CURRENT STATE AND OPTIMIZATIONS FOR PEPTIDE-BASED VACCINES AGAINST ATHEROSCLEROSIS

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1. INTRODUCTION

Atherosclerosis is a systemic disease characterized by an accumulation of low-density lipoprotein (LDL) in the wall of medium and large arteries. It is the underlying pathological process and hence the leading cause of cardiovascular disease (CVD), which has an enormous impact on global health in terms of mortality and morbidity. Although the prevention of CVD has greatly improved since the 1970s, it continues to be poorly managed with an estimated risk reduction of only 30% (1). It was estimated that a total of 17.3 million deaths were caused by CVD in 2008, clearly indicating the seriousness of the problem (2). Current treatments focus on the management of risk factors by lowering lipids, mainly LDL, however they intervene little in the pathological processes that take place in the artery wall. In addition, novel drugs targeted to lower blood lipid levels have failed in clinical trials, underscoring the urgent need for treatments with different modes of action (3,4).

Nowadays it is recognized that the pathology of atherosclerosis is much more complex than originally believed. Increasing evidence shows that atherosclerosis is highly associated with inflammation and autoimmunity, causing many research groups to direct their attention towards the immune system. The main driving force behind the inflammation is an immune response against (modified) self-antigens. These autoantigens are targeted by both humoral and cellular immune responses. However, the role of these immune responses in the onset and progression of atherosclerosis has been controversial. This was mainly caused by the seemingly opposing actions of different components of the immune system (5,6). At present, it is assumed that the activation and recruitment of Th1 and possibly Th17 cells leads to progression of the disease due to the release of pro-inflammatory cytokines. On the other hand, while not completely elucidated, it is believed that a Th2-dominant immune response results in production of antibodies by B cells that appear atheroprotective. Strong evidence also shows that progression of the disease is attenuated by regulatory T (Treg) cells that inhibit autoimmunity and suppress inflammation (5,6). Despite the complex pathology of atherosclerosis and the multifaceted immune response, in the recent decade our understanding of the underlying immune-related mechanisms has greatly increased and immunological treatments are now being pursued.

One of the most elegant approaches is an antigen-specific immunotherapy, which specifically targets disease-related antigens. By vaccination with the autoantigen or preferably subunits of the autoantigen, the immune system can be fine-tuned towards a more favorable response (i.e. antibody production or Treg cell activation). Such peptide-based vaccines are ideal alternatives for conventional pharmacological treatments and monoclonal antibodies in terms of cost-effectiveness and compliance. Therefore, the development of a vaccine is a highly attractive option to treat such a common and worldwide disease as atherosclerosis.

Over the last century, enormous successes have already been booked with vaccination programs for infectious diseases (7). In addition, peptide-based vaccination for other auto-immune diseases, allergies and cancer have also been investigated extensively (8,9). Compared to the numerous reports about vaccines in these research fields, vaccine development for atherosclerosis is still in its infancy. To date, vaccine-based treatments for atherosclerosis have shown promising preclinical results, but have not yet entered clinical

trials (10,11). In most research areas, the translation of preclinical research to a successful vaccine has been met with numerous challenges. However, in the last decades many of these obstacle have been tackled and recent developments offer new opportunities for vaccine design and development (7,12–14). The abundance of data acquired in these research areas could pave the way for investigators that try to develop a vaccine against atherosclerosis.

Therefore, this review will concentrate on how the current prototype vaccines for atherosclerosis could be enhanced with novelties in vaccine research, design and composition that have been discovered in other research areas. By doing so, we aim to save the research community valuable time and accelerate the development for a successful vaccine for atherosclerosis.

2. CURRENT STATE OF VACCINE DEVELOPMENT FOR ATHEROSCLEROSIS

In order to appreciate the potential improvements that could be applied to the prototype vaccines for atherosclerosis, it is of importance to outline the current state of vaccine development for atherosclerosis. Nonetheless, a comprehensive review of all lines of vaccine research for atherosclerosis is outside the scope of this review. Therefore, the two best characterized vaccine candidates that are close to be studied in clinical trials will be reviewed here. These vaccines target the main apolipoprotein of LDL, apolipoprotein B100 (ApoB100) and heat shock protein 60 (HSP60).

2.1. Vaccines against apolipoprotein B100

The cardinal process in the onset and development of atherosclerosis is the accumulation and oxidation of LDL in the artery wall. Oxidized LDL (oxLDL) induces the expression of proinflammatory genes, leading to leukocyte recruitment and cytokine release. In addition, it elicits strong humoral and cellular autoimmune responses and antibodies against oxLDL have been demonstrated in several animal species and humans (15,16). Since raised levels of antibodies against oxLDL were associated with a number of disorders, including hypertension and coronary artery disease, it was originally believed that the autoimmune response against oxLDL was atherogenic (17,18). Nonetheless, early research provoking an autoimmune response against oxLDL in mice and rabbits surprisingly demonstrated a reduction in atherosclerosis (19–22). These studies were the first to report the protective effects of immunization with oxLDL and laid the groundwork for further vaccine research for atherosclerosis.

Subsequently, studies were performed to identify the antigenic structures within oxLDL (23,24). Native and malondialdehyde (MDA)-modified peptide sequences from ApoB100 were screened and a number of epitopes were found to provoke immune responses (24). Atherosclerotic mice immunized with these peptides (i.e. p2, p45 and p210) showed a marked reduction in atherosclerosis, clearly showing the atheroprotective effects of a ApoB100-based vaccine (23). Further research into the underlying protective mechanisms originally pointed towards an increase in peptide specific IgG antibodies (25). More specifically, it was argued that immunization caused a shift in the Th1/Th2 balance towards the more favorable Th2 response as measured by significant increases in Th2-specific IgG1 antibodies (25). Corroborating evidence was found when passive immunization with IgG1 antibodies against MDA-modified ApoB100 resulted in significant reductions in atherosclerosis (26,27). Nonetheless, a recent phase II trial with a monoclonal antibody against oxLDL failed to meet its primary endpoint (28). Moreover, it was demonstrated that immunization with ApoB100 peptides could also reduce the development of atherosclerosis in absence of altered antibody levels, raising doubt about the protective effects of antibodies against ApoB100 (29).

Hence, an alternative theory was proposed that involved the activation of Treg cells and induction of immunologic tolerance (29). Earlier studies had already shown the importance of Treg cells in atherosclerosis and demonstrated that oral administration of oxLDL led to mucosal tolerance against oxLDL by an activation of regulatory T cells (30,31). Further efforts showed that intranasal administration with ApoB100 p210 conjugated to cholera toxin B (CTB) also induced activation of Treg cells and markedly

improved atherosclerosis in ApoE $^{-/-}$ mice (32). Direct evidence for the protective effects of Treg cells was shown by Herbin *et al.* when depletion of Treg cells completely abolished the atheroprotective effects of low subcutaneous doses of ApoB100 p210 vaccine (33).

Although nowadays most studies point to the role of Treg cells and restoration of tolerance towards LDL as the important underlying atheroprotective mechanisms, it cannot be ruled out that other immune cells mediate part of the protection as well. For instance, a recent study showed that the atheroprotective effects seen by p210 vaccination were facilitated by $CD8^+$ T cells (34). Moreover, also antigen-specific antibodies remain potential atheroprotective candidates (35). Nonetheless, it is clear that vaccines based on ApoB100 have significant therapeutic potential for the treatment of atherosclerosis.

2.2. Vaccines against heat shock protein 60

Heat shock proteins (HSPs) are highly conserved proteins that protect cells against stressful conditions, including unexpected increases in temperature and infection, by preserving the correctly folded forms of cellular proteins. They are classified according to their molecular weight into several families of which the HSP60 family appears to play a significant role in atherosclerosis. The HSP60 family is comprised of several proteins that share highly homologue sequences between species, specifically the mammalian HSP60 and the mycobacterial HSP65. Under physiological conditions, expression of HSP60 is low and exclusively present inside cells. However, when challenged by harmful conditions (e.g. accumulated oxLDL or increased levels of cytokines), cells of the artery wall respond by increasing the expression of HSP60 as a protection mechanism. However, due to the homology between the mycobacterial HSP65 and the mammalian HSP60, this could elicit aberrant autoimmune responses, especially when cells die and HSP60 is also present in the extracellular space.

Early research showed that raised levels of antibodies against HSP60/65 in humans were associated with atherosclerosis (36–38). In addition, multiple studies showed that immunization of mice or rabbits with HSP65 contributed to plaque development (39–41). Additional evidence that autoimmune reactions against HSP60 were part of the underlying mechanism of atherosclerosis was provided when HSP60 antibodies from patients with coronary heart disease caused a significant increase in atherosclerotic lesions in ApoE $^{-/-}$ mice (42). In addition, a mouse monoclonal antibody raised against a specific epitope in HSP60 also induced atherosclerosis (42).

Taking advantage of the increased knowledge about the autoimmune responses against HSP60 and the concept of immunological tolerance, several efforts were made to induce mucosal tolerance against HSP60 (43–45). Two studies in 2002 showed that both oral (43) and nasal (44) administration of mycobacterial HSP65 resulted in reduced plaque development and autoimmune responses in mice. In accordance with the earlier ApoB100 vaccine research, this was partly attributed to a shift towards a more Th2-dominated immune response. A more recent study showed that induction of oral tolerance with HSP65 caused Treg cell activation and a corresponding reduced plaque size (45). Additionally, they demonstrated that a similar response was achieved with a specific peptide of HSP65.

Taken together, these studies clearly show the importance of the HSP60 proteins in the pathology of atherosclerosis and that mucosal vaccination against HSP60 proves an attractive method for the treatment of atherosclerosis. Currently, investigators are searching for additional peptide sequences within HSP60 with the ideal properties to develop a prototype vaccine (46).

3. VACCINE OPTIMIZATION

Although the vaccines based on ApoB100 and HSP60 have shown promising preclinical results, the translation of peptide-based vaccines from the preclinical phase to clinical trials has often been hampered by several issues. Firstly, the antigens utilized in animal models for autoimmune diseases might not be appropriate for human autoimmunity, warranting the identification and validation of novel antigenic peptide sequences. Secondly, many peptide-based vaccines have stranded in clinical trials due to a limited

efficacy. This is often due to a restricted immunogenicity in the human situation or because of the limited bioavailability of the vaccine. The general approach to restore tolerance in autoimmune diseases or allergies is by introducing the peptide in a non-inflammatory environment, usually mucosal tissue through oral or intranasal administration (13). Due to the absence of proinflammatory signals in those areas, the peptide activates antigen-specific Treg cells that will home to the inflamed tissue where they recognize their cognate antigen. The key point in introducing tolerance is that the range of Treg cells that are activated in the mucosal tissue is powerful enough to skew the immune response in the inflamed areas to an anti-inflammatory phenotype. However, peptide-based vaccines often lack a strong immunogenicity resulting in an insufficient activation of Treg cells and poor efficacy.

Despite these obstacles, vaccines have been developed for over a century and a vast array of improvements in vaccine design, composition and delivery have been developed to overcome these problems. Some of the more recent vaccine concepts and developments that are being employed in other research areas could also be applied to the ApoB100 or HSP60 vaccines in order to improve their clinical outcome. Four important issues to address in this context are (1) peptide selection, (2) type of vaccine, (3) adjuvants and (4) vaccine delivery.

3.1. Peptide selection

To develop an effective vaccine against atherosclerosis in humans, it is of crucial importance that specific disease-driving epitopes are identified. However, rational selection of the appropriate antigen for immunotherapy has proven a major challenge in the past. The current epitopes from ApoB100 were identified by their specific binding to antibodies from pooled human plasma and have shown promising results in mice (23,24). The one HSP60 epitope that has been used in a vaccination experiment was selected because of its shared homology between species (45) and other epitopes were identified by antibodies from patients with carotid atherosclerosis (47,48). Although at first the atheroprotective effects were thought to be mediated by B cells, current opinion is that T cells are the major cell type involved in the protective effects of the immunotherapy. A prerequisite for T cell activation is that the antigen is presented by an APC through MHC-II molecules to the T cell receptor. However, in the case for ApoB100 p210, it has been shown that it does not bind with any clinical significant affinity with the murine homologue of MHC-II (I-A^b) and can thus not directly interact with T cells (49). Nonetheless, the presence of T cell reactive epitopes in ApoB100 has been shown (50,51) and a recent study in mice showed the atheroprotective effects of two MHC-II restricted peptides of ApoB100 (52).

In spite of these recent findings, the translation of such results to the clinical setting will still prove a major hurdle. The primary question remains which epitopes drive the autoimmune process in the human situation and will thus be suitable to use in

Table 1. *Predicted ApoB100 peptides with the best binding scores to HLA-DR3, DR7 and DR8 molecules using the computational TEPITOPEpan analysis (54). Peptides were selected as described previously (23). To compare, the peptides that were identified by their binding to human antibodies (p2, p45 and p210) are listed as well and score relatively low.*

Peptide	Sequence	HLA-DR3	HLA-DR7	HLA-DR8
26	ANPLL IDVVT YLVAL IPEPS	20,9	4,3	25,2
271	TGVLY DYVNK YHWEH TGLTL	20,0	1,8	22,4
139	NLKHI NIDQF VRKYR AALGK	11,5	3,8	28,1
177	MKVKI IRTID QMQNS ELQWP	15,6	4,1	21,7
31	CTGDE DYTYL ILRVI GNMGQ	13,9	3,7	23,0
288	INTIF NDYIP YVFKL LKENL	16,7	3,6	20,2
250	LDFRE IQIYK KLRTS SFALN	10,4	2,7	27,1
228	IPILR MNFKQ ELNGN TKSKP	17,6	4,9	15,3
203	VRFPL RLTGK IDFLN NYALF	15,7	7,0	14,7
2	ATRFK HLRKY TYNYE AESSS	6,2	2,2	10,2
210	KTTKQ SFDLS VKAQY KKNKH	5,6	1,2	0,4
45	IEIGL EGKGF EPTLE ALFGK	2,0	1,4	-1,5

a vaccine. To this end, it is necessary to identify peptides from either ApoB100 or HSP60 that are naturally presented by human leukocyte antigen (HLA) molecules and recognized by T cells in human atherosclerosis. One of the reasons that this is a difficult task, is the extreme polymorphism of MHC molecules. Therefore a number of very useful computational tools have been developed in the last decade to predict MHC-II restricted T cell epitopes (53–55). Especially when there is limited experimental data these models may provide initial starting points that can be further examined in biochemical experiments. For example, a very early study has shown that peptides of ApoB100 are mainly presented by HLA-DR3, DR7 and DR8 molecules (56). When utilizing this information with one of the more recent computational tools (55), potential HLA-binding peptides of the ApoB100 molecule can be identified (see table 1).

Although these models offer fast and cheap predictions, there is often a gap between the predictions and the actual binding properties of these epitopes. Other modern approaches often include mass spectrometry analysis of naturally HLA-presented peptides. For example, HLA-peptide complexes were immunoprecipitated from post-mortem brain tissue of multiple sclerosis (MS) patients followed by mass spectrometry to identify MS-related peptides (57). Another study used mass spectrometry analysis to identify I-A^b derived peptide sequences from B cells and macrophages and identified two ApoB peptides (58). However, whether these peptides are atherosclerosis-related is unknown. A very attractive method for atherosclerosis would be to obtain cells from human plaques and analyze them with these powerful approaches.

Another hurdle that has to be taken is to design a valid and useful animal model to test prototype vaccines based on HLA-restricted T cell epitopes. The mice should more or less mimic the human immune system if the results obtained from preclinical research are to be translated to the human situation. The majority of vaccine research has used the HLA transgenic mice model. While this has proven a suitable model for the validation of T cell epitopes in human disease (59), the question is which HLA allele the mice model should express. Nonetheless, the mice model should contain additional transgenes (hApoB100) and knockouts (ApoE, LDLR) to be used as a fully functional atherosclerosis model. Although this is a very complex and time-consuming model to set up, it should prove an invaluable tool for researchers trying to develop a vaccine against atherosclerosis.

Taken together, the selection and validation of a suitable HLA-restricted disease-driving epitope within ApoB100 or HSP60 will prove a difficult but necessary task to develop a rationally designed vaccine for atherosclerosis. Computational tools and mass spectrometry could offer researchers valuable tools to complete this mission.

3.2. Type of vaccine

Originally, complete proteins were utilized as antigen-specific immunotherapy, however, these have several drawbacks in terms of side-effects and manufacturing. To reduce the side-effects, peptide-based vaccines that have an increased specificity and are free of microbial products were developed. Moreover, peptides are much more cost-effective to produce on a large scale when compared to a complete protein. This line of development from protein to peptide immunotherapy can be seen in the development of vaccines for atherosclerosis as well. Research started out by immunization with oxLDL and HSP60 and has culminated to the point that vaccines are now based on specific peptide sequences of ApoB100 and HSP60. Nevertheless, immunization with single peptides are often limited in their potency to induce an appropriate immune response in the clinical situation. Therefore, in the last few years multiple types of next generation peptide-based vaccines have been developed that can enhance the immunogenicity and clinical outcome of peptide immunotherapy (60). Especially a lot of progress has been made in the field of oncology, where the immunotherapy strategy usually is to break tolerance towards specific antigens. However, if the administration route and dosage regimen are tailored to induction of tolerance, most of these novel vaccines could also be applied to autoimmune diseases.

3.2.1. Multivalent long peptides

One of these recent vaccine types are synthetic multivalent long peptides. Although complete protein vaccines have some demerits, one of their advantages is that they usually contain multiple epitopes that are recognized by T helper cells, generating a strong immune response or a broader range of tolerance against the protein. In contrast, the classical short peptides (e.g. the peptides derived from ApoB100 and HSP60) contain only one immunogenic epitope. To overcome the potential side effects with full-length protein and maintain their multivalent characteristics, synthetic long peptides have been developed. These peptides contain multiple antigenic epitopes and are usually taken up by APCs that present the processed epitopes to appropriate immune cells. This therapy has shown to be particularly conducive for the treatment of different types of cancer, where a robust immune reaction is favorable (61). For instance, a synthetic long peptide was developed against p53 for the treatment of metastatic colorectal cancer (62). The vaccine contained 10 overlapping peptide sequences that together covered amino acids 70 to 248 of the full p53 protein. The vaccine was well tolerated and in 90% of the patients a specific p53 T helper cell response was induced to multiple epitopes.

To this date, there have only been two reports with a somewhat similar approach in the field of atherosclerosis (63,64). In both studies a recombinant protein was constructed with peptide sequences derived from ApoB100 and HSP60 (64) or peptides derived from ApoB100, HSP60 and *Chlamydophila pneumonia* (CPN*)* (63). The CPN-derived peptide was chosen because of its sequence homology with HSP60 and its known effects on atherosclerosis (65). Both experiments convincingly showed that immunization with the combined epitopes resulted in a greater reduction in atherosclerotic lesions than immunization with only one of the epitopes (63,64). However, the approach taken by these researchers is strictly seen not that of a synthetic long multivalent peptide. To the best of our knowledge it has not yet been demonstrated whether such a peptide can be utilized to induce mucosal tolerance in an autoimmune diseases. Instead, in the field of cancer research one of the main reasons that immunization with short peptides is abandoned in favor of long peptides is the tendency of the short peptides to induce tolerance (61). On the other hand, it remains interesting to investigate if a multivalent long peptide against ApoB100 and/or HSP60 could elicit an even stronger Treg response than the monovalent peptides when the administration route and dosage is designed to achieve mucosal tolerance.

3.2.2. Multiple peptide vaccines

Another option that is being deployed nowadays is to combine multiple short peptides into one vaccine. Again, the advantage of this approach is that tolerance towards multiple disease-related antigens in theory would be more effective than tolerization against a single antigen. Moreover, this way it would be very easy to administer a large number of different antigenic epitopes, which could be more difficult with the long peptide approach. Such a vaccination cocktail has been used effectively in a number of clinical trials in patients with various types of cancer. For example, a cocktail of 14 different disease-related synthetic peptides was administered to patients with prostate cancer resulting in a reduced disease progression (66).

Several efforts have been made to investigate the effects of immunization with both a ApoB100 and HSP60 peptide on atherosclerosis (67,68). In 2010, Lu *et al.* subcutaneously immunized mice with ApoB100 p45 or HSP60 (amino acids 153-163) or a combination of both peptides (68). Interestingly, they showed that although both peptides alone were able to reduce the early atherosclerosis, the combination of both peptides resulted in an even greater reduction in atherosclerotic lesion. The same research group reported more recently that the same combination of peptides also reduced plaque progression and stabilized mature plaques to a greater effect than one of the peptides alone (67). Both experiments showed that vaccination resulted in dampened inflammation and immune cell infiltration and an expansion of Treg cells, all consistent with mucosal tolerance.

These experiments add further evidence to the theory that immunization against multiple antigens associated with the pathology of atherosclerosis can have synergistic effects. It is recommended that future research should explore which approach to induce this kind of tolerance is the most viable option in terms

of efficacy, safety and manufacturing.

3.2.3. Hybrid peptides

A third method is coupling the peptide to a functional group or protein construct that could improve the peptide by several mechanisms. One promising option that is broadly explored for use in autoimmune diseases is to bind the autoantigenic peptide to a soluble MHC molecule. It is widely known that for a sufficient T cell response, co-stimulatory signals are essential. These signals often arise from professional APCs that have processed the antigen. However, in absence of the appropriate co-stimulatory signals, presentation of an antigen to a T cell results in anergy of tolerance towards the antigen. Thus when the peptide is bound to a soluble MHC molecule that directly binds to the T cell receptor, without triggering co-stimulatory receptors, the response will be antigen-specific tolerance or T cell unresponsiveness. The recombinant T cell receptor ligand (RTL) is a MHC molecule that has been used successfully in several experimental auto-immune disease models and is now evaluated in clinical trials for MS (69). Other forms of soluble MHC complexes have also been constructed by linking two MHC molecules together with an IgG heavy chain, which has also recently shown promising results in the experimental mouse model of MS (70). However, in order to design an appropriate recombinant MHC molecule, an in-depth knowledge of the disease-related HLA molecule is essential.

A second interesting option is to link peptides to a specific part of the Ii protein called the Ii-key segment (71). The Ii-key segment has a high binding affinity for an allosteric site on MHC-II molecules which influences the antigen binding groove within these molecules. Upon binding to a MHC-II molecule on the cell surface by peptides hybridized to the Ii-key segment, endogenous bound antigen on the MHC-II molecule is replaced by the coupled peptide. Importantly, this also partly sidesteps the HLA-DR restriction of peptides, since the Ii-key segment binds to all HLA molecules and thus increases the affinity of the hybrid peptide for all HLA-DR molecules (72). Thus, coupling peptides to the Ii-key segment increases the loading of antigen to MHC-II molecules and has been used to increase the immunogenicity of various peptide-based vaccines in oncology and could be applicable to atherosclerosis as well (73).

Other possibilities include the use of cell penetrating peptides that increase the uptake of the antigen by immune cells (74). In particular for vaccines with a cytotoxic T lymphocyte (CTL) epitope this can be a beneficial solution, because of the limited capacity of the MHC-I pathway to process and present extracellular antigens. Nonetheless, cell penetrating peptides also hold potential for other vaccine types. For instance, a phase I trial with the main cat allergy antigen linked to TAT, which is a cell-penetrating peptide derived from the human immunodeficiency virus-1 (HIV-1), has been shown to reduce cat allergy with a greatly lower number of vaccine injections (75).

3.2.4. Altered peptide ligands

An alternative peptide-based treatment for autoimmune diseases is that of altered peptide ligands (APLs) (76). APLs are essentially similar peptides as the autoantigen except for one or several amino acid substitutions that are at critical binding positions for the T cell receptor (77). To induce the complete range of T cell effector functions, a full agonistic binding between the peptide-MHC complex and the T cell receptor is necessary. Partial or antagonistic binding by a presented peptide can inhibit or deviate the immune response (77). The APLs utilized for autoimmune disease are usually designed as partial agonists or antagonists to shift the immune response and induce the activation of Treg cells. This approach has shown promising results in animal models for several autoimmune diseases, including myasthenia gravis (78), rheumatoid arthritis (79) and MS (80), in which administration of the APL resulted in Treg expansion and tolerance to the antigen.

Interestingly, one study designed an APL based on HSP60 for the treatment of rheumatoid arthritis in mice (81). Others had already shown that treatment with HSP60-derived epitopes could induce Treg activation and reduce experimental arthritis. Therefore they hypothesized that by altering the epitope of

HSP60 to increase the binding affinity of that peptide to MHC class II molecules, the effects could be enhanced. Although they did not directly compare the APL with the non-altered peptide, intradermal administration of the APL resulted in an activation of Treg cells and reduced the experimental arthritis in mice. This was consistent with an upregulation of Treg cells after stimulation of peripheral blood mononuclear cells from rheumatoid arthritis patients with the APL.

Although the exact underlying mechanisms of APL-induced tolerance are unknown, it still offers an attractive therapeutic option. When the most promising epitopes of ApoB100 and HSP60 are identified, more detailed investigations into the binding characteristics of these epitopes to the T cell receptor or HLA molecules could provide additional options to enhance the efficacy of the vaccines.

3.3. Adjuvants

Even though much of the clinical potential depends on the selected peptide and type of peptide, the final package in which the vaccine will be administered is at least equally important. Despite that the often limited immunogenicity of peptide-based vaccines can be enhanced by employing a next-generation design, chances are high that the elicited immune response remains insufficient without the addition of one or several immunostimulants (adjuvants) to the vaccine. For decades it has been a well-established practice to include adjuvants in vaccine formulations to generate a more robust immune response to the antigen. Additionally, this could reduce the required amount of antigen and number of vaccine administrations.

Generally, adjuvants (in)directly stimulate APCs to create a proinflammatory environment in which a strong immune response against the administered antigen is elicited (12). However, inclusion of adjuvants to vaccines that aim to restore tolerance in autoimmune disease is a relatively new concept (82). It appears that in this context a more subtle approach would be favorable, since overstimulation by adjuvants might shift the immune responses away from the desired induction of Treg cells (83). Nonetheless, research into adjuvants that are immunomodulating rather than immunostimulating has attracted a lot of attention in the last few years (12,83,84).

One of the best examples of an adjuvant that can be used to modulate the efficacy of tolerance inducing vaccines is CTB (83). This subunit of the cholera toxin binds to the GM1 ganglioside receptor that is present on a large number of cells. It thereby increases the uptake of the bound antigen across the mucosa and greatly enhances the presentation to APCs, reducing the required antigen by more than a 1000-fold (85). An oral vaccination approach with CTB and insulin resulted in a shift from a Th1 to a Th2 phenotype and the induction of Treg cells that delayed the onset of diabetes in NOD mice (86). More recently the effects of CTB were also demonstrated for vaccination with ApoB100 p210 by Klingenberg *et al.* (32). Intranasal immunization of ApoE $^{-/-}$ mice resulted in a 35% reduction of atherosclerotic lesions that was consistent with suppressed effector T cells and increased Treg activity (32). Since CTB has also been tested clinically as adjuvant to antigen-specific vaccines, this represents a very promising option to continue using in combination with atherosclerosis vaccines (87).

Another group of adjuvants that has been investigated extensively is that of toll-like receptor (TLR) ligands. TLRs are pathogen recognition receptors that are highly expressed on APCs and can be stimulated by a wide range of pathogenic structures. Many TLR ligands have been used in vaccines against infections, however only a few have shown potential for inducing tolerance. Among these are ligands for TLR9 that demonstrated to increase the tolerogenic potency of vaccines for anthrax in mice (88). Apart from TLR9, one of the most versatile receptors in the TLR family in terms of ligand recognition is TLR2. Different agonists for this receptor are able to skew the T cell response in several directions including towards the induction of Treg cells and tolerance (89). For example, Treg expansion and tolerance to antigen was achieved with the TLR2 agonist Pam_3Cys in a mice model of asthma (90). Another study also convincingly showed that co-administration of a TLR2 agonist with antigen resulted in tolerance to the antigen due to increased expression of regulatory cytokines (91). However, it is worth noting that TLR2 can form heterodimers with a large number of other cell surface receptors and it seems that while several of these heterodimers induce Treg cells, others elicit a more proinflammatory phenotype (83). Hence, before using

a TLR2 ligand as adjuvant it is important that an agonist is identified that can induce the desired immunological effect.

Instead of activation of specific immune responses, another approach that could be taken to induce tolerance is that of immunosuppression. By suppressing the general inflammation, non-specific immunosuppressive drugs could induce a more favorable environment for the activation of Treg cells. In addition, some of these immunosuppressives, mainly rapamycin and retinoic acid, have the intrinsic capability to preferably induce a tolerogenic response (83). Especially since these drugs are already prescribed for some autoimmune disease, these drugs could be ideal candidates to be included in vaccines that target antigen-specific tolerance.

A somewhat similar immunosuppressive approach is the use of monoclonal antibodies against the T cell co-receptor CD3. Treatment with these antibodies resulted in restoration of self-tolerance by the induction of Treg cells in the $LDLR^{-/-}$ mice model for atherosclerosis (92). Likewise, a fusion protein of CTLA-4, which is an immunosuppressive receptor protein, and the Fc region of IgG1 is used to treat rheumatoid arthritis (83). The inclusion of such immunosuppressive monoclonal antibodies could enhance the effects of peptide-based vaccines. However, this would also greatly increase the costs of production and treatment, therefore other more cost-effective options would be more beneficial.

One attractive possibility is the inclusion of specific helper epitopes that are able to induce the expansion of Treg cells. Recently, specific regulatory T cell epitopes have been discovered on IgG antibodies and these have been termed Tregitopes (93). After recognition of Tregitopes by MHC molecules on circulating Treg cells, expansion and activation of these cells follow. Via secretion of regulatory cytokines, nearby APCs are driven towards a tolerogenic state, leading to antigen-specific tolerance when these APCs present the administered antigen to T cells (93). Although these Tregitopes are only recently discovered, they seem to offer a great therapeutic potential for the treatment of autoimmune disease for which the target antigen is known. In this context, it would be highly interesting to investigate the effects of co-administration of a Tregitope together with for example ApoB100 p210 to atherosclerotic mice.

Taken together, the inclusion of an immunomodulating substance in a vaccine for atherosclerosis could markedly improve its clinical efficacy. However, particularly for the treatment of autoimmune disease it remains important to be cautious of the risk-benefit ratio when selecting an adjuvant. Strong immunogenic adjuvants might overstimulate the already disturbed immune system in atherosclerosis, whereas a weak immunostimulant could result in poor atheroprotection. Nevertheless, the use of an appropriately designed and selected adjuvant represents one of the best options to improve peptide-based vaccines and could be key to a successful atherosclerosis vaccine.

3.4. Vaccine delivery

At present, there are several delivery routes for a vaccine to induce antigen-specific tolerance of which mainly the oral, intranasal and sublingual methods are applied (94). Inevitably, administered peptides will encounter the first line of defense by our immune system, the mucosal epithelium. This has two major consequences that could limit the vaccines efficacy. First, the mucosal epithelium poses a physical barrier for the antigen to get into close contact with relevant immune cells. A second hurdle, mainly for oral application, is the susceptibility of peptides for rapid degradation by enzymes present in these environments. Nonetheless, several possibilities for effective peptide delivery have been developed to overcome these obstacles.

Most of these options solve both problems by providing some kind of protective environment for the antigen, while also increasing the mucosal translocation of the antigen. Well-known delivery mechanisms that could also be used for atherosclerosis vaccination include the use of mucoadhesive polymers, liposomes, microspheres and virus-like particles and have been reviewed comprehensively elsewhere (94–97). Multiple delivery forms using nanoparticles have also been investigated extensively the last few years and represent promising options for future delivery systems (98).

An alternative, very cost-effective approach that started to attract attention in the last few years is the

plant-based production and bioencapsulation of antigens (99–101). Pharmaceutical proteins and peptides that are produced in plants (e.g. lettuce or rice) are protected from enzymatic degradation by the cell wall. Additionally, CTB-hybrid peptides can be produced in plants in order to increase the mucosal uptake and bioavailability of the antigen (102,103). Interestingly, one study with a plant-based vaccine showed that the introduction of a furin cleavage site between CTB and the protein enabled the mucosal translocation of protein, while CTB remained in the intestines (102). The many advantages of plant-produced vaccines, among which cold-chain free transport, easy scale-up and storage, would be of particular benefit to world-wide diseases with a large impact on global health such as type I diabetes and atherosclerosis. It has already been shown that a CTB-proinsulin fusion protein produced in lettuce and tobacco plants is able to delay the onset of type I diabetes in NOD mice (99). Similarly, plant-produced vaccines for atherosclerosis are now also seriously considered (11).

Next to the many existing adjuvants and delivery systems, bioencapsulated peptides linked to CTB could be a novel addition with many practical advantages. However, this alternative approach needs to be elaborated before it can be established as a realistic platform to produce effective vaccines for atherosclerosis.

4. REGULATORY AFFAIRS

The establishment of an effective prototype vaccine in animal models is the first step in a long and complex road to register a vaccine for clinical use. The first challenge that will be encountered before a prototype may be tested in a first-in-human trial, will be to convince the appropriate regulatory agencies that the product is safe. These agencies have issued several guidelines to advise researchers and developers on what types of studies should be performed to establish safety of the product. For vaccines against atherosclerosis, the appropriate guidelines would be S6(R1) and M3(R2) from the ICH (104,105). The main goal of these non-clinical toxicity studies is to have a complete picture of the potential toxic effects and to rationally select a safe starting dose for using in clinical trials.

Nonetheless, these guides should not be followed to the letter as the required studies may differ for each drug candidate. When a thorough knowledge of the pharmacodynamics is known, the outcome of many toxicology studies is to a certain degree predictable and may not have to be performed. For example, the first approved vaccine for cancer Sipuleucel-T is a personalized treatment for prostate cancer, in which a patient's own dendritic cells are pulsed with a fusion protein after which the activated dendritic cells are re-infused into the patient. Due to the autologous nature of this procedure, the developers of this treatment could convince regulatory authorities that preclinical toxicology studies did not have to be conducted. When the final vaccine for atherosclerosis is based on non-modified subunits of human proteins, it may very well be that many of these preclinical safety assays could also be skipped. Targeting oxLDL has also been showed a safe strategy by the use of a monoclonal antibody against oxLDL in 80 human volunteers (28). It could thus be hypothesized that mucosal immunization by ApoB100 peptides would not introduce any unforeseen toxicities. Nonetheless, this should be validated with safety experts and regulators before moving to clinical trials (106). On the other hand, when non-registered adjuvants or delivery systems are used, it will also be necessary to provide a full picture of the expected toxicity of these substances to the regulatory agencies. Nonetheless, CTB is a primary candidate for use in atherosclerosis vaccines and has already demonstrated an excellent safety profile in multiple clinical studies. Additional safety assays for CTB may therefore be unnecessary.

Another issue that is important for a smooth transition from the preclinical stage to clinical trials is a clear understanding about the intended use of the vaccine. To date, this topic has been underexposed and several important questions remain unanswered. The main point will be to clearly define the target population and determine if the vaccine will be given preventive or only to patients with established CVD. The clinical trial design will mainly be based on these points and should thus be resolved before moving into the clinical phase.

In general, researchers should be able to convincingly explain to regulatory authorities that all safety

issues have been reasonably explored and that all observed toxicities are within the expected safety profile of the vaccine (106). It thus remains pivotal to have a thorough understanding of the mechanism of action in relation to its intended use, hence preclinical research into these processes continues to be of great importance.

5. CONCLUDING REMARKS

Atherosclerosis and CVD have a major impact on global health and remain serious public health problems. Current treatments for atherosclerosis mainly aim at risk factor reduction, but do not modify the actual disease. Hence, novel treatments aimed at the disease process in the artery wall are urgently needed. Vaccines represent a cost-effective and innovative approach to treat atherosclerosis. To this date, preclinical research has clearly shown proof of principle for the development of a vaccine against atherosclerosis by targeting either ApoB100 or HSP60. However, the biggest challenge for researchers remains the translation of this research into a rationally selected vaccine to enter clinical trials. In this review, several recent developments and concepts in vaccine research have been addressed in relation to the current prototype vaccines for atherosclerosis. Many of these state of the art developments could be utilized by researchers in the atherosclerosis field to design an evidence-based next-generation vaccine with high clinical potential. The first priority should be to validate and identify epitopes in ApoB100 and HSP60 that are disease-driving, presented by relevant HLA molecules and are recognized by T or B cells. Whereas computational models could provide first clues, biochemical experiments should confirm the final epitopes. Secondly, a novel peptide design could be utilized to enhance the immunogenicity of the peptide. Thirdly, an adjuvant with appropriate immunomodulatory characteristics and a delivery mechanism that increase the potential to restore tolerance should be rationally chosen. Nonetheless, the obstacles that have to be taken to enter the clinic should not be underestimated and it is likely that it will take several more years before a vaccine for atherosclerosis can be explored in clinical trials.

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