Review Article

Inflammatory and Autoimmune Reactions in Atherosclerosis and Vaccine Design Informatics

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Atherosclerosis is the leading pathological contributor to cardiovascular morbidity and mortality worldwide. As its complex pathogenesis has been gradually unwoven, the regime of treatments and therapies has increased with still much ground to cover. Active research in the past decade has attempted to develop antiatherosclerosis vaccines with some positive results. Nevertheless, it remains to develop a vaccine against atherosclerosis with high affinity, specificity, efficiency, and minimal undesirable pathology. In this review, we explore vaccine development against atherosclerosis by interpolating a number of novel findings in the fields of vascular biology, immunology, and bioinformatics. With recent technological breakthroughs, vaccine development affords precision in specifying the nature of the desired immune response—useful when addressing a disease as complex as atherosclerosis with a manifold of inflammatory and autoimmune components. Moreover, our exploration of available bioinformatic tools for epitope-based vaccine design provides a method to avoid expenditure of excess time or resources.

1. Introduction

Atherosclerosis and its pursuant complications are the leading causes of death worldwide. Its complex inflammatory and autoimmune pathogenesis has recently provided much insight for treatment and therapy. The suspicion of a role for the immune system in atherosclerosis began with the discovery that atherosclerotic plaques contain cellular and molecular mediators of innate and adaptive immunity [1–4]. Studies with cytokine gene knockout mice further confirmed the role of inflammation in mediating the innate response involved in atherosclerosis [5–8]. Moreover, experiments identifying a role for T-cells in atherogenesis indicate adaptive immune and potential autoimmune mechanisms [9–12]. Despite a myriad of available treatments for an ever-increasing list of targets and risk factors, it remains to find effective treatments with the potential for not only therapeutic but also prophylactic anti-atherosclerotic benefits. Success of vaccination campaigns against infectious diseases has demonstrated the proof-of-principle that identification

of antigens and pathologically associated immune responses makes vaccination an attractive therapeutic option. While the idea of a vaccine against atherosclerosis has a seemingly mythic origin, the demonstrated inflammatory and autoimmune components of atherosclerosis provide a convincing rationale to investigate such a vaccine. Vaccines have the potential to induce and/or enhance protective immune responses generated by antigens without pathogenic consequences. In fact, active investigation during the past decade has led to clinical trials for anti-atherosclerosis vaccines with some positive results. Nevertheless, it remains to develop a vaccine against atherosclerosis with high affinity, specificity, efficiency, and minimal undesirable pathology. This effort can potentially consume excessive time and resources. However, a great boon to this field is the wealth of bioinformatic tools available to design such vaccines. In this brief review, our aim is to summarize the most recent developments in designing vaccines against atherosclerosis with a focus on how to apply a variety of useful bioinformatic tools and approaches.

2. Risk Factors for Atherosclerosis

Atherosclerosis is an inflammatory disease characterized by endothelial activation and dysfunction, lipid accumulation, monocyte infiltration and differentiation, T-cell infiltration and activation, foam cell formation, and fibrosis in the lesion area. Two of its common final results, coronary and cerebrovascular disease, are the major causes of morbidity worldwide. Nowadays, atherosclerosis is increasingly considered an immune-mediated inflammatory process of the vasculature in which intense immunological activity is occurring. Hypertension, hyperlipidemia, hyperglycemia, diabetes, obesity, cigarette smoking, age, and sex are well-established risk factors for atherosclerosis [13, 14]. Recent research from our team and others' laboratories has also identified hyperhomocysteinemia as an independent risk factor for atherosclerosis [15–17]. However, these well-defined risk factors do not account for all incidences of the disease. Infectious disease, such as *Chlamydia pneumoniae* might also contribute to atherosclerotic plaque formation [18]. This latter suggestion opens the door to the further complexity of atherogenesis—a process involving inflammatory and immune responses to a number of targets derived from both foreign and self antigens.

3. Inflammatory Mechanisms: Roles of Endothelial Cells, Monocytes, and Macrophages

A mature atherosclerotic plaque contains macrophages, vascular smooth muscle cells laden with lipid, endothelial cells, and extracellular matrix. The initial pathophysiological step in atherosclerosis is endothelial injury and monocyte infiltration. Endothelial injury manifests itself as increased adhesion molecule expression on the cell surface and cytokine or chemokine expression and secretion. In atherosclerotic animal models, vascular cell-adhesion molecule 1 (VCAM-1) appears in arterial endothelial cells during the initial vascular response to cholesterol accumulation in the intima. VCAM-1 expressed in endothelial cells and very late antigen 4 (VLA-4) expressed in leukocytes are important interacting mediators for leukocytes to infiltrate into the subendothelial compartment of the vessel. In addition, the patchy distribution of adhesion molecules progresses into fatty streaks. Low-density lipoprotein receptor deficient (Ldlr$^{-/-}$) mice that express a truncated nonfunctional VCAM-1 develop less atherosclerotic plaques [19]. Intercellular adhesion molecule 1 (ICAM-1) is constitutively expressed in endothelial cells, and its expression increases in the plaque. However, there are conflicting results of ICAM-1 having proatherosclerotic effects [19, 20]. In addition to integrins, selectins are also involved in the initial steps of the process. Apolipoprotein E deficient (ApoE$^{-/-}$) mice lacking both endothelial cell selectin (E-selectin) and platelet selectin (P-selectin) have less severe atherosclerosis [21]. Monocyte chemoattractant protein 1/Chemokine (C-C motif) ligand 2 (MCP-1/CCL2) and its receptor, CC-chemokine receptor 2 (CCR2), are noted to play an important role in the

initiation of atherosclerosis, probably due to the increased monocyte and T-cell attraction and infiltration into the plaque. Several studies have demonstrated that oxidized low-density lipoprotein (oxLDL) is chemoattractant and that its oxidized phospholipid components can induce endothelial activation as judged by upregulated expression of MCP-1 [22, 23]. Other chemokines are also detected in atherosclerotic plaques, such as the cell-surface anchored CX3-chemokine ligand 1 (CX3CL1), which is expressed on vascular smooth muscle cells. MCP-1 is crucial for recruiting monocytes to atherosclerotic lesions. Crossing ApoE$^{-/-}$ or Ldlr$^{-/-}$ mice with mice lacking MCP-1 or CCR2 leads to significant lesion decrease [24–26]. Many therapeutic drugs that have anti-atherosclerotic effects may work via their anti-inflammatory effects that specifically target leukocyte adhesion and/or chemotaxis. CX3-chemokine receptor 1 (CX3CR1) is expressed on monocytes and macrophages. The results from the compound deficient mice made by crossing ApoE$^{-/-}$ mice with CX3CR1 deficient mice suggest that CX3CR1 may be involved in monocyte recruitment to the vessel wall, thereby promoting atherosclerotic plaque formation [27]. These studies all suggest that chemokines and chemokine receptors are strongly involved in atherogenesis because they increase monocyte attraction and infiltration into the lesion areas in vessels. Our recent study found that hyperhomocysteinemia accelerates atherosclerosis by promoting inflammatory monocyte differentiation/macrophage accumulation in lesions in a new hyperhomocysteinemia mouse model with an inducible human cystathionine β-synthase (CBS) transgene in the background of ApoE$^{-/-}$ and CBS$^{-/-}$ compound knock-out (Tg-hCBS/ApoE$^{-/-}$/CBS$^{-/-}$) mice fed a high fat diet [17]. Thus it is seen that the risk factors for atherosclerosis serve to promote any of a number of inflammatory processes involved in the formation or progression of atherosclerotic plaques.

Vascular cells and infiltrated cells have to be activated in response to stimulation by risk factors for atherosclerosis. In the last ten years, identification of pathogen-associated molecular patterns (PAMPs) and their receptors (PRRs) has provided a bridge to link risk factors to initiation of inflammation and secretion of proinflammatory cytokines. Toll-like receptors (TLRs) are a group of PRRs that can sense a broad range of PAMPs. In addition to TLRs, PRRs also include Nod-like receptors (NLRs) [28]. The PRRs may have implications in the development of an anti-atherosclerotic vaccine for two reasons: (1) they initiate innate immune responses and inflammation by sensing exogenous and endogenous PAMPs; and (2) they regulate adaptive immune responses. There may be many TLRs present in atherosclerotic plaques, mainly on macrophages and endothelial cells [29]. Crosses of TLR4$^{-/-}$ and ApoE$^{-/-}$ mice show reduced atherosclerosis and altered plaque phenotype [30]. Other studies also show that oxLDL and endogenous heat shock protein 60 (HSP60) can bind to the TLR4-CD14 complex and trigger inflammatory reactions with the features of pro-inflammatory cytokine secretion [31, 32], suggesting that PRRs not only recognize exogenous PAMPs but also may be activated by endogenous metabolic stress. To determine the expression of components

in the TLR/NLR/inflammasome/caspase-1/interleukin (IL)-1β pathway, our team examined the expression profiles of those genes. Among 11 tissues examined, vascular tissues and heart express fewer types of TLRs and NLRs than immune and defense tissues including blood, lymph nodes, thymus, and trachea. Based on the expression data of three characterized inflammasomes (NALP1, NALP3, and IPAF), the examined tissues can be classified into three tiers: the first tier tissues include brain, placenta, blood, and thymus express inflammasome(s) in constitutive status; the second tier tissues have inflammasome(s) in nearly ready expression status (with the requirement of upregulation of one component); the third tier tissues, like heart and bone marrow, require upregulation of at least two components in order to assemble functional inflammasomes. This original model of three-tier expression of inflammasomes would suggest a new concept of tissues' inflammation privilege and provides an insight into the differences among tissues in initiating acute inflammation in response to stimuli. This model also suggests the possibility that atherogenic risk factors induce the upregulation of PRRs and inflammasome components to trigger the chronic process of inflammatory atherogenesis [33]. Innate immune system activation is a critical step in the initiation of an effective adaptive immune response; therefore, activation of a class of receptors for PAMPs is a central feature of many adjuvant systems in vaccine preparations. One member of an intracellular PRR, the NALP3 inflammasome, is activated by a number of classical adjuvants including aluminum hydroxide and saponins [34, 35]. Inflammasome activation in vitro requires signaling of both the TLR and NALP3 in antigen-presenting cells (APCs) [36].

After infiltration, monocytes undergo differentiation into macrophages, which then become foam cells that contain a lipid-laden cytosol. Although it is unclear how these monocytes differentiate and according to what signal they differentiate, scavenger receptors, a group of proteins that mediate internalization and lysosomal degradation of lipid, lipopolysaccharide, and apoptotic bodies [37], are considered to be crucial for lipid accumulation in the macrophage to finally form the foam cell. These scavenger receptors include CD36, CD68, CXCL16, scavenger receptor A (SR-A), SR-B1, and lectin-type oxLDL receptor 1 (LOX1). The internalization and digestion of self and foreign proteins will facilitate major histocompatibility complex (MHC) class II antigen presentation [38]. Their MHC II is directly involved in antigen presentation to CD4$^+$ T-cells and CD4$^+$ T-cell activation. MHC II is widely expressed or upregulated in the lesion area on macrophages, endothelial cells, and smooth muscle cells [39]. This basic function of macrophages is antigen presentation, which triggers adaptive immunity and potentially contributes to the autoimmune character of atherosclerosis. Thus, macrophages are considered to be professional APCs. Among the internalized materials, oxLDL is one of the most important contributors to atherosclerosis and pathologically foam cells laden with cholesterol and fatty acids are composed of degraded oxLDL cholesterol ester content. Studies suggest that SR-A [40], CD36, and CXCL [41] have important functions in mediating uptake of oxLDL and promotion of atherosclerotic development.

In addition, MHC class I molecules, which present antigenic epitopes to CD8$^+$ T-cells, are constitutively expressed in macrophages. MHC molecule/antigen epitope complexes bind T-cell antigen receptors to constitute the first signal in stimulating T-cells, whereas activated macrophages in lesions also upregulate T-cell costimulation molecules on the cell surface to form the second signal for T-cell activation. Presumptive dendritic cells (DCs) bearing the CD11c integrin and other markers have previously been identified in normal mouse and human aorta. DCs are proved to be particularly abundant in the cardiac valves and aortic sinus. In all aortic locations, the CD11c$^+$ cells are localized to the subintimal space with occasional processes probing the vascular lumen. Aortic DCs express little CD40 but generate low levels of CD1d, CD80, and CD86. Aortic DCs can cross-present two different protein antigens on MHC class I to CD8$^+$ TCR transgenic T-cells. In addition, after intravenous injection, aortic DCs can capture anti-CD11c antibody and cross-present ovalbumin to T-cells. These results indicate that *bona fide* DCs are a constituent of the normal aorta and cardiac valves [42]. Studies from ApoE$^{-/-}$ mice also show the involvement of T-cell costimulation through the B7s (CD80 and CD86)/CD28 pathway in atherosclerotic plaques. The other costimulatory factor binding pair, CD40 and CD40 ligand (CD40L), is also widely expressed in the lesion cells [43]. Inhibition of CD40 signaling, including genetic disruption of CD40L in ApoE$^{-/-}$ mice or treating Ldlr$^{-/-}$ mice with CD40L antibody, reduces atherosclerotic lesion formation [44]. Treatment with CD40L antibody also inhibits the progression of formed plaques and maintains their stable phenotype [45]. Thus, macrophages are involved in both innate and adaptive immune responses during atherosclerosis.

4. Autoimmune Mechanisms: New T-Helper Cell Subsets, Tregs, and Autoantigens

The discovery that the innate immune system had an active role in the inflammatory process of atherosclerosis was critical in reinventing the established paradigm [7]. When it was discovered that the adaptive immune system also had a significantly more complicated role in atherosclerosis—namely an autoimmune component—the paradigm was revolutionized yet again [1]. It is now seen that some immune responses protect against atherosclerosis whereas others promote it.

Classically, the adaptive immune response follows from a scenario in which a pathogen escapes elimination by the innate immune system. The lymphocyte effectors of the adaptive immune system are activated by interactions between MHC/antigen complexes, T-/B-cell antigen receptors, and costimulatory molecules on the surface of innate immune system cells [46]. Professional APCs—dendritic cells, B-cells, and macrophages—take up, process, and present antigen epitopes to T-cells, thereby activating them (Figure 1). B-cell activation requires additional involvement

of CD4$^+$ T-helper cells (Figure 1). Further, the cytokines produced during the innate immune response determine the nature of the adaptive immune response—whether cellular (T-cell mediated) or humoral (B-cell mediated) [46]. Most CD4$^+$ T-cells are T-helper (Th) cells, which may be further classified on the basis of their induction and the cytokines that they secrete—an indication of the Th cells' roles and effects. For example, interferon-γ (IFN-γ) and IL-12 will induce activated CD4$^+$ T-cells to become type 1 Th (Th1) cells that secrete IFN-γ and IL-2 in promotion of cell-mediated immunity. Similarly, IL-4 will induce activated CD4$^+$ T-cells to become type 2 Th (Th2) cells that secrete IL-4, IL-5, IL-10, and IL-13 and promote humoral immunity via B-cell activation [1, 47]. These two Th subsets further cross-regulate each other. Subsequent characterization of T-cell differentiation has additionally identified IL-9-producing and IL-17-producing Th9 cells and Th17 cells subsets, respectively [48, 49]. Th9 have been observed to enhance T-cell proliferation while Th17 mediate defenses against bacteria as well as various autoimmune responses [48, 49]. Most recently, T follicular helper cells (Tfh) have been identified as having the unique ability to home to B-cell follicles where they can induce B-cell antibody production [50]. In contradistinction to the effectors of the innate immune response, the effectors of the adaptive immune response operate with a great deal of specificity to eliminate foreign pathogens and generate immunological memory. T-/B-cell receptors recognize epitopes with great specificity and affinity. It has been estimated that there are as many as 10^{18} B-cell and 10^{14} T-cell receptors [47]. While the purpose of the adaptive immune response is to eliminate agents containing antigens perceived as nonself, the scenario in which an adaptive immune response is mounted against self antigens does sometimes occur. It is now believed that atherosclerosis proceeds from such an autoimmune mechanism [12].

Atherosclerotic plaques are seen to contain elements of both innate and adaptive immunity—mostly macrophages and T-cells with few B-cells [2]. To confirm a role for adaptive immunity in atherogenesis, atherosclerotic plaque formation was observed in T- and B-cell deficient hypercholesterolemic mice and seen to be attenuated [51–54]. Further, it was observed that CD4$^+$ T-cell transfer to these deficient mice restored lesion formation to that of the control [55]. It was also noted that atherosclerotic plaques contain numerous cells producing IFN-γ, IL-12, IL-15, IL-18, and tumor necrosis factor (TNF) with few in contrast producing IL-4 [56–58]. This suggests a role for Th1 cells and not Th2 cells in promoting atherosclerosis. Further support is provided by attenuation of atherosclerotic plaque formation in animal studies targeting mediators of T-cell differentiation into Th1 cells—for example, IFN-γ, IL-12, IL-18, and TNF [10, 59–63]. Administration of IFN-γ in order to induce Th1 differentiation was seen to increase atherosclerosis in mice whereas pharmacological inhibition of Th1 cells was seen to decrease atherosclerosis in these mice [11, 64]. In contrast, Th2 cells seem to mediate anti-atherosclerotic effects in animal studies. Genetically modified mice prone to Th2 immune responses (as opposed to Th1 immune responses) developed reduced fatty streak formation, which

was reversed by preventing T-cell differentiation into Th2 cells [65, 66]. Moreover, overexpression of Th2 cytokine IL-10 in hypercholesterolemic mice reduced lesion size by 50% [67]. Similarly, Th2 cytokine IL-5 deficiency has been shown to increase atherosclerosis [9]. Nevertheless, studies with Th2-inducing cytokine IL-4 overexpression show inconsistent results [45, 68]. Thus, Th1 cells have a clear proatherogenic role, whereas Th2 cells have a more complex role in atherosclerosis that is yet to be fully elucidated.

Th1 cytokines are proinflammatory and largely promote atherosclerosis by perpetuating the inflammatory mechanisms discussed earlier (Figure 1). IL-12, IL-18, IFN-γ, and TNF, found in atherosclerotic plaques, not only induce Th1 differentiation to produce still more cytokines but also activate macrophages and vascular cells to accelerate atherosclerosis. IFN-γ activates macrophages and inhibits endothelial cell and smooth muscle cell proliferation [69, 70]. This produces pro-inflammatory cytokines, prothrombotic factors, and vasoactive mediators. Meanwhile, TNF activates the NF-κB pathway in vascular cells to trigger still more inflammation and the generation of reactive oxygen species, proteolytic enzymes, and prothrombotic factors [71–73]. These pathways all implicate Th1 adaptive immune responses in the process of atherosclerosis. On the other hand, potentially pro-atherosclerotic pathways, associated with Th2, Th9, Th17, Tfh, and CD8$^+$ T-cells, are not as well defined [12, 74]. Nevertheless, these cells have obvious implications in modulating pro- and anti-atherosclerotic pathways since they differentiate from a common precursor, which means that the nuances governing their differentiation ultimately determine the course of pro- or anti-atherosclerotic responses. Tfh cells modulate B-cell response and antibody production (Figure 1); Th2 cells modulate Th1 cell differentiation and activity; Th17 cells regulate and are mutually exclusive of immunosuppressive regulatory T-cells [49, 74]. Further, IL-17 production by Th17 may also play a role in modulating autoimmune responses in atherosclerosis (Figure 1) [75–77].

CD4$^+$CD25^{high} regulatory T-cells (Tregs) have an important role in suppressing both innate and adaptive immune responses (Figure 1) [28]. Our lab among others has shown that loss of Treg function results in autoimmune responses [78, 79]. We have characterized a Treg-specific, IL-2-dependent apoptotic pathway and demonstrated that Treg apoptotic/survival pathways are therapeutic targets for Treg-based immunotherapy. Moreover, we and others have shown that Treg inhibition accelerates vascular inflammation, with the obvious link to exacerbate atherosclerosis [80–82]. Like effector T-cells, Tregs require T-cell receptor activation and costimulation for activation. However, it remains to distinguish a pathway that exclusively regulates Tregs as opposed to effector T-cells [83]. Though it was seen that superagonistic CD28 antibody preferentially activated Tregs in animal models, phase I clinical trials showed a dangerous lack of specificity that triggered a cytokine storm [84–86]. Nevertheless, direct Treg transplant has proven more successful [87]. As atherosclerotic plaques contain a depleted number of Tregs (1%–5% of all T-cells versus normally 25% of all T-cells), this may prove to be a useful strategy for
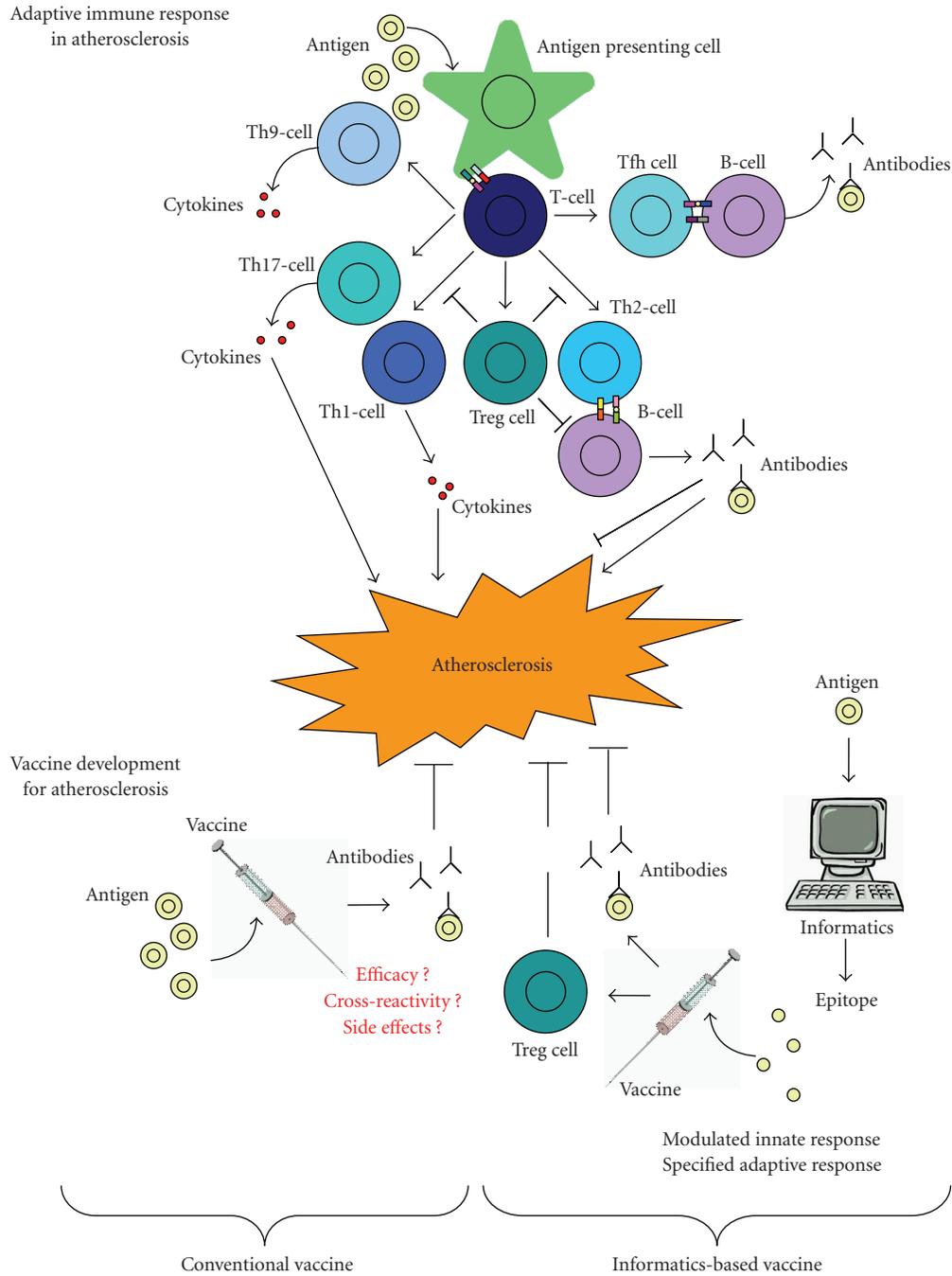
FIGURE 1: Approaches for vaccine development. This schematic diagram illustrates the adaptive immune response in atherosclerosis (top) as well as the conventional approach (bottom left) and our proposed bioinformatic approach (bottom right) to vaccine development for atherosclerosis.

regulation of T-cell dysfunction [88]. It should be cautioned that excessive T-cell suppression can compromise cellular defense systems. Ideally, Treg therapy should be targeted to specific autoantigens involved in atherogenesis and localized in atherosclerotic plaques.

In addition to T-cell mediated cellular responses, the adaptive immune response also includes humoral responses mediated by B-cells. Studies with T- and B-cell deficient mice

confirmed a net pro-atherogenic role for these mediators of adaptive immunity [51–54]. However, other studies demonstrated that splenectomy and consequent loss of some T- and B-cells led to increased atherosclerosis that could be reversed by adoptive transfer of B-cells [89–91]. The primary role of B-cells in atherosclerosis is thought to consist of antibody production, which may be either pro- or anti-atherogenic [1, 74]. B-cells can also secrete cytokines

and serve as APCs, thereby modulating the T-cell adaptive immune response, though such a role for B-cells has not been sufficiently investigated in atherosclerosis. It has been suggested that a major limitation in appreciating the cellular responses mediated by B-cells is the failure to appreciate the existence of B-cell subsets that can mediate specific responses [92, 93]. While specific B-cell subsets have been identified in mice, it still remains to identify them in humans [93]. It is thought, however, that cells from a B-cell subset termed "B1-cells" produce "natural antibodies" of the IgM subtype in a T-cell and antigen independent manner [94]. Primarily located in the peritoneal cavity, they provide a first line of defense against foreign pathogens [92, 94]. Intriguingly, IgM natural antibodies specific for oxLDL are found in the circulation of humans without significant levels of atherosclerosis [95].

CD4$^+$ T-cells from atherosclerotic plaques have been shown to have specificity for oxLDL and HSP60 among other potential exogenous and endogenous antigens [57, 96–98]. oxLDL is one of the earliest and best characterized autoantigens involved in atherogenesis [99]. As an initiating step in atherogenesis, LDL particles that are trapped within arterial intima become oxidized and begin to accumulate as a fatty streak [100–102]. APCs internalize oxLDL via the scavenger-receptor pathway and, after proteolytic processing, bind MHC class II molecules to present peptide fragments to T-cells [1]. As T-cells do not react with native LDL components, it is thought that LDL oxidation leads to a loss of immunogenic tolerance. It has also been suggested that hypercholesterolemia impairs DC mobility with a consequent loss of immunogenic tolerance as they are not able to perform their tolerogenic clearance functions [12, 103–105]. Moreover, the existence of antibodies specific for oxLDL particles further indicates that an adaptive immune response to oxLDL occurs during atherosclerosis [97]. It has been suggested that the nature of this adaptive immune response is a combination of pathological autoimmunity and protective immunity [12].

HSPs' expressions are widespread amongst different organisms, and HSPs largely function as chaperones. They have also been implicated in autoimmune atherosclerosis [106]. Interest in HSPs as targets, developed from an observation that *Chlamydia pneumoniae* have HSPs that are found in atherosclerotic plaques [18, 107]. Epidemiologically, elevated HSPs have been found in patients with systemic hypertension, coronary artery disease, carotid atherosclerosis, myocardial infarction (MI), and ischemia [107]. Further, high levels of antibodies against mycobacterial HSP65 independently predict MI, stroke, and cardiovascular death [108]. The argument for HSPs as autoantigens relates to their cross-reactivity with other bacterial HSPs (e.g., *Mycobacteria*, *Chlamydia*) and potential recognition by antibodies against infection by those bacteria [107]. Such autoimmune recognition has been considered a consequence of a molecular mechanism termed "molecular mimicry" [109]. Other infectious agents like herpes simplex and cytomegalovirus have also been found in atherosclerotic lesions [47]. Though infection is not a necessary contributor to atherosclerosis, it may certainly play a role in activating PRRs that initiate innate immune responses leading to subsequent adaptive immune responses. For example, it has been demonstrated that TLR9 activation on DCs in atherosclerotic plaques leads to an autoimmune T-cell attack on vascular smooth muscle cells [110]. This study demonstrates the mechanism of how a pathogen can generate a pro-atherogenic autoimmune response.

5. Vaccine Development for Atherosclerosis

Historically, vaccines have been proved to be a safe and efficient tool for protection against infectious diseases [111]. Following decades of conceptual and technological breakthroughs, the concept of vaccination has been extended to a range of diseases not strictly limited to those caused by infection. Not surprisingly, vaccine design for the treatment of atherosclerosis has been an actively investigated field for a number of years. Several antigen targets have been proposed as well as several approaches to vaccine design, implementation, and efficacy. In some cases this has already led to funded clinical trials. Vaccines for atherosclerosis are somewhat different from traditional vaccines for infectious diseases. Ideal vaccines for atherosclerosis should provide recipients with (1) protective immunity against infection-derived pro-atherogenic antigens and (2) immune tolerance for autoimmunogenic self-antigens.

In the previous section, we discussed the immune responses to pro-atherogenic autoantigens. In this section, we focus on the research progress using these autoantigens as the targets of vaccine development. As LDL is a major mediator of atherosclerosis, it is not surprising that this was the earliest target for anti-atherosclerotic vaccine therapy [112–116]. These early studies provide conflicting, yet encouraging results. Subsequent studies focusing on knock-down of oxLDL showed a decrease in atherosclerotic lesion size [117, 118]. The complexity of LDL has, however, made identification of antigenic epitopes difficult. To this end, it has been necessary to develop several different experimental models. Early experiments targeted modified LDL with some success [119–121]. Still other experiments targeted components involved in LDL metabolism [122–124]. Targeting oxidized phospholipid components of oxLDL such as phosphatidyl choline (PC) generated enthusiasm in the field since both oxLDL and apoptotic cells express PC which promotes targeted removal by receptor-scavenger and IgM pathways [125]. Binder et al. were able to demonstrate that immunization with PC-containing *Streptococcus pneumoniae* vaccine generated oxLDL-specific antibodies that also correlated with reduced atherosclerosis [126]. Caligiuri et al. further showed that immunization with PC linked to a carrier protein was sufficient to induce specific antibodies and significantly reduce atherosclerosis [127]. Collectively, these studies confirm that antibodies against oxLDL epitopes have atheroprotective effects. A major consideration remains, however, as to what potential cross-reactivity will occur with non-oxLDL endogenous products containing oxidized phospholipids. For greater oxLDL specificity, more recent studies have targeted ApoB-100 peptide fragments following aldehyde modification and proteolytic degradation, which

are specific for LDL [12]. Immunization of mice with several of these ApoB-100 peptides was seen to reduce atherosclerosis [128–131].

Studies in animal models have confirmed the potential antigenicity of HSPs. Xu et al. have demonstrated that mycobacterial HSP65 elicits a pro-atherogenic immune response [132, 133]. Based on similar modes of delivery, other studies corroborate that the immune response against HSP65 is a pro-atherogenic most likely because of the innate immune response and Th1 activation [134, 135]. A different strategy using mucosal delivery in order to elicit a Th2 response showed decreased atherosclerosis [136, 137]. Immunization of patients with HSP70 also showed decreased atherosclerosis [138]. Following the idea that molecular mimicry is the mechanism responsible for human cross-reactivity with foreign HSPs, vaccination against infectious agents like influenza virus has been examined in the context of atherosclerosis. Though some results are encouraging, the benefits of the clinical trials have not yet proven convincing [107, 139]. Similarly, vaccination against hepatitis A was not seen to change atherosclerotic development in animal models [140]. Vaccines against still more risk factors for atherosclerosis have been examined like nicotine [141, 142], angiotensin I [143], ghrelin [144], and periodontitis [145] with some interesting results. While all avenues of thought have serious implications for preventing atherosclerosis, it is unrealistic to believe that a vaccine constructed from a single underlying molecular target of a clearly multifaceted disease process will effectively prevent it. In light of recent advances in knowledge and technology, it is useful to reevaluate the delivery and mode of action of vaccines in addition to simply choosing its targets.

Adjuvants are agents administered along with vaccines in order to generate an increased immune response to the vaccine. They often enhance the potency and longevity of a specific immune response without themselves inducing toxicity or initiating long-lasting immune effects [146, 147]. Aluminum salts—aluminum hydroxide, aluminum phosphate, or simply "Alum"—have been used as adjuvants since 1926 [148]. In fact, Alum is still used in a number of US FDA-approved vaccines for diphtheria-pertussis-tetanus, *Haemophilus influenzae* type b, hepatitis B, hepatitis A, inactivated poliovirus, *Streptococcus pneumonia*, and human papilloma virus [146, 149]. Despite its widespread use, Alum's mechanism for enhancing immune response has only recently begun to be understood. Numerous suggestions abound the following: (1) depot formation facilitating continuous antigen release, (2) promotion of phagocytosis by APCs, (3) induction of chemokine secretion by monocytes and macrophages, (4) inflammatory monocyte recruitment, (5) Th2 persistence and cytokine secretion to facilitate humoral response, (6) NALP3-dependent caspase-1 activation with IL-1β secretion [34, 150–154]. Each mechanism offers a conceivable route by which Alum may enhance different specific immune responses, though it is not certain under which circumstances which pathways are activated. A recent study of Alum in hypercholesterolemic mice showed that conditions of hypercholesterolemia affect Alum-induced immune responses. Wigren et al. demonstrated that Alum

induced Treg expansion and inhibition of T-cell proliferation in hypercholesterolemic mice, but not in normal control littermates [155]. Aside from Treg induction being an important finding in its own right, this result points to a very important consideration in vaccine design—proper adjuvant formulation is necessary for efficacy. For more details, Reed et al. provide an excellent review of adjuvant design and development [146].

In addition to adjuvants, the route of vaccine delivery is also important to consider in order to elicit a specific immune response. Mucosal (oral or intranasal) immunization is often used to induce tolerogenic responses [156]. It is thought that this mode of delivery induces a Th2 mediated response, resulting in Th1 suppression, decreased inflammation, and reduced development of delayed-type hypersensitivity. Van Puijvelde et al. have shown that orally administered oxLDL can suppress atherosclerosis via Treg induction [157]. As an additional consideration, the time of delivery can also affect the efficacy of the vaccine. The neonatal immune system is distinctly different from the adult immune system with a Th2 bias and an abundance of Tregs [158]. Consequently, it has been shown that immunization of neonatal mice with oxLDL results in suppressed T-cell responses to oxLDL and inhibition of atherosclerosis [159].

Thus, while vaccine development for atherosclerosis is already underway, it's still important to explore a myriad of possibilities in order to anticipate future obstacles and in order to avoid a slow stepwise progression from one innovation to the next. Identification of a single antigen target is not a sufficient end. It would be better to identify a process that determines how many other targets there may be and how and when to deliver vaccines against them. Immunomodulation involves working with the entirety of the immune system—both innate and adaptive. Determination of target antigens should be made with the types of induced immune responses in mind—immunosuppressive versus immune-provocative, cellular immune responses versus humoral immune responses, and so forth.

The great availability of databases and bioinformatic tools characterizing B- and T-cell epitopes allows for evaluation of sequences among known autoantigens to identify suitable epitopes for inducing humoral and/or cellular immune responses. We previously found that the transcripts of all the autoantigens involved in various autoimmune diseases including atherosclerosis are highly modulated by alternative splicing. In contrast, the RNA transcripts of only 42% ± 5% randomly selected human genes are modulated by alternative splicing. However, housekeeping splicing of RNA transcripts could not generate autoantigen epitopes encoded by alternatively spliced exon(s). The only splicing events that respond to inflammatory/pathological stimulation include extra-exon sequences in mRNA transcripts, which then generate antigen epitopes previously untolerized in thymic T-cell development. In support of our observation via database mining, our experimental reports demonstrated that (1) the expression of an essential alternative splicing factor ASF/SF2 is modulated in the autoimmune/inflammation affected tissue in patients with autoimmune disease [160], (2) unmutated tumor antigens are actually a special group

of autoantigens that elicit antitumor immune responses in patients with tumors [161], and (3) the alternatively spliced long isoform of unmutated self-tumor antigen CML66 is immunogenic but not the alternatively spliced short form of CML66 in patients who developed anti-CML66 immune responses [162]. Based on these analyses, we proposed a new "stimulation-responsive splicing" model for generating untolerized autoantigenic epitopes (see our invited review for the details) [163]. We propose a similar analysis of known pro-atherosclerotic antigens in order to determine ideal autoantigenic epitopes rather than mere guesswork as to what moieties and modifications are potentially good targets. Moreover, by generating a systematic approach to autoantigen epitope identification, we can also lend additional support in identifying candidate targets hypothesized by present methods.

6. Bioinformatic Approach for Vaccine Development

We would like to suggest a bioinformatic approach for vaccine design that utilizes the vast array of epitope data available from prediction algorithms and databases. The idea is to use sequences of known autoantigens to characterize autoantigenic epitopes recognized by either/both B- and T-cells. As these recognized autoantigenic epitopes are generated from experimentally verified autoantigenic sequences, it follows that their homology may be characterized. We can then make statistical comparisons with candidate sequences to assess their antigenicity. This is a novel approach to vaccine design as it uses well-characterized information in order to generate a systematic approach of characterizing new antigenic candidates. Moreover, this approach has a structural basis as epitope recognition largely proceeds from sequence and structure rather than purported pathology. Presently, most available vaccines utilize weakened or inactivated forms of pathogens to either raise neutralizing antibodies or stimulate secondary immune responses specific to the antigen of interest (Figure 1) [111]. This method of vaccinology aims to induce immunity similar to that elicited by natural infections, without the pathogenic autoimmune consequences. This approach, however, has not been able to yield effective vaccines for diseases including most types of cancers, human immunodeficiency virus (HIV), tuberculosis, and atherosclerosis [166]. To improve the specificity, efficacy and safety of traditional vaccines, the concept of "epitope-based vaccines" was developed, the execution of which relies on the availability of genomic sequence databases as well as modern bioinformatic technologies (Figure 1). Since protein antigens mutate periodically (especially in viruses implicated in diseases—e.g., influenza virus, HIV), it is advantageous to target common epitope sequences that are conserved across subtypes. The identification of conserved genetic variants can be performed using public databases such as the NCBI Entrez protein database (http://www.ncbi.nlm.nih.gov/entrez) [163]. Consequently, a polypeptide that contains genetic information of multiple epitopes could be engineered to protect against a broad spectrum of microbial antigenic strains [167]. Utilizing bioinformatic approaches, combined with biological tools such as microarray, epitope-based studies of vaccines for HIV, malaria, and *B. meningococcus* have produced positive results [168–170]. Long-term studies in determining the relationship between antigen structure and antigenicity have generated numerous antigenicity prediction algorithms. Once the antigenic sequences of interest are identified, the prediction and mapping of relevant T-cell and B-cell epitopes is the next critical step in the creation of vaccines for atherosclerosis [107].

B-cells mediate a humoral immune response through the secretion of antibodies that neutralize invading microbes and pathogenic antigens. B-cells are stimulated when their antigen receptors recognize an antigenic epitope, which could be linear continuous amino acid sequences with about 15 amino acids or discontinuous amino acids connected via tertiary structures. The conformational aspect of discontinuous epitopes complicates the accurate prediction of B-cell epitopes [164, 171]. The algorithms developed for the prediction of B-cell epitopes have not been as effective or accurate [164]. B-cell epitope prediction tools fall into three categories: prediction based on primary amino acid sequences, based on conformational structure, and based on phage-display data. For continuous B-cell epitopes, prediction algorithms analyze physicochemical properties of primary sequences including amino acid hydrophilicity, flexibility, polarity, and turns. Sequence-based tools for the analysis of continuous B-cell epitopes include ABCprep [172], Bepipred, BEPITOPE [173], and IEDB B-cell epitope tools [164] (Table 1). For discontinuous B-cell epitopes, conformational structures play a more important role in epitope prediction compared to continuous epitopes, and structure-based tools have been developed such as Epitopia [174] and Ellipro [175] for the prediction of continuous epitopes (Table 1). Programs such as Epitope Mapping Tool (EMT) and EPIMAP utilize phage libraries to screen epitopes specific for known antigens. By analyzing many experimentally identified antigenic epitopes, we have generated statistical confidence intervals of antigenicity scores in order to ascertain the antigenicity of predicted B-cell epitopes [176]. This may be used to characterize potential vaccine targets with the aim of inducing a B-cell immune response in two ways. First, it can be applied to determine if a candidate antigen can be recognized by B-cells. Also, this method may be used to generate candidate epitopes within candidate antigens.

Compared to B-cell epitopes, T-cell epitopes are simple, short linear 9-15 amino acid sequences. As a result, the prediction tools for T-cell epitopes are better defined and give consistent high-quality results. T-cell epitopes are presented by MHCs for recognition by specific T-cell antigen receptors (TCRs). The interactions between epitopes and MHC as well as ones between epitope and TCR are critical in antigen recognition [177]. The binding of epitopes with MHC is a necessary step in dominant epitope generation and has been used as a predictor for epitope identification. Epitopes bind to special grooves in MHC molecules, which consist of specific sets of amino acids called anchor motifs

TABLE 1: B-cell epitope prediction tools. This table, adapted from Table 1 [164] summarizes available websites for B-cell epitope prediction. The prediction mechanisms employed, URLs, or authors' email addresses are included. Detailed descriptions and references are included in the paper.

Name	Description	URL
ABCpred	Based on sequence using recurrent neural network module	http://www.imtech.res.in/raghava/abcpred/
BEPITOPE	Based on sequence to predict continuous epitopes	jlpellequer@cea.fr
Bepro	Based on structure of the antigen to predict discontinuous B cell epitopes	http://pepito.proteomics.ics.uci.edu/
CEP	Based on structure for the prediction of continuous and discontinuous epitopes	http://bioinfo.ernet.in/cep.htm
COBEpro	Based on primary sequence of continuous B cell epitopes. Secondary structure and solvent accessibility information can be incorporated to improve prediction	http://scratch.proteomics.ics.uci.edu/
DiscoTope	Based on sequence and structure for the prediction of discontinuous epitopes	http://www.cbs.dtu.dk/services/DiscoTope/
Ellipro	Based on solvent accessibility and protein flexibility	http://tools.immuneepitope.org/tools/ElliPro/iedb_input
EMT	Based on Phage-display for the prediction of continuous and discontinuous epitopes	elro@novozymes.com/
EPIMAP	Based on Phage-display for the prediction of continuous and discontinuous epitopes	mumey@cs.montana.edu
Epitopia	Based on either protein 3-D structure or linear sequence using training data	http://epitopia.tau.ac.il
IEDB B-cell epitope tools	Predict continuous B cell epitope based on amino acids scales and discontinuous epitopes based on 3D structures	http://tools.immuneepitope.org/main/html/bcell_tools.html

TABLE 2: T-cell epitope prediction tools. This table, adapted from Table 1 [165], summarizes available websites for T-cell epitope prediction. The prediction mechanisms employed, URLs, or authors' email addresses are included. Detailed descriptions and references are included in the paper.

Name	Description	URL
BIMAS	Half time dissociation to HLA class I molecules	http://thr.cit.nih.gov/molbio/hla_bind
EpiMatrix	Binding efficiency with MHC Class I and II	http://www.epivax.com/
FRAGPREDICT	Binding score of Proteasome Cleavage Sites	http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html
Immune Epitope Database and Analysis Resource (IEDB)	Analyze proteasomal processing, TAP transport, and MHC I&II binding to produce an overall score for a peptide's potential for of being a T cell epitope	http://www.immuneepitope.org/
MHCPred	Based on MHC/peptide or TAP/peptide IC50 binding values	http://www.jenner.ac.uk/
MMBPred	Mutated high affinity MHC binding peptides	http://www.imtech.res.in/raghava/mmbpred/
NetChop	Proteasome or immunoproteasome cleavage sites	http://www.cbs.dtu.dk/services/NetChop/
NetCTL	Combined scores for MHC subtype binding, TAP transport and NetChop proteasome scores	http://www.cbs.dtu.dk/services/NetCTL/
NetMHC	MHC binding propensity of peptides	http://www.cbs.dtu.dk/services/NetMHC
ProPred-1	Efficiency of MHC I peptide binding, optional proteasome/immunoproteasome cleavage filter	http://www.imtech.res.in/raghava/propred1
SYFPEITHI	Binding motifs to MHC Class I and II	http://www.syfpeithi.com/
TAPPred	Binding affinity of TAP proteins	http://www.imtech.res.in/raghava/tappred/
TEPITOPE	Promiscuous MHC II epitopes	http://www.vaccinome.com/

[165]. We found that in addition to the primary anchor amino acid residues E2 (the second in the epitope) and E9, some secondary residues including E3, E4, E6, E7, and E8 as well as some residues in the N-terminal and C-terminal flanking regions also contribute to binding to MHC molecule and epitope cleavage [178]. Predictions of epitopes based on analysis of anchor motif sequences are the most widely used including SYFPEITHI, BIMAS, and EpiMatrix (Table 2). The high affinity binding grooves accommodate peptides in a highly promiscuous manner such that each

MHC molecule could bind approximately between 1,000 and 10,000 peptide sequences with different affinities [179]. Moreover, MHC anchor motif sequences are prone to mutation and result in polymorphisms. To accommodate variations, programs such as TEPITOPE (Table 2) have been created. Furthermore, an assumption underlying prediction methods analyzing anchor motifs is that each amino acid residue contributes independently to the overall binding efficiency. In reality, interactions among different sites could influence the binding property of individual sites [180]. To address this discrepancy, machine learning methods such as artificial neural networks (ANNs) have been devised to address the complex relationships in the data sets [181].

Epitope-MHC binding is a prerequisite for T-cell epitope presentation. Generally, the higher the affinity binding is between the antigenic epitope and MHC molecule, the better the immunogenicity of the epitope. However, epitope-MHC binding is not the only definitive epitope indicator. Before epitope-MHC binding, the peptide needs to be processed in order to be presented by MHC molecules for T-cell recognition. This requires two steps: (1) proper protease cleavage in cytosolic ubiquitin-proteasome-peptidase systems and (2) translocation from cytosol into endoplasmic reticulum lumen by the transporter associated with antigen processing (TAP). Prediction programs such as NetCTL and IEDB (Table 2) have been designed to account for epitope proteasome cleavage and TAP binding efficiencies.

As with B-cell epitopes, we have generated statistical confidence intervals of antigenicity scores, proteasome processing scores, as well as TAP binding scores for various tumor antigen epitopes by analyzing many experimentally identified antigenic epitopes. This allows us to ascertain the antigenicity of predicted T-cell epitopes. These statistical confidence intervals suggest that over 95% of experimentally identified antigenic epitopes have the prediction scores within the intervals [163]. Similar measures could be made for the design of atherosclerosis vaccines. Proceeding from experimentally verified pro-atherosclerotic autoantigens and mediators will be possible to characterize relevant B- and T-cell epitopes. Further, candidate antigens may be analyzed using this result to determine potential antigenicity and mediation of the desired immune response. The effective utilization of B-cell and T-cell epitope prediction tools will greatly save time and effort and enhance future endeavors to design effective vaccines for atherosclerosis.

7. Discussion

In this review, we have interpolated a number of novel findings in the fields of vascular biology, immunology, and bioinformatics in order to discuss the development of a vaccine against atherosclerosis. We began with a brief discussion of traditional and novel risk factors for atherosclerosis that led to the determination of the inflammatory components of atherosclerosis. Now it is seen that the inflammatory pathways of the innate immune response set a course for an adaptive immune response—specifically an autoimmune response—that mediates the progression of atherosclerosis.

Thinking of atherosclerosis in this way makes the disease a candidate for treatment by vaccine in the traditional sense of modulating immune responses. However, with recent technological breakthroughs, vaccine development affords precision in specifying the nature of the desired immune response—a useful tool when addressing a disease as complex as atherosclerosis with a manifold of inflammatory and autoimmune components. Moreover, our exploration of available bioinformatic tools for epitope-based vaccine design affords a thorough consideration of vaccine design without expenditure of excess time or resources.

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