because vaccination is narrowly antigen-specific.

Vaccination is one of the most effective medical strategies to control and prevent infectious diseases and some virally induced cancers. Vaccines dramatically reduced the incidence of serious and life-threating infectious diseases. Smallpox was globally eradicated by vaccination in 1980. At present, at least 27 diseases are known as vaccine-preventable diseases by currently approved vaccines. Recently, preclinical and clinical research has started to address non-infectious diseases such as cancer [9], hypertension [10], Alzheimer's disease [11], and diabetes mellitus [12]. In each case, preclinical studies suggest that vaccines could prevent disease progression (Figure 1).

Both antibody-inducing and regulatory T cell (Treg)-inducing vaccines need vaccine adjuvants. Adjuvants can be aluminum salts, squalene oils, TLR ligands, nanoparticle formulations, and more [13]. An antibody-inducing vaccine will only be effective if it inhibits a

Figure 1



Atherosclerosis vaccines.
An atherosclerosis vaccine contains B or T cell epitope(s) or both, together with adjuvant, in a stable formulation. Preclinical studies: Atherosclerosis-prone mice (usually *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$) are vaccinated before being fed with a high fat or high cholesterol diet. Vaccine efficacy is evaluated by atherosclerotic plaques. Clinical study: 1) Identify at-risk patients. 2) Design the immunization schedule, dose, and vaccine formulation. 3) Establish the safety profile and biomarkers for technical efficacy (antibodies, tetramers). 4) Evaluate vaccine efficacy by clinical biomarkers, including imaging biomarkers. 5) Evaluate efficacy by hard endpoints (Major Adverse Cardiovascular Events).

biologically important function of an enzyme or other protein that is involved in atherosclerosis. A Treg-inducing tolerogenic vaccine will only be effective if the epitope(s) recognized by the Tregs are expressed and accessible and if the elicited Tregs remain stable over time.

The major difference between infectious and non-communicable diseases vaccine is the origin of vaccine antigen. Infectious disease vaccine antigens are derived from specific pathogens (non-self) to induce specific immune responses against these pathogens. In contrast, non-communicable disease vaccine antigens are derived

from self proteins. For example, hypertension and Alzheimer's disease vaccines are designed to induce adaptive immune responses against angiotensin-II and amyloid β, respectively.

Developing a vaccine against atherosclerosis is both attractive and challenging. Atherosclerosis is not categorized as an autoimmune disease, but humans and animals with atherosclerosis have clear evidence of autoimmune responses against apolipoprotein B (ApoB), one of the best-known atherosclerosis antigens [14]. ApoB is the core protein of LDL and also found in chylomicrons and remnant particles [15•,16,17•]. Patients with atherosclerosis have autoantibodies against oxidized LDL (oxLDL). These autoantibodies come from two sources: B1 cells are part of the innate immune system that mature into IgM plasma cells independent of T cell help. These plasma cells secrete germline-encoded (unmutated) IgM antibodies with modest affinity to antigens [18]. Such 'natural' antibodies are critical in protecting children from *streptococcus* infections. Some natural IgM antibodies recognize phosphatidyl-choline (PtC) [19] and epitopes on apoptotic cells [20]. PtC IgM autoantibody levels are negatively correlated with CVD [21]. The second type of B cells, B2 cells, require help by follicular helper T cells (TFH). B2 cells undergo a germinal center reaction where they switch isotypes and, through somatic hypermutation, achieve affinity maturation. B2 cells result in long-lived plasma cells that secrete high affinity antibodies [22]. In most studies, IgG levels against LDL are positively correlated with CVD [23,24].

Atherosclerotic lesions contain helper (CD4) and cytotoxic (CD8) T cells that respond to ApoB, oxLDL and other antigens. These interactions result in the secretion of cytokines, often inflammatory cytokines [25••,26]. A T cell targeted vaccination would be aimed at eliciting an anti-inflammatory immune response against atherosclerosis-related antigens. Such cells include regulatory T cells (Tregs and type 1 regulatory T (Tr1) cells) and anti-inflammatory cytokines include interleukin-10 (IL-10) and transforming growth factor-β (TGF-β).

Antibody-inducing vaccines

PCSK9 vaccines are designed to induce neutralizing antibodies (Table 1). PCSK9 modulates the LDL receptor (LDLR) expression. Upon binding LDL, LDLR is internalized into hepatocytes. LDL receptor is normally stripped of LDL in the lysosome, and recycled to the plasma membrane, where it can bind another LDL molecule. PCSK9 is a secreted serine protease that directly binds to LDL receptor and targets it toward degradation. This results in reduced LDLR surface expression and elevated plasma LDL cholesterol level [27]. Gain of function mutations of PCSK9 are associated with familial hypercholesterolemia [28••]. Loss of function mutations result in low LDL cholesterol levels and protection from CVD [29]. Statin treatment

Table 1

Summary of antibody-inducing vaccine for atherosclerosis

Target protein	Peptide name	Peptide sequence	Route	Carrier protein containing T cell epitope	Adjuvant	Species	Result	Reference
Human PCSK9	$PCSK9_{68–76}$ AKDPWRLPG $PCSK9_{153–163}$ SIPWNLERITP		Not described	VLP-Qβ	IFA	Mouse	Reductions in total cholesterol, free cholesterol, phospholipids, and triglycerides	[38]
	$PCSK9_{207–233}$ NVPEEDGTRFHRQASKC		Not described	VLP-Qβ	Alum	Macaque		
	$PCSK9_{207–233}$ NVPEEDGTRFHRQASKC					Mouse Rat	Reductions in total cholesterol (~30%) and LDLC (~50%)	[33]
	AFFITOPE	Variants of the PCSK9 fragment having amino acid sequence SIPWNLERITPPR	Subcutaneously	KLH	Alum	Mouse	Reduction of atherosclerotic lesion (~64%)	[36]
Human CETP	TT-CETi-1	FGFPEHLLVDFLQSLS	Not described	14 amino acid derived from tetanus toxin QYIKANSKFIGITE	Alum	Human	53% of patients developed anti-CETP antibodies No change lipid profile and CETP function	[47]

Abbreviation: PCSK9, Proprotein convertase subtilisin/kexin type 9; IFA, incomplete Freund's adjuvant; KLH, Keyhole Limpet Hemocyanin; TT, tetanus toxin; VLP, virus-like particle; CETP, cholesteryl ester transfer protein. Alum: a general term of aluminum adjuvant including Alhydrogel.

reduces LDL cholesterol level through the increase LDL receptor expression level but also induces PCSK9 expression. The inhibition of PCSK9 is an attractive target for atherosclerosis prevention and treatment. Monotherapies with PCSK9 blocking monoclonal antibodies (mAb) result in dramatic reductions of LDL cholesterol [30]. Two mAbs (alirocumab and evolocumab) are already FDA-approved and on the market. They were shown to dramatically reduce MACE in the ODYSSEY and FOURIER clinical trials [6,31]. PCSK9 mAb binds to PCSK9 and prevents its binding to and degradation of LDLR. Patients must inject alirocumab or evilocumab subcutaneously every two weeks or every month. Also, the cost of PCSK9 mAbs is high. Currently, payment for anti-PCSK9 treatment requires approval by the insurer, which is restricted to severe hypercholesterolemia or statin intolerance. A PCSK9 vaccine would be designed to induce blocking antibodies. This is expected to produce persistent PCSK9 blockade. Vaccination is also expected to be cost-effective.

A PCSK9 vaccine needs to induce neutralizing antibodies to prevent the interaction between LDLR and PCSK9. Several pre-clinical studies provided proof-of-concept that a PCSK9 vaccine could work. These studies used B cell epitopes as vaccine antigens. The antigen may be engineered to be devoid of T cell antigens, because self-reactive T cell responses might induce tissue damage. To induce high-affinity antibodies, B cells need help from follicular helper T (Tfh) cells in the germinal center, where the antibody VDJ gene undergoes affinity maturation by somatic hypermutation. Tfh cells are essential for the germinal center formation, affinity maturation, mature B cell proliferation, immunoglobulin class switching, and differentiation into antibody-secreting plasma cells [32]. Galabova *et al.* used short peptides (8–13 amino acids) of the N-terminal region of human PCSK9 protein conjugated to the foreign carrier protein keyhole limpet hemocyanin (KLH) that contains non-self T cell epitopes [33]. After immunization with aluminum adjuvant, they showed the induction of anti-PCSK9 antibody and reduction of total cholesterol in Wistar rats. Six months after prime immunization in Balb/c mice, total cholesterol level was still lower than in the control groups. Importantly, this vaccination did not induce PCSK9-specific T cell responses. They also used APOE*3Leiden.CETP transgenic mice as an atherosclerosis model. APOE3*-Leiden is a variant of ApoE that was identified from type III hyperlipoproteinemia male [34]. APOE3*Leiden. CETP transgenic mice are 'humanized' mice and have a lipoprotein profile similar to humans [35]. Vaccination with the PCSK9 peptides in adjuvant reduced atherosclerotic lesions and vascular inflammation and decreased plasma total cholesterol and PCSK9 concentration [36]. They used AFFITOPE technology [37] to develop non-self-epitopes. These peptides are similar to the original PCSK9 peptides but not identical. These 'neoepitopes' elicited self-protein specific antibodies. Crossey *et al.* used a Qβ bacteriophage virus-like particle (Qβ-VLP) vaccine platform for atherosclerosis [38]. They selected five regions of PCSK9 as candidate peptides predicted to be involved in LDL receptor binding based on the published crystal structure of PCSK9 and LDL receptor [39]. The immunization of PCSK9 peptide-conjugated Qβ-VLP with incomplete Freund's adjuvant and aluminum adjuvant significantly induced anti-PCSK9 antibodies, reduced total cholesterol and LDL cholesterol in both mice and macaques. Another group showed that a Qβ-VLP vaccine for Alzheimer's disease induced antibody-specific responses and very weak antigen-specific T cells responses [40]. These results suggest that a Qβ-VLP vaccine may avoid undesirable auto-reactive T cell responses. A phase I clinical trial for PCSK9-targeting vaccine is currently ongoing (NCT02508896).

Cholesterol ester transfer protein (CETP) is also a candidate antigen for an antibody-inducing atherosclerosis vaccine. CETP is an enzyme that promotes cholesteryl ester transfer from HDL to LDL and triglyceride from LDL to HDL [41]. The idea of CETP as a therapeutic target originated from the observation that genetic deficiency of CETP showed high levels of HDL in the plasma [42]. HDL is negatively correlated with the risk of CVD [43,44], but interventional clinical trials aimed at raising HDL showed no benefit in outcomes [45]. Thus, the evidence for CETP being a good target is much more tenuous than the case for PCSK9. Vaccination with CETP peptides functioning as B cell epitopes together with tetanus toxin peptide containing T cell epitope(s) increased HDL cholesterol levels and reduced atherosclerotic lesion size in rabbits [46]. However, a phase I clinical trial for CETP vaccination showed inconsistent antibody production and no difference of HDL cholesterol levels [47]. The small molecule CETP inhibitor anacetrapib was tested in a recent phase III clinical trial (REVEAL: randomized evaluation of the effects of anacetrapib through lipid modification) [48]. Patients receiving the CETP inhibitor together with statin treatment showed lower incidence of coronary heart disease and reduced level of non-HDL cholesterol.

Treg-inducing vaccines

In both mice and humans, regulatory T cells (Tregs) highly express CD25 (the high affinity IL-2 receptor) and the lineage-defining transcription factor forkhead box p3 (FoxP3) [49]. Tregs can suppress physiological and pathological immune responses by reducing effector T cell expansion and by curbing cytokine secretion [50] (Table 2). Several mechanisms of Treg suppression through cell-to-cell contact and secreted cytokines have been reported [51]. Some mechanisms require presentation of the antigenic epitope to the T cell receptor (TCR) of Tregs. Tregs express inhibitory molecules such as CTLA-4 and LAG3 on the cell surface. CTLA-4 binds

Table 2

Summary of Treg-inducing vaccine for atherosclerosis

Target protein	Peptide name	Peptide sequence	Route	MHC restriction	Adjuvant	Species	% reduction of atherosclerosis lesion	References
Human ApoB	P2	ATRFKHLRKYTYNYEAESSS	Subcutaneously followed by intraperitoneal booster	Not determined	Alum		40%	[83]
	P210	KTTKQSFDLSVKAQYKKNKH	Subcutaneously	Not determined	Alum		50–60%	[60,84]
	P45	IEIGLEGKGFEPTLEALFGK	Not described	Not determined	Alum		48%	[85]
	P143	IALDDAKINFNEKLSQLQTY	Not described	Not determined	Alum		About 60%	[60]
Human and mouse ApoB[a]	Human $P18_{3036–3050}$	SLFFSAQPFEITAST	Subcutaneously to inguinal area followed by intraperitoneal boosters	DRB1[a]01:01 DRB1[a]04:01 DRB1[a]07:01			About 35%	[66••]
	Mouse $P18_{3030–3044}$			I-A^b				
Mouse ApoB	$P3_{3501–3516}$	SQEYSGSVANEANVY	Subcutaneously to inguinal area followed by intraperitoneal boosters	I-A^b				
	$P6_{978–993}$	TGAYSNASSTESASY	Subcutaneously to inguinal area followed by intraperitoneal boosters	I-A^b	CFA and IFA	Mouse	40%	[67]
	$P6_{978–993}$	TGAYSNASSTESASY	Subcutaneously	I-A^b	Addavax		About 50%	[81••]
	$P101_{705–720}$	FGKQGFFPDSVNKALY	Subcutaneously followed by intraperitoneal boosters	I-A^b	CFA and IFA		39%	[65]
	$P102_{441–456}$	TLYALSHAVNSYFDVD		I-A^b			37%	
	$P103_{3953–3968}$	LYYKEDKTSLSASAAS		I-A^b			40%	
Mouse HSP65	$P34_{166–180}$	KVGNEGVITVEESNT	Orally	Not determined	–		About 40%	[86]
	$P67_{331–345}$	VEGAGDTDAIAGRVA					About 60%	
	$P84_{416–430}$	TLLQAAPTLDELKLE					About 40%	
	$P85_{421–435}$	APTLDELKLEGDEAT					About 50%	
Human HSP60	$HSP60_{153–163}$	CAELKKQSKPVT	Orally	Not determined	–		33.5%	[87]
Mycobacterial HSP60	$HSP60_{253–268}$	EGEALSTLVVNKIRGT	Orally	Not determined	–		83.3%	[88]

Abbreviation: CFA/IFA, complete/incomplete Freund's adjuvant; ApoB, apolipoprotein B; HSP, heat shock protein. Alum: a general term of aluminum adjuvant including Alhydrogel. All mouse models ($Apoe^{-/-}$ and $Ldlr^{-/-}$).

[a] P18 is sequence-identical in human and mouse ApoB.

to and down-regulates CD80 and CD86, two costimulatory molecules found on antigen-presenting cells (APC). LAG-3 on Tregs binds to MHC class II on APCs, resulting in decreased antigen presentation. Tregs also produce anti-inflammatory cytokines such as IL-10, TGF-β, and IL-35 that can inhibit pro-inflammatory cytokine production from effector T cells and modulate T cell survival. CD73 and CD39 are two ectonucletidases highly expressed on Tregs. These two cell surface enzymes degrade ATP to immunosuppressive adenosine [52].

Tregs have been shown to curb atherosclerosis progression in mouse models. In partially Treg-deficient mice, increased atherosclerotic lesions were observed [53••,54]. Adoptive transfer of Tregs reduced atherosclerotic lesions in several mouse models such as LDLR-deficient (*Ldlr*$^{-/-}$) and Apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice [53••]. Effector type 1 helper T (Th1) cells are known to be pro-atherogenic. Buono *et al.* showed that T-bet-deficient *Ldlr*$^{-/-}$ mice had reduced atherosclerosis [55]. However, the roles of Th2 and Th17 cells in atherosclerosis are still controversial [56]. The concept of Treg-inducing vaccines for atherosclerosis is based on idea that induction of antigen-specific Tregs would suppress atherosclerosis by curbing effector T cell expansion, especially Th1 cells, and reducing their inflammatory cytokine secretion. Treg-inducing vaccines to ApoB and Hsp60/65 have been tested in animal models. Several groups used native human LDL or modified human LDL as vaccine antigens. Indeed, immunization with either showed atheroprotective responses in mice [57–59]. On the basis of these evidences, Fredrikson *et al.* screened peptides from ApoB-100, the core protein of LDL, for atheroprotection [15•]. They identified about 100 peptides that were recognized by antibodies found in plasma from patients with atherosclerosis. Vaccination with human ApoB peptides p2, p143, and p210 peptide reduced atherosclerotic lesions in *Apoe*$^{-/-}$ mice [60]. P210 vaccination induced Tregs in the spleen. Depletion of Tregs by administration of a CD25 blocking antibody canceled the vaccine-induced atheroprotection [61,62]. The proposed mechanism of action is supported by a study showing that ApoB-peptide vaccination induces atheroprotection without elevation of peptide-specific IgG in *Ldlr*$^{-/-}$ human APOB-100 transgenic mice [63]. Conversely, when p210 antibody was injected into *Apoe*$^{-/-}$ mice through the tail vein, this resulted in significant reduction of atherosclerotic lesions [64]. Thus, it is unclear whether vaccinating mice with human ApoB p210 protects from atherosclerosis in an antibody-dependent or antibody-independent manner.

CD4 T cells recognize antigenic peptide epitopes only when presented by major histocompatibility complex-II (MHC-II). Our group recently identified 27 different I-A^{b} (the MHC-II molecule in C57BL/6 mice) restricted peptides from mouse ApoB [65]) and 30 human MHC-II (various alleles) restricted human APOB peptides [66••]. Peptide identification was based on screening by peptide binding to mouse and human MHC-II, respectively. We found atheroprotection by vaccinating *Apoe*$^{-/-}$ mice with P3, P6, P18, P101, P102 or P103 in Complete and Incomplete Freund' adjuvant (CFA/IFA) [65,66••,67] and others. Vaccinating with mouse ApoB P3 and P6 peptides (sequences in Table 1) induced IL-10 mRNA in the aorta [67]. Vaccinating with mouse ApoB peptides P101, P102, and P103 (sequences in Table 1) induced Tregs in the peritoneal lavage collected after subcutaneous and intraperitoneal immunization [65]. Recently, we tested a new candidate peptide, P18. P18 is sequence identical between mouse ApoB and human APOB and binds both mouse and (some alleles) of human MHC-II. P18 immunization induced P18-specific CD4^{+} T cells in the peritoneal lavage and spleen [66••]. Interestingly, around 40% of the P18-specific CD4^{+} T cells expressed FoxP3 and CD25, identifying them as Tregs. Vaccination with the ApoB peptides P3, P6, P18, P101, P102 and P103 in CFA/IFA strongly induced peptide-specific antibody responses and T cell responses.

HSPs are highly conserved stress proteins between different species including prokaryotes. Several studies showed that vaccination with Hsp65 reduced atherosclerosis in mice [68–70], whereas other studies showed Hsp65 vaccination increased atherosclerosis [71,72]. Subcutaneous immunization with Hsp65 with aluminum adjuvant or IFA reduced atherosclerotic lesions in *Apoe*$^{-/-}$ mice [68]. Interestingly, intranasal immunization of Hsp65 protein or Hsp65-encoding plasmid DNA without adjuvant induced IL-10 production, reduced IFN-γ production and reduced atherosclerotic lesions in rabbits [69]. Intranasal immunization with Hsp60 without adjuvant reduced atherosclerostic lesions and induced Tr1 and Treg cells in the spleen and cervical lymph nodes of *Apoe*$^{-/-}$ mice [70]. These results suggest that mucosal vaccination with Hsp60/65 could be a viable approach for an atherosclerosis vaccine.

Until recently, there was no direct evidence that APOB-specific T cells exist in CVD patients. Indirect evidence that LDL and ApoB stimulation induced inflammatory cytokine production suggested that such cells may exist. To detect antigen-specific CD4^{+} T cells in human peripheral blood mononuclear cells (PBMCs), we collaborated with Dr. William Kwok at Benaroya Research Institute to develop an APOB peptide-specific MHC-II tetramer. An MHC-II tetramer consists of biotinylated recombinant fused MHC-II A and B chains, loaded with the antigenic peptide and tetramerized by binding to fluorochrome-labeled streptavidin [73]. Multiple controls are necessary to establish specificity of binding: Separate tetramers labeled with two different fluorochromes should label the same cells, labeling of myeloid cells suggesting non-specific binding should be minimal, and tetramer binding should lead to capping of the TCR in the targeted cells. We demonstrated this for human DRB1*07:01 tetramer loaded

with the APOB peptide P18. We had expected that more APOB-specific CD4 T cells might be found in human PBMCs from subjects with (cases) than without (controls) subclinical CVD, assessed by carotid ultrasound [66••]. However, this was not the case. In both cases and controls, we found that between 0.1 and 0.2% of all CD4 T cells specifically bound P18:DRB1*07:01. This number of antigen-specific CD4 T cells suggests that these clones are already antigen-experienced and expanded (not naïve). The main difference between cases and controls was that the majority of APOB-specific $CD4^+$ T cells in CVD^- donors expressed FoxP3 only. In contrast, APOB-specific $CD4^+$ T cells from CVD^+ donors expressed FoxP3 together with T-bet and RORγt, the defining transcription factors for Th1 and Th17 cells, respectively. These findings suggest that the phenotype of auto-reactive T cells may change from Tregs to more inflammatory cells during atherosclerosis progression [66••].

Several mouse studies support this hypothesis. $T\text{-}bet^{+-}$ $FoxP3^+CCR5^+$ effector CD4 T cells were found in atherosclerotic plaques, and adaptive transfer of these cells exacerbated atherosclerosis in $Apoe^{-/-}$ mice [74•]. In addition, Butcher *et al.* found $CXCR6^+CD4^+IL\text{-}17A^+$ and $CXCR6^+IL\text{-}17A^+TCR\gamma\delta^+$ T cells in murine atherosclerotic aorta and human plaques [75]. These cells retained a limited ability to regulate effector T cells, but were unable to halt effector T cell proliferation. Another study suggested that IL-17 production accompanied by IL-10 production might be anti-inflammatory [76]. Thus, it is not clear at this time whether APOB-specific $FoxP3^+ROR\gamma t^+$ cells are still regulatory or already inflammatory. Further analyses are required to clarify the involvement of antigen-specific T cell phenotype and progression of atherosclerosis.

Adjuvant for a Treg-inducing atherosclerosis vaccine

To develop an atherosclerosis vaccine, effective adjuvants are needed to activate the immune system locally (in the lymph nodes draining the injection site) and induce the desired immune response (antibodies or Tregs). One problem to overcome is that the expansion of self antigen-specific T cells after vaccination is lower than expansion in response to non-self peptide antigen. Yu *et al.* showed that self-specific $CD8^+$ T cells could not expand after peptide with anti-CD28 stimulation [77]. Therefore, an effective vaccine adjuvant is essential to overcome self-tolerance though strong innate immune signaling. In $Apoe^{-/-}$ mice, we found a three-fold expansion of ApoB P18-specific $CD4^+$ T cells after immunization with CFA/IFA [66••]. Aluminum adjuvant can effectively induce antibody responses [78]. Several studies used either CFA/IFA or cholera toxin B subunit (CTB) for Treg-inducing vaccines [61,65,67]. CTB is used as mucosal adjuvant for several experimental infectious disease vaccines [79]. Montanide ISA-51 (also called IFA) is used for experimental peptide vaccines for several cancers in clinical trials [80].

To find a suitable adjuvant for a Treg-inducing atherosclerosis vaccine, we screened six adjuvants in mice and found that the ApoB peptide P6 in Addavax (similar to the squalene-based adjuvant MF59) can protect mice from atherosclerosis as effectively as P6 in CFA/IFA. Since MF59 is a clinically used [81••], this finding holds promise for a translational path. Interestingly, P6 with Addavax was atheroprotective, but did not induce P6-specific antibody responses, suggesting that the antibody response could be dissociated from the Treg response. It remains to be seen whether this strategy can be effective clinically. On a cautionary note, the same P6 peptide given in CFA twice (prime and boost) exacerbated atherosclerosis when given in established disease, although the same P6 CFA prime and IFA boost vaccination inhibited atherosclerosis in a prevention model [82].

Conclusions

Atherosclerosis vaccines are focused on inducing antibodies to PCSK9 or CETP, or Tregs to various atherosclerosis antigens. Many approaches have demonstrated effective reduction of atherosclerosis in animal models. However, several issues must be addressed before clinical translation can be attempted. First, any vaccine must be safe, even in subjects that already have subclinical atherosclerosis. Second, the adjuvant and the formulation must be designed to promote the desired responses and prevent other responses. Third, in the case of antibody-inducing vaccines, the B cell antigen needs to be engineered to promote the development of antibodies that can actually block the function of the target protein. Fourth, for Treg vaccines, the instability of antigen-specific Tregs needs to be addressed, because Tregs switching to other phenotypes could exacerbate atherosclerosis. Fifth, the immunization route, antigen dose and injection schedule must be determined in appropriate experimental systems and validated in clinical trials. Sixth, the subjects who would benefit most from an atherosclerosis vaccine must be identified. Finally, any vaccine inducing self protein-specific immune responses must be screened against potential autoimmune disorders. Thus, developing an atherosclerosis vaccine faces enormous challenges, but at the same time holds enormous promise. Vaccination is in the best position to mitigate the residual inflammatory risk in atherosclerosis without compromising host defense.

Conflict of interest statement

K.L. is a founder and co-owner of Atherovax, Inc.

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