Cardiovascular disease is still a leading cause of death in the European Union (EU) accounting for nearly half of all deaths in Europe (48%). In addition, cardiovascular disease complications lead to a vast number of hospitalizations and thus to a great burden of health care costs in the EU. Atherosclerosis is the main underlying pathology of cardiovascular disease and it is estimated that atherosclerosis is responsible for 70% of all cases of cardiovascular disease. Atherosclerosis is an inflammatory process that proceeds in the context of dyslipidemia and leads to the narrowing of medium and large sized arteries. The final complication of atherosclerosis is plaque rupture leading to a heart infarct in heart or stroke. More than 30 years of research into the pathology of atherosclerosis shows that its etiology is found in a combination of dyslipidemia and a related inflammatory response with an established autoimmune component. The major cause of acute cardiovascular disease events, plaque rupture, is the consequence of an inflammatory destabilization of the atherosclerotic lesion. Cardiovascular disease is therefore an autoimmune-like disease in the context of a metabolic disorder: dyslipidemia.
Thus far, therapeutic approaches to treat cardiovascular disease have been focused at normalizing dyslipidemia in order to lower and normalize plasma cholesterol levels. Statins are widely used drugs that inhibit the cholesterol synthesis in the liver and achieve a significant beneficial reduction in the plasma (Low Densitiy Lipoprotein, LDL) cholesterol level, in combination with additional surgical approaches such as angioplasty and bypass surgery, an impressive 30% risk reduction for cardiovascular disease has been achieved during the last 10-15 years. However, additional approaches focusing on the treatment of dyslipidemia by for instance improving the level of the anti-atherogenic lipoprotein High Density Lipoprotein (HDL) have failed in a number of clinical trials, although PCSK9 directed therapies may be applicable in the future for patients suffering from familial hypercholesteremia.
These findings indicate that new therapeutic approaches are urgently needed to narrow down the remaining 70% risk for cardiovascular disease and based on the outcome of clinical trials addressing IL-1ß it is evident that a therapy aimed at correcting the derailed inflammatory response during atherosclerosis development, progression and plaque destabilization is a valid approach to limit the impact of cardiovascular disease.
Within the present VIA consortium, we searched to develop a new immunomodulatory treatment, a therapeutic vaccine, that may permanently restore the immune balance within the arterial wall by inhibiting the inflammatory responses during atherosclerosis. We have focused on two types of vaccines, one vaccine will be directed at improving the function of regulatory T cells, while the second vaccine will improve the antibody response towards modified lipids present LDL. The first approached was performed in experimental models for atherosclerosis and resulted in new formulations of peptides derived from LDL combined with various types of adjuvants and immunomodulatory proteins. The immediate outcome of the studies is to develop monoclonal antibodies against apoB100 peptides to identify the optimal epitopes that can subsequently be used in the development of an atheroprotective vaccine. The second approach led to a First-in-Humans clinical trial in which an existing streptococcus vaccine was tested for its ability to induce antibodies that can neutralize modified LDL, which underlies the inflammatory response in atherosclerosis.

We foresee that managing the inflammatory response in atherosclerosis will receive large attention of the pharmaceutical industry and the development of anti-inflammatory response specifically addressing the immune response, such as therapeutic vaccines, will be a main focus point in future cardiovascular drug treatment.

Summary description of the project context and the main objectives

Atherosclerosis is a major cause of mortality and morbidity worldwide and currently available medication achieves only a 25-30% reduction in cardiovascular disease risk. Despite tremendous efforts and investments over the last decades, a number of novel drugs to treat cardiovascular disease (CVD) have failed in clinical trials due to an unexpected lack of efficacy over the last 20 years, specifically with respect to HDL cholesterol management. During the course of this consortium two new developments have occurred in relation to the treatment of cardiovascular disease: firstly a monoclonal antibody against PCSK9 is now used in the clinic for treatment of dyslipidemia and is effectively and efficiently lowering LDL cholesterol levels and has a beneficial effect on the outcome of CVD (ref). Secondly, the CANTOS trial has recently proven that inhibiting the inflammatory component of CVD by using a monoclonal antibody to block interleukin-1ß (IL-1ß) effectively lowers the risk of a secondary event in patients having a higher level of inflammation (ref). The CANTOS trial underlines the overall approach of our **V**accination **I**n **A**therosclerosis (VIA) consortium, that was aimed to develop a ground-breaking treatment for atherosclerosis following an original and novel strategy: vaccination to treat atherosclerosis and in this approach predominantly target the inflammatory component of atherosclerosis.

Cardiovascular disease

Cardiovascular disease is still the leading cause of death in the European Union (EU) accounting for 37% of all deaths in Europe (Eurostat 2018). During their lifetime 2 out of 3 people in Europe will eventually suffer from a cardiovascular event. In addition, complications from cardiovascular disease lead to a vast number of hospitalizations with a huge clinical and socio-economic impact.

Atherosclerosis is the main underlying pathology of cardiovascular disease and is responsible for 70% of all cases of cardiovascular diseases. Atherosclerosis is the process of the progressively narrowing of medium

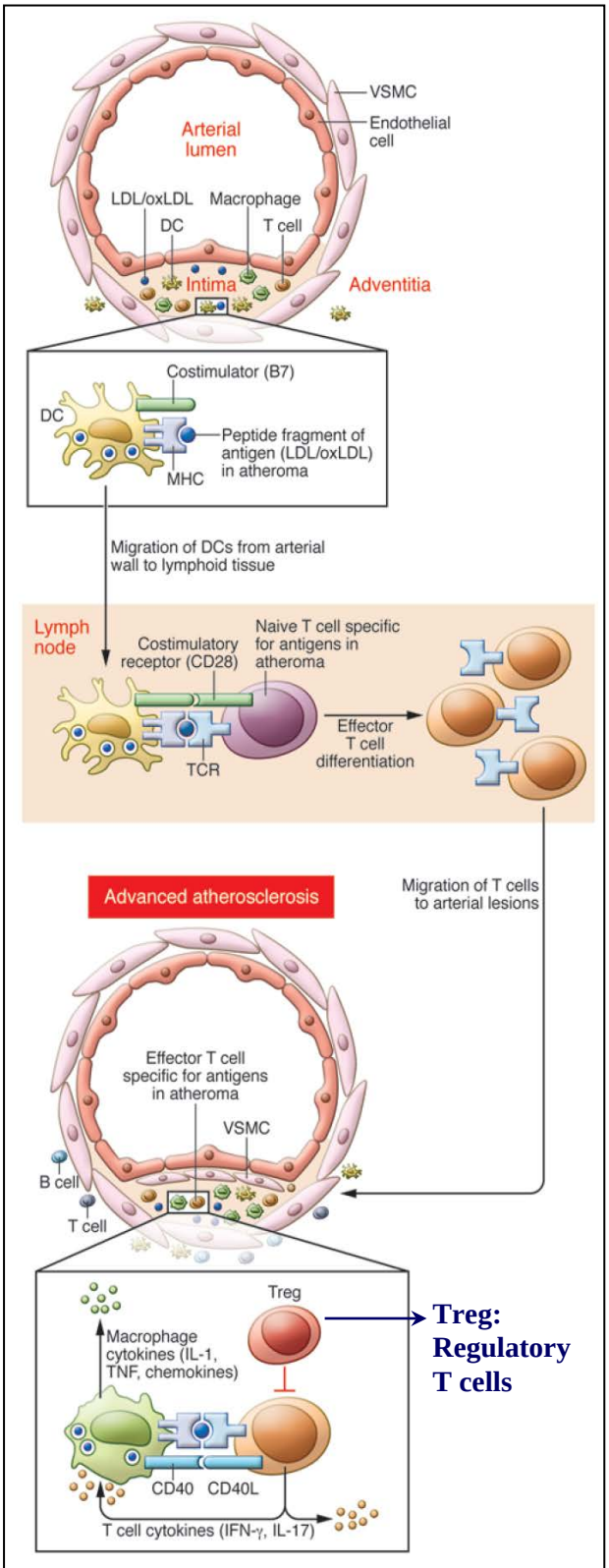


Figure 1: Immune activation during atherosclerosis: *Antigen uptake, presentation by dendritic cells (DC) and activation of the effector T cells takes place in lymphoid tissues. Effector T cells migrate to the atherosclerotic plaque, where they enhance the inflammatory process. Of importance are regulatory T cells (Treg) that have the unique capacity to inhibit the immune process in the atherosclerotic plaque and can be addressed for therapeutic applications.*
(From: Lichtman, Binder,J Clin Invest. 2013;123(1):27–36.

and large sized arteries by the formation of plaques. During a persons' lifetime plaques increase in size and occlude the affected arteries leading to cardiovascular complications. Eventually atherosclerotic plaques may rupture and result in catastrophic heart and brain infarcts. Extensive studies into the pathophysiology of atherosclerosis show that its etiology is found in a combination of dyslipidemia and an inflammatory response with an autoimmune component. Furthermore the major causes of acute cardiovascular events, plaque rupture and plaque erosion, are directly linked to an inflammatory destabilization of the atherosclerotic plaque.

Atherosclerosis is identified as a chronic, autoimmune-like inflammatory disease that progresses in the context of chronically elevated plasma cholesterol (LDL) levels[1]. Both innate and adaptive immune responses strongly affect atherosclerosis and plaques are characterized by the infiltration of leukocytes from the blood such as macrophages and T cells. The immune response underlying atherosclerosis results from the uptake of (modified) LDL by antigen presenting cells in the plaques[2] (Figure 2). The primary activation of effector T cells occurs within lymphoid tissue (adventitial tertiary lymphoid organs, lymph nodes draining the atherosclerotic arterial segment or spleen), where dendritic cells present plaque derived antigens, including LDL, to naïve T cells. The activated effector T cells subsequently migrate to the site of plaque formation, where they can exert their effector function directly on vascular stromal cells by the activation of plaque macrophages, which may subsequently produce cytokines such as Il-1ß. This type of immune response contributes to plaque progression and plaque de-stabilization and the CANTOS trial also shows the pathological role of this type of immune response. Regulatory T cells (Treg) are important adaptive immune cells that are able to inhibit atherosclerosis[3] via the inhibitopn orf antigen presenting cells such as macrophages and pro-inflammatory T cells and these Tregs form an important target for the development of a therapeutic vaccine[4,5] (figure 2).

In parallel to the induction of effector T cells, B cells (B1 and B2) are activated during atherosclerosis and B cells produce antibodies that interact with oxidatively modified LDL. B1 cells are known to produce innate IgM types, such as the prototypic PC-specific germ line encoded IgM antibody T15/E06, that protects against atherosclerosis[6], while anti-oxLDL IgG is produced by B2 cell and its level correlates with disease severity[7]. Production of atherosclerosis protective antibodies by B cells both of the IgG and the IgM class is therefore also a valuable target of the VIA consortium.

Existing pharmacologic approaches:

Thus far, pharmacologic approaches to treat atherosclerotic cardiovascular disease have mainly been focusing on normalizing dyslipidemia via the use of drugs that decrease levels of plasma low-density lipoprotein cholesterol, the so-called "bad cholesterol". Pharmacologic approaches to lower LDL, primarily statins, have over the last 20 years achieved a 30% risk reduction for cardiovascular disease. During the last 5 years a new drug has entered the clinic: antibodies inhibiting PCSK9 directly lead to a vast increase in the expression of LDL receptors on the surface liver cells and to an impressive 60-70% reduction in plasma cholesterol levels. Very recent studies have also shown that this approach also leads to a reduction in clinical complications from CVD. Additional approaches focusing on PCSK9 include siRNA against PCSK9 and small molecules affecting the interaction between PCSK9 and the LDL receptor. Tremendous efforts and investments over the last two decades to increase levels of plasma high-density lipoprotein (HDL) cholesterol, the so-called "good cholesterol", via the introduction of so-called CETP inhibitors as a novel class of drugs have not been successful

In addition, small molecules affecting lipid metabolism resulted in progression of atherosclerosis in man[8] and increased cardiovascular event rates in patients[9]. In addition, safety issues have led to serious drawbacks: e.g. the use of apoB antisense strategies to lower LDL led to liver steatosis in man[10], while liver-selective LXR agonists led to a triglyceride increase and liver steatosis in man in contrast to anti-atherosclerotic effects in animal models[11]. Also other approaches to improve the treatment of cardiovascular disease by increasing HDL have so far failed in a number of clinical trials[9].

Lately, the outcome of the CANTOS trial has shown that addressing the inflammatory pathology in atherosclerosis can beneficially lower the incidence of CVD complications in secondary prevention in a group of patients that showed an enhanced level of inflammation as monitored by the level of hsCRP. As figure 1 shows IL-1ß is one of the main pro-inflammatory cytokines produced by macrophages in the lesion as a consequence of activation of these cells due to inflammasome activation and/or activation by antigen-specific T cells. Another trial that addressed the inflammatory component of atherosclerosis was the "Low-Dose Methotrexate for the Prevention of Atherosclerotic Events" CIRT trial. In this trial in patients with a previous myocardial infarction or suffering from multivessel coronary disease who additionally had either type 2 diabetes or metabolic syndrome were treated with a low dose methotrexate. Unfortunately no beneficial effect was observed from the treatment, which may be a consequence of the fact that in this trial in contrast to the CANTOS trial, no selection was made for CVD patients with a higher level of inflammation as indicated by hsCRP.

New therapeutic approaches that focus on other causes of cardiovascular disease are urgently needed in order to further reduce the remaining 70% risk for CARDIOVASCULAR DISEASE. Since mechanistic observations indicate that the inflammation in atherosclerotic plaques is driven by a combination of innate immune responses activated by (modified) lipid accumulation and adaptive autoimmunity against (neo) self-antigens in the plaque such as (modified) LDL, we conclude that new therapies focusing on normalizing the immune response represent a promising new approach to treat atherosclerotic cardiovascular disease. The VIA project takes this one step further, by targeting the specific component that is central in the inflammatory pathway in atherosclerotic cardiovascular disease, the immune response elicited by native as well as oxidatively modified (ox)LDL particles.

Pre-clinical findings formed the basis for the VIA proposal

Over the years VIA partners have gathered ample evidence to support the paradigm that atherosclerosis is a chronic disease in which inflammatory and immune responses contribute to disease. As with many other biologic systems, they have shown that the immune system displays both athero-promoting and athero-protecting effects. Pre-clinical studies using appropriate models for the development of atherosclerosis have shown that the immune balance can be restored and this approach is a potent means of treating atherosclerosis.

Studies on the development of an atheroprotective vaccine started with exploring the effects of administering mice and rabbits autologous oxidized LDL and the induction of both IgG and IgM recognizing oxLDL epitopes might form an important protective mechanism. Extensive studies demonstrated the possibility that epitopes of the main protein part in the LDL particle, apoB100, elicit a specific activation of effector T cells and B cells. Subsequently, the identification of immunogenic peptides, epitopes, led to their use in vaccination protocols to limit atherosclerosis, which confirmed their usefulness in inducing an atheroprotective response (figure 3).

The most recent finding on the peptide-based vaccines is that they can induce a state of tolerance towards the antigen LDL through the generation of antigen-specific regulatory T cells. Additional studies from VIA partners have shown that tolerance induction towards LDL epitopes can be

improved by using different routes of antigen administration and the use of specific adjuvants. For example, mucosal administration of apoB100 peptides linked to cholera toxin B subunit can strongly enhance a tolerogenic response towards apoB100 and improve the beneficial effects of such a vaccine on atherosclerosis. Understanding the mechanisms of atheroprotection on the cellular and molecular levels using a range of formulations and routes of administration will be important for the construction of an efficient atheroprotective vaccine.

A new immunomodulating method to treat atherosclerosis that has recently been explored is the use of antigen-pulsed dendritic cells that are loaded ex vivo with epitopes from (modified) LDL. Administration of such antigen-pulsed dendritic cells to atherosclerosis-prone mice induced antibodies against modified LDL and antigen-specific regulatory T cells leading to protection against progression of atherosclerosis. Finally, the lipids in (modified) LDL are also immunogenic and these epitopes can be used in a vaccine to induce a protective immune response towards modified LDL. Phosphorylcholine (PC)-epitopes, which are major antigenic lipid epitopes in oxidized LDL, can induce the production of an innate type of IgM. This IgM binds oxidized LDL and this leads to protection against atherosclerosis. Interestingly, individuals with low anti-PC IgM levels have been found to have a significantly higher risk of developing cardiovascular disease.

Overall objective of VIA:

Develop an atheroprotective vaccine based on epitopes derived from LDL and evaluate the preliminary safety and efficacy of a selected, optimized vaccine in a First-in-Humans clinical trial thereby advancing the development of a widely applicable vaccine for cardiovascular disease

Objective 1: **Identify epitopes in (modified) LDL and use them for prototype vaccine development**

Objective 2: **Translate the prototype vaccines into clinical candidate vaccines using novel *in vitro* (artificial human lymph node) and *in vivo* (humanized mouse models) tools**

Objective 3: **Optimize the selected vaccines in terms of formulation, use of adjuvant and route of administration**

Objective 4: **Assess the preliminary safety of the selected clinical candidate vaccine**

Objective 5: **Conduct a First-in-Humans clinical trial with the selected clinical candidate vaccine**

Overview of the results of experimental studies:

The Vaccination in Atherosclerosis project consisted basically of two parts, a preclinical and a clinical part. The preclinical part was directed towards identifying the best possible epitope in human low-density lipoprotein, the formulation of this epitope into a vaccine and its preclinical testing in humanized mouse models of disease and translational models of the human lymph node.

The preclinical part was performed in work package (WP) 2 and WP 3. Based on the findings in the two work packages we performed a clinical trial to study the effect of a lipid containing registered vaccine (WP5, as planned in the first amendment). During the course of the project, we came to the conclusion that probably the induction of antibodies towards apoB100 derived peptides would be preferable over the induction of regulatory T cells towards apoB100. This approach is in line with vaccines in development against Alzheimer and Parkinson's disease, where a similar approach is chosen to induce antibodies against peptides derived proteins critically involved in these disease. An epitope derived from apoB100 that can be safely used to permanently induce antibodies towards this epitope in CARDIOVASCULAR DISEASE patients is however not identified. To address this problem, we have developed new vaccination strategies to induce anti-apoB100 and put efforts in the production of monoclonal antibodies specific for previously identified apoB100 peptides (as stated in the second amendment) and these monoclonal antibodies can be used in the near future to test their effect and safety in a First-in-Human clinical trial focusing on the effects in CARDIOVASCULAR DISEASE patients. The results obtained with these monoclonal antibodies can thereafter be used to apply the target epitope of the monoclonal antibody in the development of a vaccine directed to induce antibodies.

The scientific outcome of the strongly related WP2 and 3 are reported together to provide an optimal overview of the results.

WP2 (Translation of prototype vaccines into human-based vaccines) and WP3 (Formulations and route of administration)

Objectives:

1. **Construct a humanized mouse model that can be used to optimize the selection of an atheroprotective vaccine**
2. **Identify the best LDL-derived epitopes able to elicit an atheroprotective immune response and select a limited number of approaches to pursue based on their mode of action and their effectiveness in the new mouse model**
3. **Optimize selected epitopes for vaccine preparations with respect to formulation, use of adjuvant and route of administration to achieve desired immune modulation.**
4. **Optimize the use of selected vaccine preparations to achieve maximal and long-lasting effect on atherosclerosis.**

1. Humanized mouse model:

The development of the humanized mouse model was focused at using available mouse strains expressing the human HLA-DR3 or the human HLA-A2. In the end we only succeeded in breeding the HLA-A2 mice and used these mice for a proof-of-concept study to target peptides presented via class I antigen presenting molecules.

Human HLA-A2 transgenic (HHD), H-2Db$^{-/-}$ β2m$^{-/-}$ C57BL/6 mice, a kind gift from Institut Pasteur (Paris, France) were crossbred with human ApoB100 transgenic LDLr$^{-/-}$ mice to generate hApoB100 and HLA-A2 transgenic H-2D$^{b-/-}$ β2m$^{-/-}$ LDLr$^{-/-}$ (without murine MHC-I expression) and hApoB100 and HLA-A2 transgenic H-2D$^{b+/+-}$ β2m$^{+/+-}$ LDLr$^{-/-}$ mice (with murine MHC-I expression).

Using a prediction model we identified peptides that were HLA-A2 restricted epitopes, shown to stabilize HLA-A2 and induce peptide specific CD8 T cell responses in the humanized mouse model using dendritic cell vaccination protocols. Although our vaccination strategy is able to induce peptide specific CD8$^+$ T cells, we were unable to modify the course of atherosclerosis upon vaccination (Schaftenaar, Kuiper submitted). Additional strategies to skew the CD8 T cell response towards regulatory CD8 T cells are underway, but the essential finding is that it is possible to modulate the CD8 T cell mediated immune response to apoB1000 in the context of a human HLA-A2 antigen presenting molecule. This indicates at least that it is the possible to elicit a CD8 T cell response towards apoB100 in the context of the human antigen presenting system and opens the possibility to vaccinate against apoB100 in CARDIOVASCULAR DISEASE patients.

2. **Identify the best LDL-derived epitopes able to elicit an atheroprotective immune response and select a limited number of approaches to pursue based on their mode of action and their effectiveness in the new mouse model**

Several lines of research have been performed to identify within LDL the best epitope for vaccination and to test the approaches in relevant mouse models of atherosclerosis, including LDLr and apoE knockout ($^{-/-}$) mice as well as LDLr$^{-/-}$ mice expressing human apoB100.

a. **Identification of apoB100 derived peptides as targets for B cell directed vaccination approaches**

p45 and p210 are present in human plasma and to determine the possible association of such autoantibodies with cardiovascular risk. Patients with systemic lupus erythematosus (SLE) have a particularly high risk of cardiovascular disease (CVD) and represent a possible target population for testing atherosclerosis vaccine therapy. Immune responses against oxidized low density lipoproteins (LDL) play a key role in atherosclerosis development. The purpose of the first sub-study was to investigate associations between autoantibodies against the apolipoprotein B-100 (apoB-100) peptides p45 and p210 and risk of CVD.

In a prospective study, including 5400 individuals belonging to the cardiovascular arm of the Malmö Diet and Cancer cohort, apoB-100 autoantibodies were analyzed by ELISA. The analysis revealed significantly lower levels of IgM autoantibodies recognizing the native and malondialdehyde apoB-100 peptides p45 and p210 and also lower IgG levels recognizing native p210 in individuals with a subsequent incidence of CVD (15 years mean follow-up time) compared to controls. The autoantibodies were further analyzed in relation to coronary artery and stroke events. The same pattern was detected for coronary events, whereas the differences disappeared for incidence of stroke. No significant correlations between the autoantibodies and common and bulb carotid intima-media thickness were detected after adjustment for common risk factors (age, sex, LDL/HDL ratio, triglycerides, systolic blood pressure, smoking and diabetes). On the other hand, in a logistic regression model a significant association was found between high levels of IgG-p210native (OR = 0.811, 95% CI 0.69-0.94, P=0.007) and the occurrence of carotid plaques after adjustment for the risk factors. When tertiles of autoantibody levels were entered into a Cox proportional hazard regression model, a significant association was identified between high levels of IgM-p45MDA (Hazard ratio (HR) [95%CI]: 0.73 [0.56, 0.96], P =0.022) or IgG-p210native (Hazard ratio (HR) [95%CI]: 0.74 [0.56, 0.97], P =0.030) and a lower risk of incidence of coronary events after adjustment for the risk factors. Taken together, *the present findings suggest that high levels of apoB-100 autoantibodies protect against incidence of coronary events*.

Increased production of autoantibodies is a characteristic feature of SLE and there is evidence that several of these autoantibodies may contribute to the increased CVD in SLE. Autoantibodies against the apolipoprotein (apo) B-100 peptides p45 and p210 have been associated with a lower CVD risk in non-SLE cohorts. The aim of the second sub-study was to investigate how SLE affects the occurrence of these potentially protective autoantibodies. The study cohort consisted of 434 SLE patients and 322 age and sex-matched population controls. Antibodies against native and malondialdehyde (MDA)-modified p45 and p210 were measured by ELISA. SLE patients had significantly lower levels of p210 IgG and p45 IgM (both the native and MDA-modified forms). SLE patients with manifest CVD (myocardial infarction, ischemic cerebrovascular disease or peripheral vascular disease) had lower levels p210 IgG and p45 IgM than SLE patients without CVD. Decreased levels of these autoantibodies were also observed in SLE patients with permanent organ damage as assessed by the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (ACR) Damage Index (SDI). The present findings show that patients with SLE, a condition generally characterized by abundance of autoantibodies of multiple specificities, have reduced levels of antibodies against the apo B-100 antigens p45 and p210 and that the levels of these antibodies are further reduced in SLE patients with CVD. These observations suggest the possibility that an impaired antibody-mediated removal of damaged LDL particles may contribute to the development of vascular complications and organ damage in SLE.

The present findings have several important for the development of an apoB peptide-based atherosclerosis vaccine:

- Immune response against the apoB peptides p45 and p210 can be detected in human plasma and linked to CVD.
- Subjects with high levels of autoantibodies against p45 and p210 have a lower risk of developing acute coronary events including myocardial infarction. This observation strongly implies that inducing p45 and p210 antibody responses with a vaccine does not lead to an increased cardiovascular risk for the patient, which is important from a safety perspective.

- The observations provide indirect support for a protective role of antibodies against p45 and p210.
- Plasma levels of autoantibodies against p45 and p210 are reduced in patients with SLE and SLE patients with prevalent CVD have lower antibody levels than those without CVD.

b. **Identification of new immunodominant *CD4* epitopes of human ApoB100**

The huB100tg x LDLr deficient (*HuBl*) mouse is a humanized model of atherosclerosis, which expresses full-length human ApoB100 in the liver and gut, thus allowing us to study human LDL derived antigens in atherosclerosis. In the first year of the project, in order to identify epitopes recognized by auto-reactive T cell clones, we immunized *HuBl* mice with human carotid plaque homogenates. Spleen cells were retrieved and challenged *in vitro* with 302 native ApoB100 peptides (obtained in collaboration with Partner 2), which cover the whole sequence of ApoB100. Thirty-two native peptides in ApoB100 were identified with T cell specificity, as registered by induction of T cell proliferation. Two peptides, designated as P1 and P2, also induced the secretion of IFNγ (Figure 1; Dotted line, threshold), and were selected for further investigations. In the last 6 months, *HuBl* mice were immunized subcutaneously (s.c.) with these peptides. Effects in T cell activation, antibody production, inflammation, and atherosclerosis are ongoing.

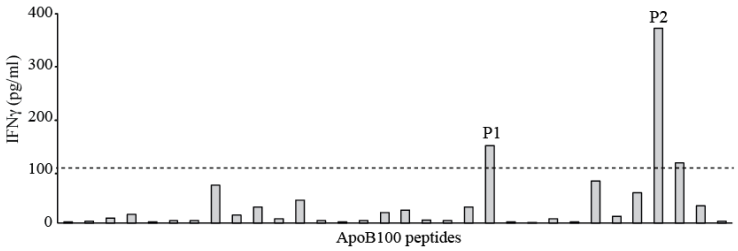


Figure 1. Screening of ApoB100 T cell epitopes. *HuBl* mice were immunized and boosted s.c. with human plaque homogenates. One week after boosting, splenocytes were cultured with 10 µg of single ApoB100 peptides. IFN supernatants from 32 cultures displaying increased proliferation (stimulation index > threshold).

In 2010, we showed that T cells carrying the TCRbeta chain TRBV31 respond to human LDL, and that inhibition of TRBV31+ T cell responses protected *HuBl* mice against atherosclerosis (Hermansson et al., 2010). We have used this knowledge and generated mouse strains transgenic for TRBV31 that will be used to identify immunodominant epitopes of ApoB100, and evaluate specific T cell responses in atherosclerosis. ApoB100 reactive TCRs were cloned into vectors, placed under transcriptional control of CD2 promoter. Transgenic mice were generated using standard microinjection techniques (Karolinska Core Facility). Six strains comprising 3 major ApoB100 autoreactive T cells were generated. Of them, three transgenic strains, named BT1, BT2, and BT3, have been recently characterized *in vitro*. T cells from BT1, BT2, and BT3 strains proliferate in response to human but not mouse LDL (Figure 2A). Further characterization of BT1 T cells showed that they upregulate CD40L and secrete TNF-alpha upon stimulation with human LDL (Figure 2B).

Recently, T cells from BT1, BT2, and BT3 were used to screen a library of ApoB100 peptides predicted to bind mouse and human MHC-II molecules. Preliminary data suggest that one peptide from ApoB100 activates BT2 and BT3 T cells. This sequence has been selected for further investigations, including in vitro assays with human T cells, and vaccination using HuBl, and HuBl x HLA-DR1 mice. Moreover, BT1 and BT3 mice are being intercrossed with *HuBl* mice to be used as tools for a better understanding of specific T cell responses in atherosclerosis, and in the selection of suitable therapy targets derived from ApoB100.

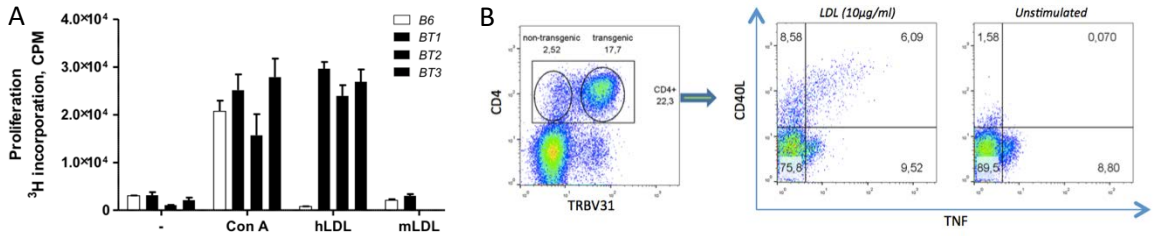


Figure 2. In vitro characterization of TCR transgenic T cells. A) Spleen cells from *BT1, BT2, BT3* or C57Bl6/J mice were cultured with 10 µg of human (h) or mouse (m) LDL. Unstimulated (-) and ConA were used as negative and positive controls, respectively. After 72 hours, cultures were pulsed with 1 µCi (3H-thymidine) for 18 h. Results are expressed as mean ± SEM of CPMs from duplicate wells. B) TNF intracellular staining of CD4+ TRBV31+ T cells from BT1 mice stimulated or not with human LDL.

The T-cell response to low-density lipoprotein (LDL) in the vessel wall plays a critical role in atherosclerotic plaque formation and stability. Partner 3 and Partner 2 have conducted a study where they used a new translational approach to investigate epitopes from human apolipoprotein B100 (ApoB100) which trigger T-cell activation. They also evaluated the potential of two selected native ApoB100 epitopes to modulate atherosclerosis in the human ApoB100-transgenic Ldlr$^{-/-}$ (*HuBL*) mice. *HuBL* mice were immunized with human atherosclerotic plaque homogenate to boost cellular autoimmune response to tissue-derived ApoB100 epitopes. *In vitro* challenge of splenocytes from immunized mice with a library of overlapping native peptides covering human ApoB100 revealed several sequences eliciting T-cell proliferation, including P2, P17, P38, P44, P64, P66, P109, P110, P116, P146, P147, P152, P165, P177, P183, P212, P222, P223, P235, P260, P265, P277, P283, P286, P287, P290, P291, P294, P295, P299, P301, and P302. Of these sequences, peptide (P)265 and P295 were predicted to bind several human leukocyte antigen (HLA)-haplotypes (Table 1) and induced high levels of interferon (IFN)γ. Vaccination of *HuBL* mice with these peptides mounted a strong adaptive immune response to native ApoB100, including high levels of epitope-specific plasma IgGs. Interestingly, P265 and P295 vaccines significantly decreased plaque size (Figure 3).

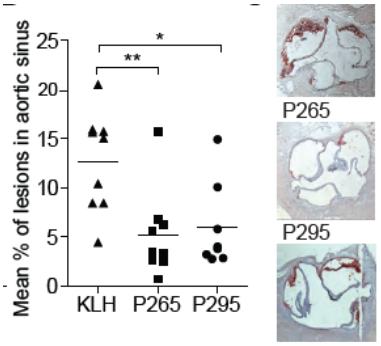


Figure 3. Vaccination against P265 and P295 protects *HuBL* mice from atherosclerosis. *Graph shows the mean percentage lesion in the aortic sinus (KLH n=9, P265 n=9, P295 n=8); Micrographs depict representative samples from the three different groups. *) P<0.05; **) P<0.01.*

This study identified novel human ApoB100-derived vaccine candidates to treat atherosclerosis. The promiscuity of our peptides, which were predicted to bind several HLA-II alleles, suggests that a successful vaccination strategy could affect disease in a large segment of the population. P3 and P9 now collaborate to obtain IP protection for the use of P265 and P295 to treat and prevent atherosclerosis.

LDL-reactive T cells protect against atherosclerosis

Three tg mouse strains with strong anti-LDL reactivity, termed BT1, BT2, and BT3 were used for experiments. They all expressed tg TCR with β-chain TRBV31 together with α-chain TRAV12, 4, or 14, respectively. Nearly all these cells were naïve in the tg mice, but exposure to human LDL *in vitro* evoked a strong T-cell response. The long-term effects of a strong cellular immune response to LDL were studied in HuBL mice receiving BT cells twice over a 5-week period. BT cells remained detectable in the spleen five weeks after the last injection. Atherosclerotic lesion burden, analysed at the end of the experiment, was reduced by 30% in the HuBL/BT3 and HuBL/BT1 animals, with a similar trend also for HuBL/BT2 mice (Figure 4).

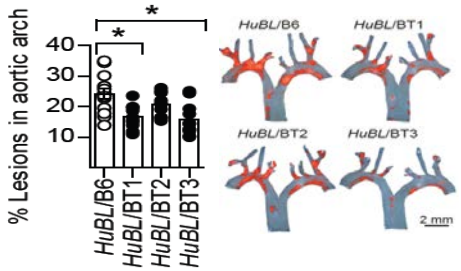
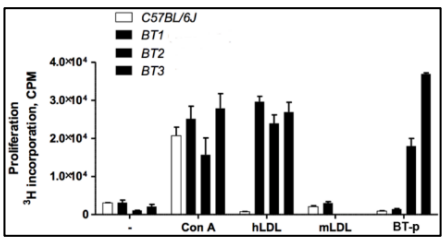


Figure 4. LDL-reactive T cells protect from atherosclerosis. *Atherosclerotic lesion burden in the aortic arch o HuBL/B6 n=14, HuBL/BT1 n=10, HuBL/BT2 n=10, HuBL/BT3 n=11. Right panels show En face preparations of aortic arches with lipid-laden plaques stained with Sudan IV (orange color).*

In silico prediction methods were employed for the prediction of IA^b binding peptides in human apolipoprotein B-100 precursor, taking open source webtools RANKPEP, IEDB SMM, and ANN as base methods. Using a strict combination of these methods scanning through the full-length sequence of human ApoB100, nonamer peptides were identified as potential IA^b binders. One peptide was able to activate ApoB100-reactive BT cells in *in vitro* cultures, a nonamer peptide was named BT-p (Figure 5).

Figure 5. T-cell proliferation to different stimuli. *White bars shows splenocytes from regular a C57BL/6J mouse. From the left, the x-axis starts with unstimulated cells, showing non-specific proliferation, Concanavalin A (positive control). The black bars show the response of the three different TCR transgenic strains to human LDL (hLDL), mouse LDL (mLDL), and BT-p. Splenocytes were incubated with the antigen for 60 hours, and thymidine incorporation during the last 12 hours was measured and recorded as counts per minute (CPM).*



ApoB-reactive T-cells reduce atherosclerosis through activation of B cells and generation of anti-LDL/ApoB IgG antibodies

P3 and P2 demonstrated that vaccine-elicited IgG Antibodies to peptides of ApoB (P265 and P295) could inhibit pro-inflammatory response of macrophages (Figure 6). P3 showed that ApoB-reactive T-cells (BT cells) can differentiate into T follicular helper cells that promote plasma cell and germinal center formation, and the secretion of anti-LDL/ApoB antibodies. These antibodies can enhance LDL clearance through immune complex formation (Figure 7A), resulting in lowered plasma cholesterol levels (Figure 7B) and reduced atherosclerosis (Figure 7C). In common, these studies show mechanisms by which cellular immune responses to LDL protect against atherosclerosis, by promoting B-cell responses and anti-LDL/ApoB IgG antibodies, which is an important step towards development of a clinically admissable vaccine.

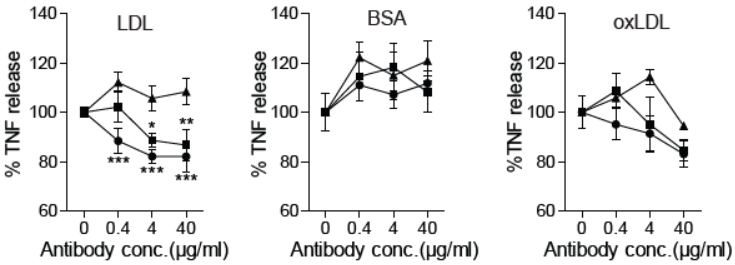


Figure 6. Antibody-mediated inhibition of TNF-α release by macrophages. *Panels show the percentage TNF-α released in the supernatant of LPS-stimulated RAW264 cells co-incubated with purified IgGs from plasma of vaccinated mice in LDL pre-coated plates (n=6), in BSA pre-coated plates (n=3), and in oxLDL pre-coated plates (n=3). The data is presented as mean ± SEM. *) P<0.05; **) P<0.01; ***) P < 0.001.*

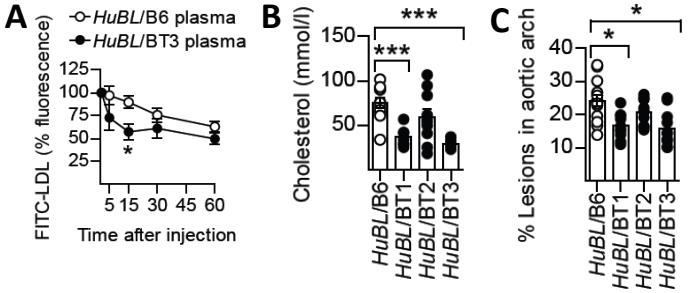


Figure 7. LDL-reactive T cells protect from atherosclerosis. A) *Clearance of injected FITC-LDL particles pre-incubated with either HuBL/B6 or HuBL/BT3 plasma (HuBL/B6 plasma n=9, HuBL/BT3 plasma n=8). B) A) Plasma cholesterol after 5 weeks on Western diet (HuBL/B6 n=11, HuBL/BT1 n=7, HuBL/BT2 n=10, HuBL/BT3 n=7). C) Atherosclerotic lesion burden in the aortic arch o HuBL/B6 n=14, HuBL/BT1 n=10, HuBL/BT2 n=10, HuBL/BT3 n=11)*

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Reduction of atherosclerosis by apo B peptide vaccines specifically designed to give a strong anti-apoB peptide IgG response

Since the studies described above provide compelling evidence that generation of antibodies against apo B peptides is an important mediator of the athero-protective effect of apo B peptide-based atherosclerosis vaccines partners 2 and 9 developed a novel vaccine formulation specifically aimed to produce such a response. We used the pan HLA DR-binding epitope (PADRE), a carrier epitope in vaccines, which previously has been demonstrated to induce strong antibody responses against PADRE coupled peptides and produced a

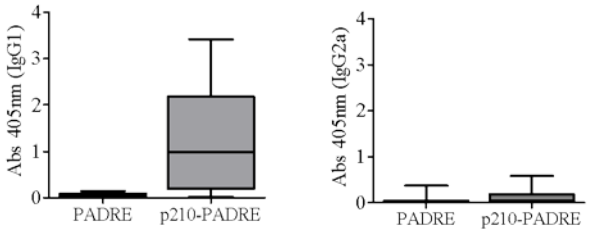


Figure 8. *Immunization with p210-PADRE induces a strong IgG1 (Th2) response against p210 but no response against PADRE. There is only a weak IgG2a (Th1) antibody response.*

synthetic peptide containing both the apoB p210 sequence and PADRE (AKFVAAWTLKAAA). Alum was used as adjuvant and apoE$^{-/-}$ mice immunized at 12, 14 and 17 weeks of age. High fat diet was given after the last booster immunization and the mice were killed six weeks later. Analysis of the antibody response at 23 weeks of age demonstrated a strong IgG1 response against p210 in mice immunized with p210-PADRE (figure 8) Competition studies demonstrated that the antibodies were specific for p210 (figure 9). There was no effect on cholesterol levels in response to immunization.

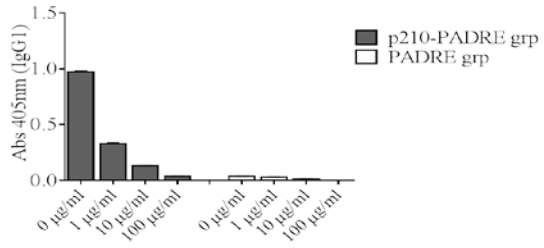


Figure 9. *Addition of p210-PADRE effectively competes binding of antibodies in plasma from immunized mice to p210 demonstrating their specificity.*

Previous formulations of apoB peptide-based vaccines have in many cases been shown to act through generation of regulatory T cells (see findings on P210-CTB complex and Il-2-oxLDL combinations). To investigate which T cells populations respond to immunization with p210-PADRE we performed flow cytometry of spleen cells from immunized mice. These analyses demonstrated that immunization with p210-PADRE leads to an expansion of Th2 cells (IL-5 expressing CD4+ cells) but does not affect the frequency of Th1 (interferon-gamma expressing CD4+ cells) or regulatory (CD25+/FoxP3/CD4+ cells) T cells (figure 10).

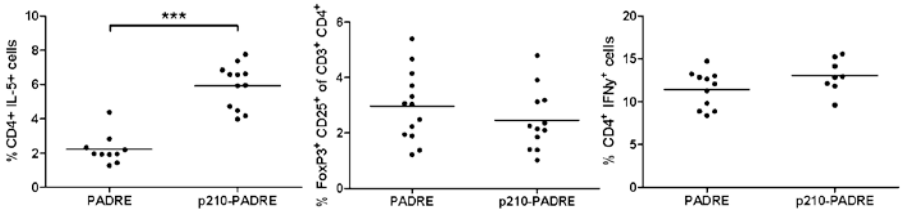


Figure 10. *Immunization with p210-PADRE induces an expansion of Th2, but not Th1 and regulatory T cells in the spleen.*

Immunization with p210-PADRE reduced atherosclerosis by more than 60% as compared with the PADRE control and there was also a significant reduction in atherosclerosis with the adjuvant alone control (figure 11). A concern in this study was that PADRE in itself appeared to have some pro-atherogenic properties.

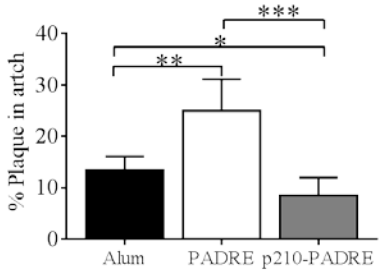


Figure 11. *Effect of PADRE and p210-PADRE on atherosclerosis in apoE$^{-/-}$ mice.*

We next repeated the studies using both apoE$^{-/-}$ and diabetic (glucokinase deficient) apoE$^{-/-}$ mice that develop more severe atherosclerosis than apoE$^{-/-}$ mice. Immunization with p210-PADRE reduced atherosclerosis in both non-diabetic and diabetic apoE$^{-/-}$ mice as compared with the PADRE control (figure 12). Moreover, in this experiment PADRE did not have a pro-atherogenic effect in non-diabetic apoE$^{-/-}$ mice. However, an increased atherosclerosis was observed in diabetic apoE$^{-/-}$ mice immunized with PADRE alone.

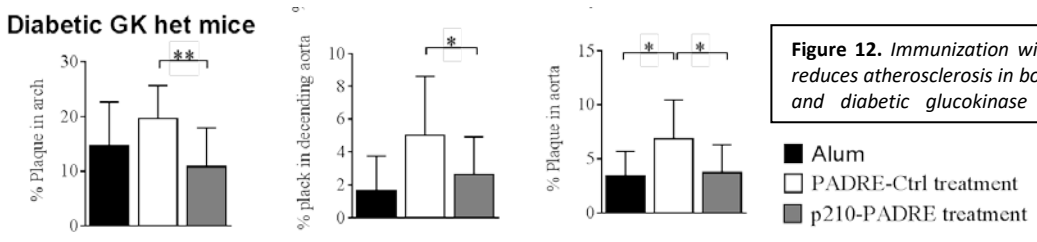


Figure 12. *Immunization with p210-PADRE reduces atherosclerosis in both non-diabetic and diabetic glucokinase (GK) deficient*

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by autoimmune responses against a number of different tissues. The incidence of cardiovascular disease is significantly increased in SLE patients with a 5-10 fold increased risk of myocardial infarction after adjusting for the Framingham Risk Factors. The pathogenic mechanisms responsible for the increased CVD risk in SLE remains to be fully understood. There is evidence that a dysfunctional clearance of autoreactive lymphocytes and apoptotic material in SLE is an important cause of autoimmunity in SLE by inducing autoantibodies against various self-antigens. However, in spite of this we have previously shown that autoantibodies against p210 are reduced in SLE patients. This suggests that immunization with p210-PADRE could be of particular benefit in SLE. To test this hypothesis we immunized hypercholesterolemic mice with a lupus-like phenotype (B6.lpr ApoE$^{-/-}$ mice) with p210-PADRE using PADRE/adjuvant and adjuvant alone as control groups. The first immunization was given when the mice 9 weeks of age with booster immunizations given 2 and 4 weeks later. The mice were then kept of high-fat diet for 10 weeks until the study was terminated. Interestingly, p210-immunization did not induce a p210 antibody response and did not reduce atherosclerosis in B6.lpr ApoE$^{-/-}$ mice (data not shown). These results show that hypercholesterolemic mice with a lupus-like phenotype are unable to respond to immunization with p210-PADRE and suggest that this is unlikely to represent a potential therapy for reducing the cardiovascular risk in SLE.

We (P1) also tested the p210-PADRE vaccination in a more humanized mouse model. We immunized HuBL mice with p210-PADRE and used alum as an adjuvant to stimulate a humoral response and a conventional effector CD4 and CD8 T cell response against p210. Mice were subcutaneously vaccinated 3 times in 37 days with PADRE-p210 (25 µg, 6.7 nmol) in alum, PADRE (9.1 µg, 6.7 nmol) in alum, or PBS (Fig. 13A). Increased levels of p210 IgG antibodies were observed in the p210-PADRE treated group compared to PADRE and PBS treated groups after the second immunization and were further boosted with a third vaccination, after which mice received WTD until sacrifice (Fig. 13A). We did not detect differences between treatment groups in levels of CD8 T cells, Ki67^{+} proliferating CD8 T cells, CD44^{+} antigen experienced CD8 T cells, and FoxP3^{+} CD8 Tregs in mediastinal lymph nodes (data not shown) and spleen. The effect of PADRE-p210 on atherosclerosis was assessed by histological analysis of aortic root tissue sections (Fig. 13B) p210-PADRE treatment did not affect aortic root lesion size (Fig. 13C) (PBS: 490517 ± 118811 µm^2 PADRE: 495962 ± 154416 µm^2 PADRE-p210: 436183 ± 102236 µm^2). Anti-p210 IgG levels of PADRE-p210 treated animals did not correlate with plaque size (Fig. 13D), similar to what we observed in CTB-p210 treated mice. The difference with the previous data is the fact that LDLr$^{-/-}$ mice were used

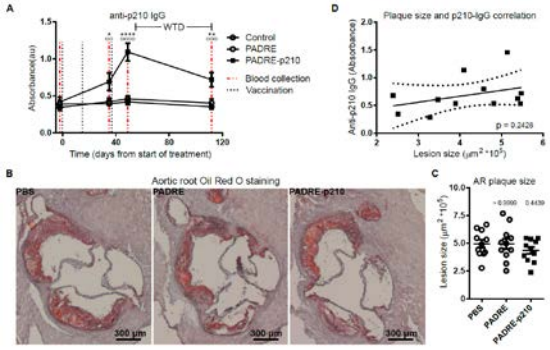


Fig. 13 PADRE-p210 vaccination induces high p210 specific IgG levels but does not act atheroprotective in HuBL mice. A) Female HuBL mice received subcutaneous PBS, or alum adjuvanted PADRE or PADRE-p210 injections at day 0, 15, and 37. From day 55 mice were fed western type diet B) Representative sections of the aortic root stained with ORO C) used for the quantification of the average atherosclerotic lesion size D) anti-p210 antibodies in blood serum obtained at sacrifice from PADRE-p210 treated animals, were plotted against the average atherosclerotic lesion size in the atherosclerotic root. to assess whether a correlation between antibody levels and atherosclerotic lesion size existed.

c. **Induction of Tregs**

Next to the activation of B cells, the induction of regulatory T cells was one of the main goals of the VIA project and we applied several ways to address this topic. One of the main goals was to make a prototype of vaccine consisting of CTB in combination with apoB100 derived peptides, which were prepared and optimized by P? and tested by P1 in humanized mouse models of disease and tested by P9 in in vitro models of human lymph nodes. Next to that several other approaches were tested to optimize the induction of regulatory T cells to apoB100 derived antigens using apoB100-Il-2 combinations. .

ECDI coupling of immunogenic ApoB100-derived peptides to reduce atherogenesis in mice:

Th1 responses in atherosclerosis are mainly associated with the aggravation of atherosclerotic plaques, whereas Th2 responses may lead to a less pronounced disease in mouse models. The attachment of disease-specific antigens on cells that were fixed with ECDI, and successive injection has been successfully used to improve other Th1-driven diseases such as type 1 diabetes or experimental encephalomyelitis. We analyzed this approach in a mouse model for atherosclerosis.

Methods and results: To understand the effects of antigen-coupled splenocytes (Ag-SP) in our models for immune responses, OTII-transgenic mice were i.v. treated with OVA-coupled splenocytes (OVA-SP).

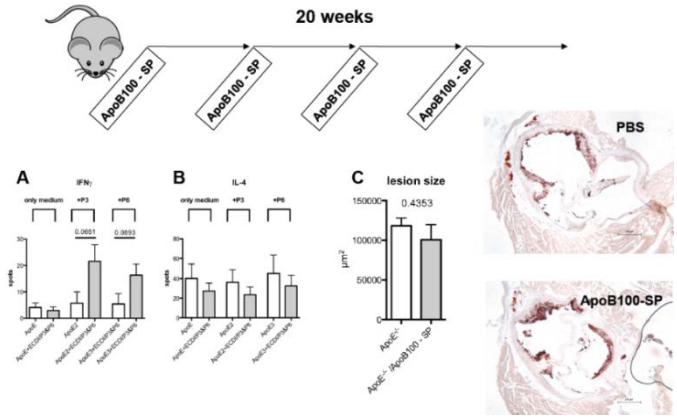


Figure 14. ECDI coupling of Apob100 peptides (P3 and P6) does not reduce Th1 immunogenic responses and does not limit atherogenesis in Apoe$^{-/-}$ mice.

At sacrifice, the proliferation of total splenocytes was as expected decreased, however IFNγ in cell culture, as well as *in vivo* CD4 cell activation as measured by flow cytometry were increased. In order to test self-antigens for atherosclerosis, we separately used oxidized LDL, and highly immunogenic Apolipoprotein B100 peptides P3 and P6. In B6 mice, we found an increase of IFNγ production was detected in the P3&6-SP treated mice, and also after oxLDL-SP treatment. Flow cytometry analysis of cytokine secreting spleen cells revealed CD4 positive T cells to be mainly the source for IFNγ.

Finally we tested this approach with injections every 5 weeks in male Apolipoprotein E - deficient mice. IFNγ was elevated in cultured splenocytes after *in vitro* recall. However, the atherosclerotic plaque burden in aortic roots as well as total aorta was unchanged compared to PBS treated controls.

Conclusion: Though the treatment with antigen-coupled splenocytes in its present form already impacts the immune responses in our model, we did not observe a modification of atherosclerotic plaques. However, additional treatment protocols and antigens should be explored.

Induction of Tregs against oxidized LDL in combination with IL-2-anti-IL-2 complex

Previously P1 published that the induction of antigen specific Tregs against oxLDL results in a reduction of atherosclerosis, while in an unrelated study P1 also showed that treatment of LDLr-/- mice with an IL-2-anti-IL-2 complex induced massive amounts of Tregs and also lowered the degree of atherosclerosis. In this study we combined the two approaches to optimize the induction of oxLDL specific Tregs.

To induce atherosclerosis male LDLrKO mice were fed WTD from age 10-12 weeks for 8 weeks. To induce oxLDL specific Tregs, oral tolerance to oxLDL was induced by oral administration of oxLDL (30 µg) 4 times in 8 days in oxLDL treated groups, starting when WTD feeding was initiated. To induce expansion of Tregs, mice received intraperitoneal IL-2 complex injections (1µg IL-2, 5µg anti IL-2 mAb, clone JES6-1A12) for 3 consecutive days, 2 days after the final oral oxLDL or oral control/PBS administration. To prevent elevated Treg levels from declining after the 3 consecutive days of IL-2c administration, mice received IL-2c injections every 10 days after the 3 initial IL-2c injections. To assess whether the administration of oxLDL and IL-2c acted immunosuppressive, we assessed immune populations in circulation, spleen, HLN and MLN. Separate IL-2c treatment reduced white blood cell counts (-37.7%, p = 0.0001) compared to the control treated group, similar to separate oxLDL treatment (-33.9%, p = 0.0005). Oral oxLDL and IL-2c combined treatment (-58.0%, p = 0.0001) seemed to have an additional effect (vs oxLDL, p = 0.0209; vs IL-2c, p = 0.0593) on reduction of overall circulating leukocyte numbers.

To assess whether the immunosuppressive effects of the different treatment regimens translated in reduced atherosclerosis, aortic root tissue sections were stained with oil red o (ORO) and trichrome to assess plaque size and stability. The average lesion size in the control group was small (167,582 ± 84,079 µm^2) (Fig. 15) and low in collagen content (4.133 ± 2.775 %) (Fig. 15), characteristic for early atherosclerotic lesions. Surprisingly, despite increased Treg levels and clear signs of immunosuppression in IL-2c treated groups, only separate oral oxLDL treatment significantly reduced plaque size compared to the PBS group (48.2%, 86675 ± 45311 µm^2, p = 0.0051) (Fig. 15). A trend towards decreased plaque size was observed in IL-2c treated group (27.5%, 121342 ± 49079 µm^2, p = 0.2255), and the IL-2c + oxLDL combined group (33.9%, 110527 ± 39027 µm^2, p = 0.1034) compared to control treated (Fig. 15). Plaque size from the oxLDL treated group was not significantly different from the IL-2c treatment

group (p=0.4714) or the IL-2c + oxLDL treatment group (p = 0.7602) (Fig. 15). We therefore conclude that the combination of IL-2-anti-IL-2 treatment with oxLDL tolerance induction does not provide additional protection compared to oxLDL treatment alone and do not further explore the possible clinical application of this approach.

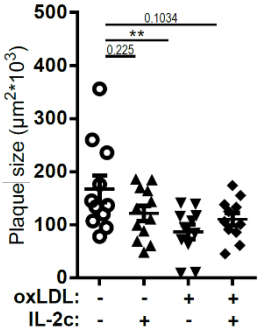


Fig. 15 Only separate oral oxLDL administration reduces aortic root plaque size. To assess the effect of oral oxLDL and IL-2c administration aortic root sections were assessed. Representative microscopic images of neutral lipid staining with ORO used for quantification of atherosclerotic lesion size and vessel occlusion. Expressed as mean ± SEM, one-way ANOVA with Tukey posttest, ** p < 0.01.

Peptide loaded liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice

To identify a relevant ApoB100 peptide for immunization, we eluted MHC-II restricted peptides from Bone marrow derived DCs exposed to hypercholesterolemic serum from LDLr$^{-/-}$ mice. We identified several ApoB100-derived peptides using our peptidomics strategy. Based on the predicted MHC-II binding, we selected the peptide ApoB1003500–3514 (p3500) and successfully loaded it into DSPG-liposomes which we previously identified for its capacity to induce Tregs. LDLr$^{-/-}$ mice were fed a WTD for 10 weeks, during which they were injected i.p. four times with PBS, 10 nmol of free p3500 or 10 nmol of p3500 encapsulated in DSPG-liposomes (DSPG/p3500-liposomes). Oil-Red-O staining of the aortic valve area of the heart showed that treatment with p3500-loaded DSPG-liposomes significantly reduced the lesion area by 50% (Fig. 16A and B). Interestingly, only the group of mice that received the DSPG/p3500 treatment had significantly lower levels of serum cholesterol compared to the PBS control group (Fig. 6D). The aortic sections were further stained for macrophage content, which is an indicator of immune activation [60].

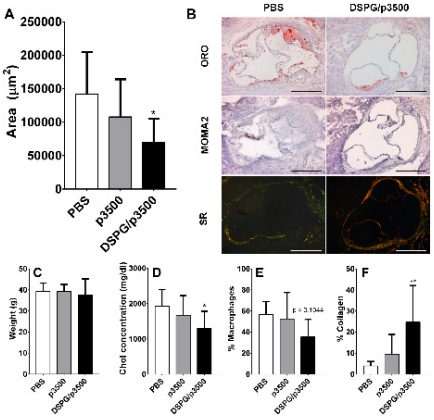


Fig. 16. Histological analysis of lesion formation in the aortic valve area of LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice on a WTD were administered either with PBS, free p3500 or p3500 encapsulated in DSPG-liposomes i.p. every 3 weeks for 10 weeks on a WTD. (A) Lesion area as determined by Oil-Red-O staining. (B) Representative images of sections of the aortic valve area in a mouse receiving PBS or DSPG/p3500-liposomes. (C) The weight of mice at sacrifice. (D) Serum cholesterol levels of mice at sacrifice. (E) Percentage of macrophage area relative to total lesion area as determined by MOMA2 staining. (F) Percentage of collagen area relative to total lesion area as determined by Sirius Red staining. Graphs show mean ± SD (n = 9), *p < .05, **p < .01 determined by one-way ANOVA and Bonferroni's multiple comparisons tests

There was a trend towards lower macrophage content in the mice immunized with liposomes (Fig. 16B and E),. Furthermore, there were significantly fewer CD8^{+} T cells
present in the aorta of mice injected with liposomes. Levels of the inflammatory CCR2+Ly-6Chi monocytes were unchanged in the blood of mice in all groups, further indicating that there was no increased inflammation. Finally, the collagen content in the lesions was assessed, as an indication of lesion stability [51]. Only the mice receiving DSPG/p3500 presented with a higher collagen content in their lesions (Fig. 16B and F), suggesting a more stable plaque.

Production of apoB100-peptide CTB vaccines for tolerance induction studies

The focus of the work of P5 has been on optimizing methods for genetic fusion of LDL ApoB100 peptides p210, p45 and p2 to the non-toxic Cholera Toxin B (CTB) protein and production and purification of the resulting gene fusion proteins.

The rationale for this is that induction of peripheral immunological tolerance ("oral tolerance") by oral or sublingual mucosal administration of selected epitopes is an attractive approach to prevent or treat selected autoimmune or allergic disorders. The currently known most effective way to induce oral tolerance for immunotherapeutic purposes is to administer the relevant epitope or antigen together with, and preferably linked to the non-toxic Cholera Toxin B (CTB) protein. As shown by WP2 and previous work by partner 2, the selected ApoB100 peptides, especially p210 and p45, have shown a protective "vaccine" effect when coupled to a carrier protein in the prevention of atherosclerosis. Previous work has also shown that in a mouse model intranasal administration of either a chemically conjugated or genetically engineered CTB-p210 fusion protein could significantly protect against atherosclerosis.

Since engineered gene fusion proteins much better than chemical conjugates can be tailored to homogeneity and may also be easier to produce at industrial scale, we have focused our efforts in WP3 on fusing p210, p45 and p2 to CTB by genetic means and producing the resulting gene fusion proteins in fermentor-grown E. coli bacteria with purification of the proteins from bacterial inclusion bodies. Effective vectors, expression systems and purification methods have been developed together with successful fusion constructs. Using these tools CTB fusion proteins have been achieved for each of the three selected peptides displaying the desired features of being pentameric proteins binding to GM1 ganglioside (the receptor for CTB) and reacting with antibodies to both CTB and the appropriate fused ApoB peptide. However, the fusion proteins are produced at markedly different yields in the expression system and also display differential solubility in physiological buffers. As a result the best protein CTB-p45 is isolated with excellent purity at almost a log higher yield than the two other proteins.

This fact together with recent considerations of the properties and surface accessibility of the p210 and p45 peptides on LDL and oxidized LDL may suggest that it might be an attractive strategy to combine parenteral immunization with p210 to induce LDL-uptake blocking ("neutralizing") antibodies and mucosal tolerization based on p45. Thus we have paid main attention to optimizing procedures for high-yield production and purification of the CTB-p45 protein and provided such protein to our partners (Partner 1 and Partner 9) for testing the atheroprotective efficacy after oral and other routes of mucosal administration. The results from partner 9 are promising demonstrating a significant atheroprotective effect of weekly oral administration of CTB-p45 fusion protein; results from partner 1 are underway. Further dose-response and alternative route studies are being planned by both partners as are studies of the combination of parenteral p210+adjuvant immunization and mucosal administration of CTB-p45.

Effect of oral tolerance induction using p-210 and p-45-CTB complexes on atherosclerosis:

P1 initiated experiments to validate the atheroprotective properties of the apoB100 peptide-CTB complexes produced by P5 in HuBL mice. These experiments were carried out in close collaboration with P2, P5 and P9.

The main setup that the HuBL mice orally received a low dose of the P45-CTB and P210-CTB complexes and CTB alone as control. The scheme for tolerance induction is based on previous findings of P1 and P5: 30 µg of peptide complex is administered orally to the mice at the first week of the experiment every other day and thereafter once every week.

There was no difference in cholesterol level between the groups as can be seen in Figure 17: all groups displayed on a western type diet (0.25% cholesterol) a rather high level plasma cholesterol in the range of 2500 to 3000 mg/dl. This high level of cholesterol resulted in very advanced lesions in the aortic valve area of the aorta. At this site we monitored huge lesions, which had reached what we interpret as a maximal lesion size of more than 700000 μm^2. Not to our surprise we were unable to detect significant differences between the various groups of mice. We therefore focussed

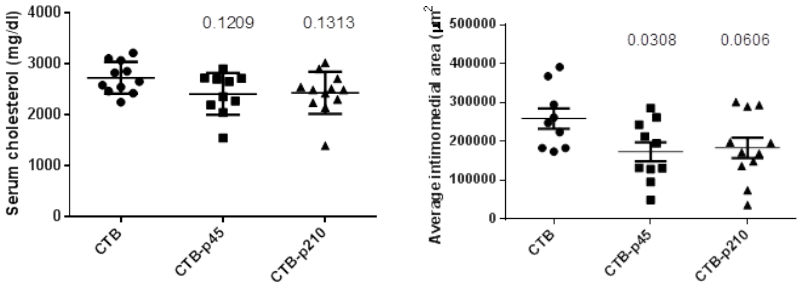


Figure 17: *Effect of CTB alone (control), p45-CTB and P210-CTB complexes on cholesterol levels (left panel) and degree of atherosclerosis in the brachiocephalic artery (right panel). Each group contained 11 HuBL mice and differences between the groups (p values) are illustrated.*

on another site in the arterial bed: the brachiocephalic artery (innominate). At this site lesions did not yet reach the maximal size and we were able to detect clear differences between the groups and we identified that p45-CTB significantly reduced the lesions size, while p210-CTB almost reached a significant effect.

During the experiment and at the end of the experiment we were however unable to identify antigen-specific regulatory T cells, limiting the possibility to use them as a readout in clinical trials. P2 however did determine the levels of antibody (figure 18) and we can clearly see that oral administration of the peptide complexes induced specific antibodies: p45-CTB induced significant anti-p45-IgG levels, whereas p210-CTB complexes induced significant anti-P210-IgG levels. As expected p45 did not induce anti-p210 antibodies and vice versa, while CTB had no effect on antibody levels at all.

In conclusion the p45-CTB complex had the best effect, while the antibody levels in concordance with the findings of P2 using peptide-padre complexes may form a good marker for monitoring the effectiveness of vaccination (D2.4 and D2.5).

These encouraging results in the humanized mouse model led to a second set of experiments in which we basically used the same setup but focussed on varying the dose and introducing a second control group which only received PBS. We therefore used four groups of HuBL mice that received PBS, CTB (73µg per treatment), p45-CTB (30 µg) and CTB-p45 (90µg). The study was performed in male and female HuBL mice. The dosing regimen was exactly the same as in the previous study. The study has been finished and initial results show that again we cannot find a significant effect of treatment on cholesterol levels (figure 19), but also not on atherosclerosis in the valve area, although the mean lesions size was more moderate than in the previous experiment (figure 20).

Preliminary conclusion is that the tolerance induction predominantly affects atherosclerosis predominantly in the aorta, which is however the more relevant site to analyze atherosclerosis for the human situation.

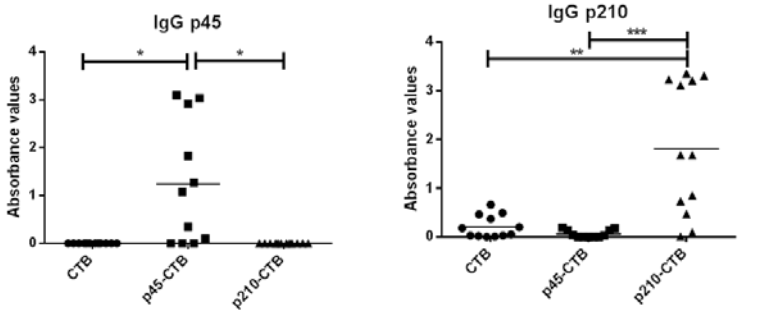


Figure 18: *Effect of CTB alone (control), p45-CTB and P210-CTB complexes on antibody levels*

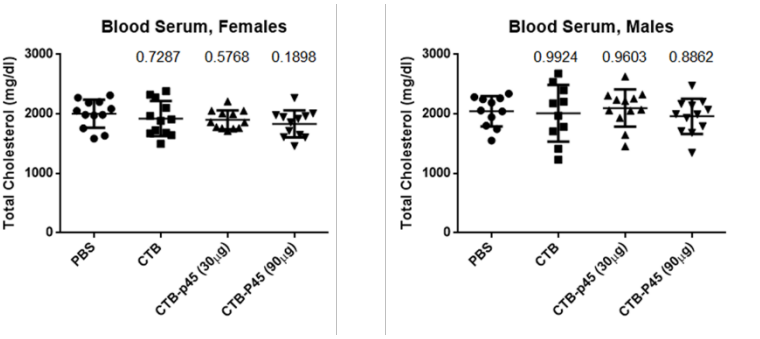


Figure 19: *Effect of PBS (control), CTB alone (control), p45-CTB complex in two doses on cholesterol levels.*

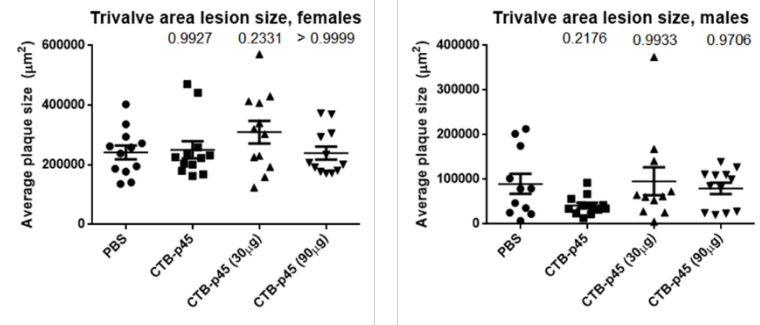


Figure 20: *Effect of PBS (control), CTB alone (control), p45-CTB complex in two doses on atherosclerotic lesion size in the aortic root in female and male HuBL mice.*

Effect of oral tolerance to p45-CTB on atherosclerosis

Following completion of this study P1 in collaboration with collaboration with P5 and P9 performed a second study in which hypercholesterolemic mice with a lupus-like phenotype (B6.lpr ApoE$^{-/-}$ mice) were treated with p45-CTB conjugate (5 μg, n=8), CTB (30 μg, n=13) or PBS (n=13) started at 18 weeks of age at day 0 and were orally administered at day 0, 2, 5, 7, 14, 21, 28, 35, 42 and 49. Weight was monitored at day 0, 28 and 56 (figure 21).

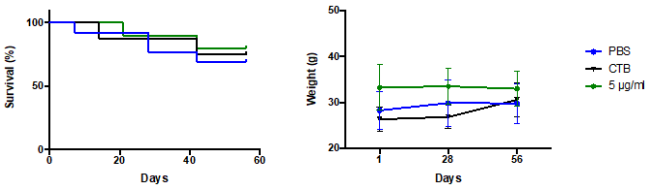


Figure 21. Oral immunization with p45-CTB in B6.lpr ApoE$^{-/-}$ mice. (B) survival and (C) body weight.

Aortas were analyzed with Oil Red O (ORO) where the aortic arches exhibited significantly lower lipid prevalence after P45-CTB treatment compared to CTB and PBS control (figure 22 A). Immunization with p45-CTB reduced the ORO positive staining for lipids with 41.8 % and 38.2% for PBS and CTB, where P45-CTB had a mean ORO content of 12.1 ± 5.0% compared to 20.8 ± 4.9% and 19.5 ± 6.7% respectively ($p<0.01$ and $p<0.05$). However, there were no difference in subvalvular plaque area or plaque collagen between the groups (figure 22B and C). (The inflammatory cell composition in the subvalvular plaques was evaluated using antibody detection of CD68 in the subvalvular heart region (figure 22D), where a reduction of 35.2% CD68 in the p45-CTB group compared to CTB control (5.12± 2.97% versus 8.59 ± 2.36%, $p<0.05$) was observed. There were no effects of treatments on plasma lipid levels or p45 antibody levels. *Taken together these studies demonstrate a modest effect of oral immunization with p45-CTB on atherosclerosis in B6.lpr ApoE$^{-/-}$ mice.*

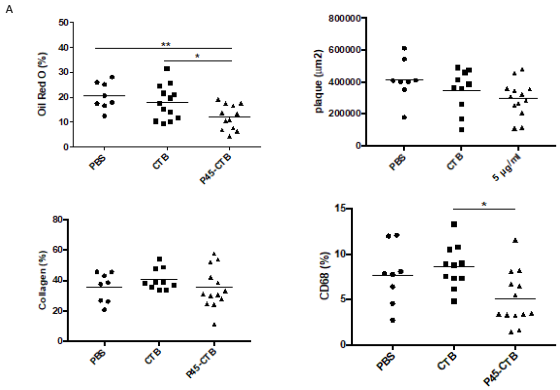


Figure 22. Effect of oral immunization with p45-CTB on (A) aortic atherosclerosis, (B) subvalvular plaque area, (C) plaque collagen and (D) plaque macrophages in B6.lpr ApoE$^{-/-}$ mice.

TGFβ$_2$-treated ApoB100-pulsed DC-based Vaccine

TGFβ$_2$ induces a potent tolerogenic phenotype in DCs

Bone marrow-derived DCs from HuBL mice were incubated in the presence or absence of TGFβ$_2$ and pulsed with or without ApoB100. Upon TGFβ$_2$ treatment, the DCs exhibited a decreased surface expression of the co-stimulatory molecule CD86 and a modest reduction in the I-A^b MHC-II levels, compared to the controls. Based on an analysis of the supernatants from these cells, we show that TGFβ$_2$-induced tolerogenic DCs secrete lower levels of IL-12, TNF and produce higher levels of IL-10 independently of being loaded with or without ApoB100. Next, we evaluated the capacity of the TGFβ$_2$-induced tolerogenic DCs to induce the de novo expression of Foxp3 in naïve CD4$^+$CD25$^-$ T cells *in vitro*. Compared to the untreated or IL-10-treated DCs, the TGFβ$_2$-treated DCs showed a superior capacity to induce Tregs.

Injection of TGFβ$_2$-treated ApoB100-pulsed DCs increases Treg numbers in atherosclerotic plaques

HuBL mice were divided in four groups that received a single intravenous injection of (i) untreated, (ii) ApoB100-pulsed, (iii) TGFβ$_2$-treated and ApoB100-pulsed, or (iv) TGFβ$_2$-treated DCs. The immunohistochemistry analysis of the lesions revealed that only the TGFβ$_2$-treated ApoB100-pulsed DCs substantially increased the FoxP3$^+$ Treg numbers in the plaques (Figure 23).

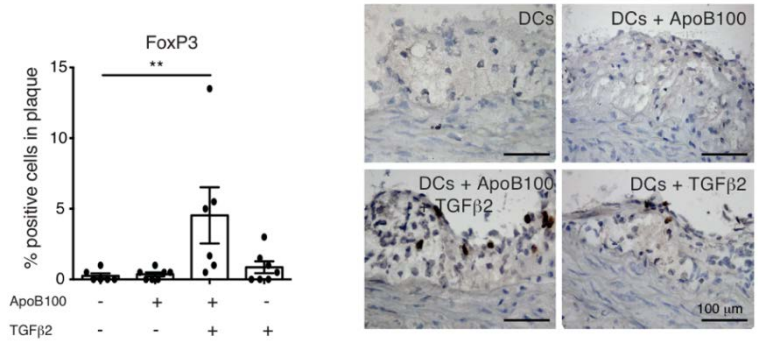


Figure 23. TGFβ2-ApoB100-loaded DCs increase Treg numbers in atherosclerotic plaques. A: Analysis of immunostaining for FoxP3-positive cells in atherosclerotic plaques from mice injected with DCs alone (n=6), DCs loaded with ApoB100 (n=7), DCs loaded with ApoB100 and treated with TGFβ2 (n=6), DCs treated with TGFβ2 alone (n=7). Right panels: Representative microphotographs of staining of FOXP3-positive cells. **P<0.01.

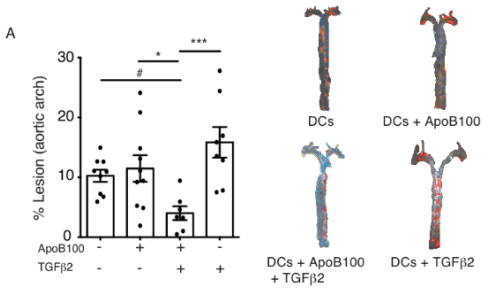


Figure 24. Activation of IDO-Treg axis reduces atherosclerosis lesions and vascular inflammation
A: Quantitative analysis of atherosclerotic lesion areas in thoracic aortas from mice treated with DCs alone (n=9), DCs loaded with ApoB100 (n=10), DCs loaded with ApoB100 and treated with TGFβ2 (n=7), DCs treated with TGFβ2 alone (n=8). Right panels: Representative images of mouse thoracic aortas showing Sudan IV plaque lipid staining. B: Analysis of CD68-positive cell immunostaining in atherosclerotic plaques from different DC-treated mice.

Injection of TGFβ$_2$-treated ApoB100-pulsed DCs reduces atherosclerosis and vascular inflammation

We evaluated whether TGFβ2-treated ApoB100-pulsed DCs could influence plaque development. The mice receiving the TGFβ$_2$-treated ApoB100-pulsed DCs exhibited a 75% decrease in the surface lesion area in the thoracic aorta compared with that in the other groups (Figure 24A). The reduction in the lesion area was associated with a significant reduction in plaque CD68 macrophage infiltration and VCAM-1 expression (Figures 24).

We showed that IL-10 renders DCs a tolerogenic phenotype and the capacity to induce antigen-specific Tregs *in vivo* and prevent disease (Hermansson, Johansson et al. 2011). Because TGFβ2 is highly expressed in immune-privileged sites and demonstrates potent immunosuppressive effects, we hypothesized that treating DCs with this cytokine could yield more Tregs in vivo. Indeed, the TGFβ2-induced tolerogenic DCs showed a robust capacity to induce Tregs in vitro and in vivo, suggesting that TGFβ2 can promote a more potent tolerogenic phenotype in DCs than IL-10. Despite increased Treg numbers, the degree of atheroprotection induced TGFβ2-induced tolerogenic DCs (Forteza, Polyzos et al. 2018) resembles that of IL-10-induced tolerogenic DCs (Hermansson, Johansson et al. 2011). This is an important finding suggesting that even low numbers of ApoB100-specific Tregs, induced by tolerogenic DC-based vaccines, can be sufficient to combat deleterious immune responses in the artery wall. This work has been recently accepted for publication at the *Frontiers in Immunology* (Forteza, Polyzos et al. 2018).

B cells treated with p210-CTB acquire a regulatory phenotype *in vitro* and reduce atherosclerosis in apolipoprotein E deficient mice

Intranasal immunization with a fusion protein of the ApoB100-derived peptide p210 and the cholera toxin B subunit (p210-CTB) has previously been shown to induce mucosal tolerance and reduce atherosclerosis development, but the exact mode of action remains to be elucidated. Recent studies have indicated an important role for B cells in mucosal tolerance, in particular by induction of regulatory B (Bregs) and T cells (Tregs). In this study, we aimed to investigate if transfer of B cells pulsed with p210-CTB can protect against atherosclerosis.

Ovalbumin peptide fused to CTB (OVA-CTB) has previously been reported to induce Bregs that express membrane bound TGFβ/latency-associated peptide (mTGFβ) and subsequently turn ovalbumin-specific T cells (OTII T cells) into antigen specific Tregs[11]. To study if p210 fused to CTB (p210-CTB) have the same effects *in vitro*, we first wanted to verify the effect of OVA-CTB on cells isolated from OTII mice. In accordance with the previous study, when pulsing B cells with OVA-CTB and co-culturing them with OVA-specific CD4$^+$CD25$^-$ T cells we observed an increased frequency

of mTGFβ expressing B and T cells as well as an increased amount of CD25^{high}FoxP3^{+} Tregs (Fig. 25A-B and data not shown). To test the effect of p210-CTB, we isolated B cells from HFD fed *Apob/ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice and pulsed them with p210-CTB, OVA-CTB, CTB or control buffer as above. After 48 hours of co-culture, we observed increased frequency of mTGFβ positive B cells among the p210-CTB treated B cell cultures as well as increased levels of Tregs compared to OVA-CTB and CTB treated cells (Fig. 25C-G). To test if p210-CTB can induce Bregs and Tregs in blood cells from healthy human donors, B cells were isolated from human peripheral blood mononuclear cells and treated with p210-CTB as described for mouse cells. After 48 hours of co-culture, we observed an increased amount of mTGFβ positive B cells in all three donors but no effect could be found on Treg levels (Fig. 25H and data not shown).

Having established a regulatory role for p210-CTB treated B cells *in vitro* we wanted to see if the transferred B cells had the same regulatory effect *in vivo*. HFD fed *Apoe*$^{-/-}$ mice received *i.v.* transfer of 2 x 10^{6} OVA-CTB or p210-CTB stimulated B cells or PBS only at 8, 10 and 12 weeks of age (Fig. 26A). The Oil Red O stained area and percentage in the aorta was reduced in mice receiving p210-CTB treated B cells (Fig. 26B). Transfer of p210-CTB treated B cells to *Apoe*$^{-/-}$ mice did not affect plasma lipid levels indicating that the observed decrease in atherosclerotic lesions was not due to an effect on lipid metabolism (Supplement 1A-B). Moreover, we did not observe any differences in subvalvular plaque area or for subvalvular plaque macrophage, CD3^{+} cells, collagen and smooth muscle cell α-actin content (Fig. 26C-F and data not shown).

To further investigate if transfer of p210-CTB treated B cells affected plaque phenotype, we measured mRNA levels of cytokines in the carotid artery. Interestingly, we observed increased mRNA levels of IL-10 in mice receiving p210-CTB treated B cells compared to OVA-CTB treated B cells. No differences were observed for TGF-β, TNF-α, or MCP-1.

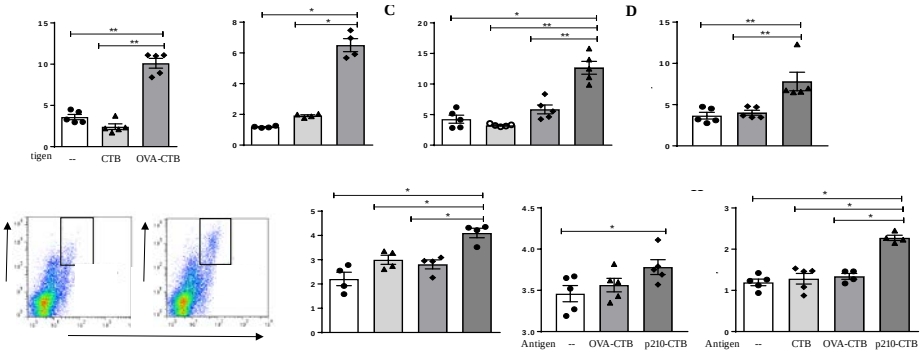


Figure 25

GFβ expression on B cells and induce Tregs *in vitro*

(A-B), high fat diet fed *Apob/ldlr*$^{-/-}$mice (C,E,F) or high fat diet fed *Apoe*$^{-/-}$mice (D,G) and
A-CTB, CTB or control buffer. Treated B cells were then co-cultured with autologous
r, the cells were assessed by flow cytometry for mTGFβ^{+} B cells (A,C,D) and CD25^{+}FoxP3^{+}
t least 3 separate experiments. H) CD19+ B cells were isolated from human peripheral blood
r mouse cells. Graph represents one donor and is representative of 3 donors tested. Each dot
an±SEM. * p≤0.05; **p≤0.01 (Mann Whitney test)

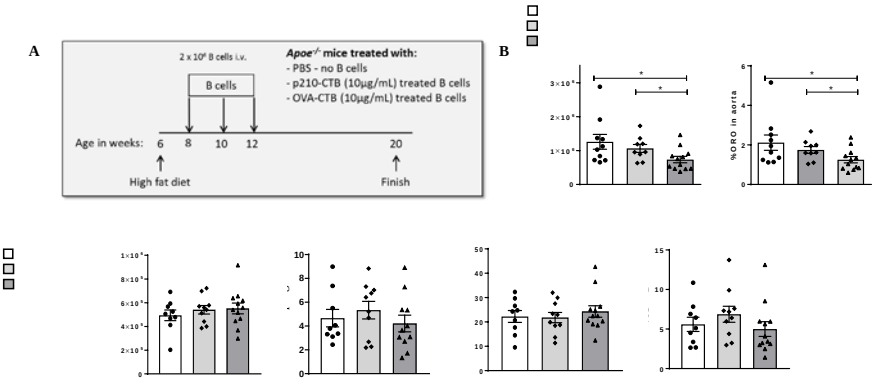


Figure 26

erosis in the aorta

reated B cells (OVA-CTB or p210-CTB)
of age. B) Oil Red O (ORO) staining of flat
ique area calculated on sections stained
), collagen (Van Gieson, E) and smooth

When analyzing the B and T cell phenotype in the spleen at the end of the experiment (8 weeks after the last B cell transfer), we could not observe any differences in the levels of mTGF-β^+ B cells or Tregs. There were no differences in the frequency of total B cells, marginal zone, transitional, $CD40^+CD86^+$ or $CD1^+CD5^+$ B cells. Moreover, there were no differences between the groups in terms of total $CD4^+$ and $CD8^+$ T cells as well as for naïve, effector or memory T cells (data not shown).

In conclusion, in the present study we show that treating B cells with p210-CTB is an efficient method to induce Bregs and Tregs *in vitro* and that transfer of p210-CTB treated B cells reduce atherosclerosis development possibly via an increased production of IL-10 in the plaque.

d. **Vaccine-elicited humoral responses against lipid moieties of modified LDL and relationship with atheroprotection in mouse models**:

A first round of preventive vaccination strategies has been performed in the apoE deficient mouse model using preparations including phosphorylcholine (PC), conjugated to either BSA or KLH and injected with or without CpG, as adjuvant. In addition to phosphorylcholine, we have tested two clinically approved pneumococcal formulations: Pneumovax®23 (Merck; stabilized by Phenol, subcutaneous or intramuscular route, does not contain adjuvants) and Prevnar®13 (GSK; contains aluminum salt, exclusively intramuscular route). INSF (P6) has performed the vaccination protocol, sampling and sacrifice as well as tissue collection. INSF (P6) has performed the vaccination, blood sampling, tissue collection and lesion size analysis and MUWI (P4) has performed the measurement of the antibody titers against oxLDL, PC and the clinical vaccine immunogens in all serum samples from the experimental mice. In addition, MUWI (P4) has performed studies to understand the regulation of the IgM response with specificity towards these lipid moieties by evaluating the role of sialic acid-binding immunoglobulin-like lectin-G (Siglec-G), a negative regulator of these responses. Global as well as B-cell specific knockout of Siglec-G was analyzed in Ldlr deficient mice fed an atherogenic diet.

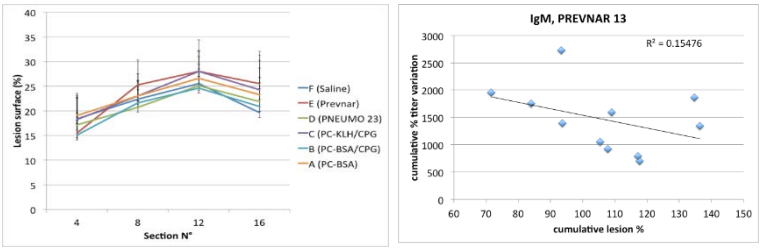


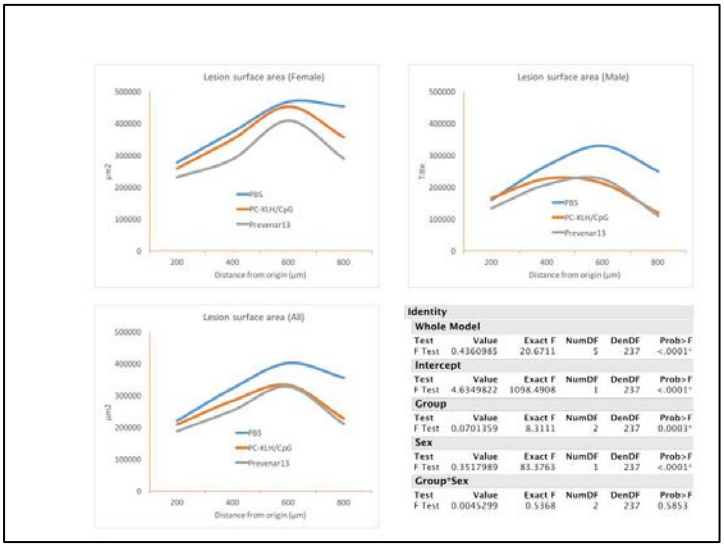
Figure 27

The data from the first experiments have shown that:

a) The most powerful immunization against PC (and oxLDL) was obtained using PC coupled to KLH, injected with the adjuvant CPG.

b) Figure 27 (left). There was no apparent difference in lesion extension among the groups, possibly because the study did shorter than 5 months, as initially planned.
c) PC-BSA/CPG was able to raise PC-specific IgG in 4 out of 10 mice, we will redo the study using a higher PC:BSA ratio.
d) PC immunization without adjuvant is ineffective
e) PREVNAR 13 but not PNEUMOVAX 23 could be suitable to elicit a PC-targeting (protective?) humoral response.
f) Figure 28. Vaccine-elicited PC-specific IgM but not IgG in response to PREVNAR 13 vaccination is inversely correlated with lesion size (could carry protection against atherosclerosis).
g) Deficiency of total as well as B cell-specific Siglec-G protects from atherosclerosis and hepatic inflammation. Analysis of the IgM repertoire in these studies revealed a bias towards model epitopes of oxLDL. Additional targeting of Siglec G could be a strategy to favor protective humoral responses.
We also assessed the importance of the VHS107.1.42 heavy chain gene in the generation of protective anti-PC antibodies. Mice deficient in VHS107.1.42 were crossed with atherosclerotic prone Ldlr deficient mice. The concentration of plasma antibodies levels reacting with oxLDL, PC as well as the T15-specific anti-idiotypic antibody AB1-2 was evaluated and atherosclerosis was assessed in these mice fed an atherogenic diet. VHS107.1.4 was found to be dispensable for the generation of atheroprotective anti-PC IgM antibodies under these conditions.
Anti-oxLDL antibodies directed against the lipid component PC are positively correlated with the extent of atherosclerosis in patients and may exert a protective role, as suggested by experimental studies in mice. The target of PC-specific antibodies is shared by both oxLDL and Streptococcus pneumoniae. In the previous report, we evaluated the potential of two commercially available anti-pneumococcal vaccine in terms of active raising PC-specific antibodies. At variance with Pneumovax 23® (a completely synthetic sugar-based vaccine, devoid of adjuvant), Prevenar 13® (in which immunogenic sugars are derived for whole bacteria and coupled to alum-derived adjuvant) is able to raise the titer of PC-specific antibodies and reduce lesion size.

Figure 28. *Lesion Density (lesion surface area expressed as percentage of vessel surface area) analysed at serial cross section levels in the aortic roots the three groups (PBS, PC-KLH/CpG and Prevenar). There was a statistically significant effect of the both vaccines (Prevenar even better than PC-KLH/CpG) in reducing lesion density. Covariance analysis showed that there was also an effect for the sex (protection greater in male mice) but the protective effect of the vaccine was independent of the sex (Group*Sex Prob>F = 0.8).*



We have repeated the preclinical evaluation of Prevenar® vaccine as well as of the PC-KLH custom formulation in younger mice.The protective effect of both Prevenar and PC-KLH/CpG was this time evident also in female mice, although to a lesser extent as compared to male mice (Figure 29).
We conclude that PC-targeting vaccine are effective in protecting against the development of experimental atherosclerosis. Protection is evident in both male and female mice if the vaccination occurs before full maturation of lesions. Although proper regression studies were not performed, this finding suggests that PC vaccine is best effective when performed in a preventive protocol (young age), which is feasible for Prevenar vaccination.

5. **Human Artificial Lymph Node model: test the recognition of peptides by human lymphocytes**
The Human Artificial Lymph Node model (HuALN) is a bioreactor-based model for long-term treatment of tissue-engineered lymph nodes (lymph node micro-organoids). It is designed to induce and monitor human immune responses induced by drugs in vitro. Using primary human immune cells and stromal cells, the perfused 3D matrix-

assisted bioreactor technology emulates an in vivo-like system to analyse, for example, immune modulation, cytokine storms, immunogenicity and immunotoxicity of pharmaceutical drugs, cosmetics, and chemical substances.

For VIA the HuALN model is used for mode of action analysis (MoA) of immune tolerance-inducing peptides, CTB-peptide fusion proteins and adjuvant formulations, and efficacy and safety assessment of the final vaccines. In addition to the HuALN model selected cell-based assays using human immune cells, e.g. dendritic cells (DCs) and DC/T co-cultures, are applied for peptide binding studies.

Human DC assay for peptide binding to MHCII

apoB100 p210 peptide binding on human MHCII was successfully confirmed for HLA-DR1 (homozygous donor) and HLA-DR3 (heterozygous donor). Human monocyte-derived dendritic cells were exposed to biotinylated peptide (p210-linker-biotin) and peptide binding to MHCII was analysed by flow cytometry using avidin-FITC and a biotinylated anti-avidin-antibody. For p210 and a MHC II peptide binding frequency on DCs of up to 80% for HLA-DR1 and 55% for HLA-DR3 was observed (Fig. 29,30).

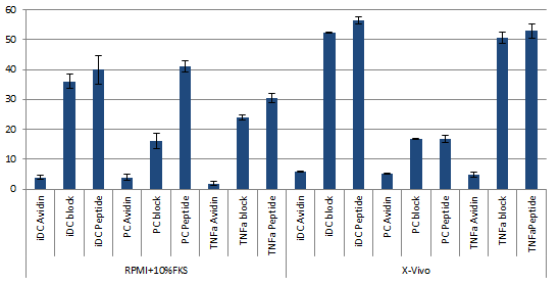


Figure 29: *MHC II binding performance of p210 for DCs of a HLA-DR3 heterozygous donor.*
Binding frequency: Peptide binding [%]) of p210 peptide preparations using different DC preparations (immature DC, Positive-cocktail matured DCs, and TNF-α-treated DCs, different cell culture media (FCS supplemented vs. serum free "X-Vivo") MHCII blocking and Avidin-background controls

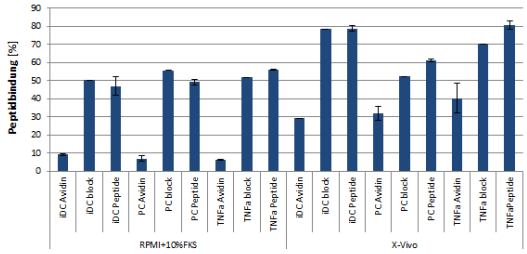


Figure 30: *Corresponding MHC II binding performance of p210 for DCs of a HLA-DR 1 heterozygous donor.*

Human DC/T cell assay to analyze peptide-specific DC and T cell induction

p45 and p210 as well as the CTB fusion proteins CTB-p45 CTB-p210 have been analyzed for the induction of human DCs and of early T cell responses using DC and DC/T cell assays. The fusion proteins show a higher induction potential than the peptides only (Fig. 31). CD80, CD83 and CD86 are upregulated only for the fusion proteins and in a clear dose dependent manner (1>10>30µg/mL). HLA-ABC (MHC I) is also upregulated. HLA-DR is slightly induced by CTB-P210 but not by CTB-p45 and the peptides only.

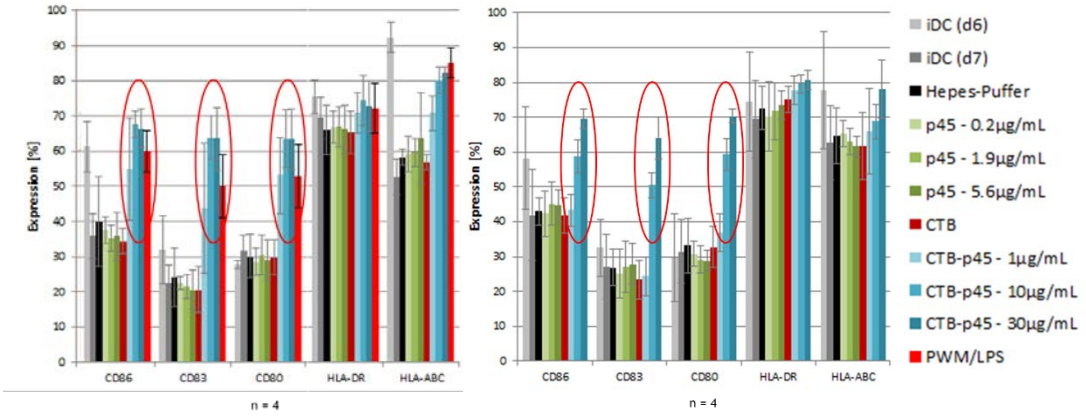


Figure 31: *DC activation by p45 and CTB-p45 (left) and p210; p210-CTB (right)*

Treatment of the Human lymph node model (HuALN)

In a first run, the HuALN model have been used for testing of p45 and p210 peptides and the CTB fusion proteins in a long-term repeated dosing study of 28 days (Fig. 32). Three different concentrations for the single peptides of 0.2, to 6.2 µg/mL and for the fusion proteins of 1, 10 and 30 µg/mL have been applied.

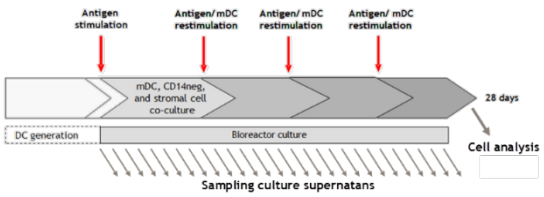


Figure 32: *Treatment regimen of the HuALN model including 4 consecutive dosings and daily sampling of culture supernatants.*

For p45 the peptide only induces in repeated treatments the highest pro-inflammatory response in a dose - dependent manner (Fig. 33) even compared to the CTB-fusion protein variant. The higher dose is showing higher effect (30 µg/mL).

For p210 the CTB-fusion protein variant is more excitatory towards pro-inflammation (Fig. 34).

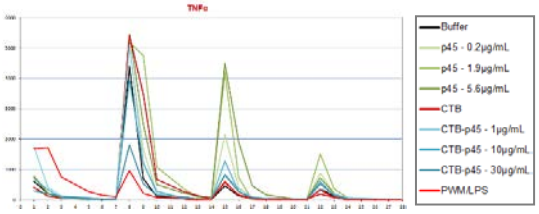


Figure 33: *Induced cytokine secretion for the p45 variants (TNF-α, exemplified)*
Drug restimulation was applied on day d7, d17 and d21

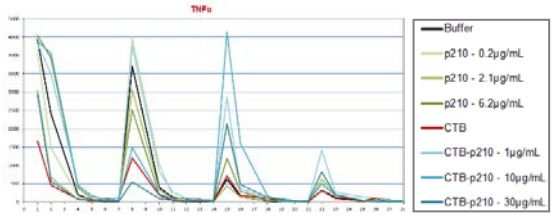


Figure 34: *Induced cytokine secretion for the p210 variants (TNF, exemplified)*
Drug restimulation was applied on day d7, d17 and d21

3, 4: Optimize selected epitopes for vaccine preparations with respect to formulation, use of adjuvant and route of administration to achieve desired immune modulation.

Vaccine testing for tolerance induction using the HuALN model

The HuALN is the most complex assay delivering important information elucidating the mode of action especially of the B cell response. Furthermore donor variability will be investigated.

Adjuvant based vaccines

Previous data showed the positive effect of p210-CTB on eliciting an immune response and reducing atherosclerosis symptoms in transgenic mouse ($APOE^{-/-}$). Therefore p210 was selected to conduct initial binding studies to aluminium-based adjuvants produced by P11. Peptide p210 carries an excess of positive charges due to 6 His residues (for 1 Asp) which gives a theoretical isoelectric point of 10.1. Thus AlPO4 micro-particles (AdjuPhos) were used, as these particles carry an excess of negative charges characterized by a zeta-potential of about -50 mV at pH 7.4 in 10 mM phosphate buffer. Physico-chemical parameters (buffer type, pH and ionic strength) were

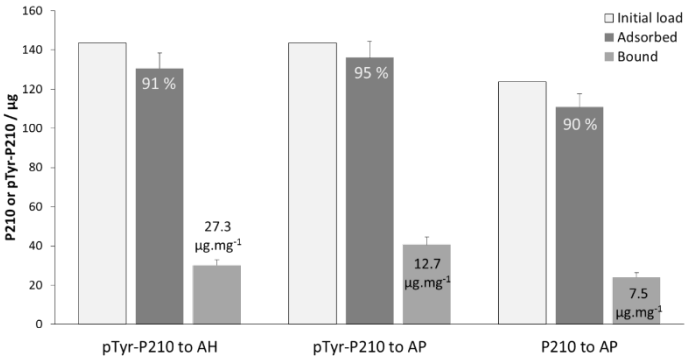
optimized in order to confer AlPO4 micro-particles the highest zeta-potential. It was assumed that attractive Coulomb forces would drive the adsorption of p210 to AlPO4 micro-particles. To better record the adsorption of p210 to micro-particle adjuvants (and later to track p210 in in vitro / in vivo studies), p210 was labelled with the near-infrared fluorophore Alexa-Fluor 647 using standard EDC-NHS chemistry. Using flow-cytometry in a competitive assay, binding of p210 to AlPO4 was shown with an apparent dissociation constant of 20 µM. The binding capacity of AlPO4 for p210 is 50 µg/mg, at least.

Due to the randomness of Alexa-Fluor 647 coupling to p210 with EDC-NHS chemistry (modification of native Lys residues, variable stoichiometry), p210 was redesigned with an extra amino-acid (N-terminus) to allow for site-specific Cu-catalyzed cyclo-addition of Alexa-Fluor 647. Coupling was performed and mass-spectrometry check is ongoing.

Design of vaccines on basis of custom-made aluminum-based adjuvants and apoB100 peptides

We chose to evaluate classical aluminum-based adjuvants as direct carriers for ApoB100 immunogenic peptides. We hypothesized that an optimal formulation of the P210 peptide/adjuvant mixture should be studied in order to reach a clinically relevant vaccine. P11 developed a method to quantitatively determine the amounts of ApoB100 immunogenic peptides adsorbed to aluminum adjuvants, since the intensity and type of immune response will also depend on the way the peptides are delivered, as freely diffusible molecules or as adsorbed to adjuvants, depending on the type of adjuvant or the route of administration. Using the fluorogenic molecule fluorescamine, we show that it is possible to determine quantitatively the amounts of peptides actually adsorbed to the adjuvant particles. It is shown that P210 adsorbs to Adju-Phos at 5 µg.mg-1 (or 23 µg.mg-1 Al) (w:w), in optimized conditions corresponding to a single dose of 200 µL for the vaccination of mice. The adsorption is driven by electrostatic forces (P210 is positively charged and Adju-Phos negatively charged at pH 7.1), as all of P210 is washed away at high ionic strength. At intermediate ionic strength corresponding to a physiological serum (1x PBS buffer), about 40% of P210 is immediately washed away. In order to provide for a stronger binding of P210 to aluminium adjuvants, a phospho-tyrosine was added to the sequence of P210 (pTyr-P210) and shown to improve the adsorption of P210 to Adju-Phos by 69% and to Alhydrogel by 360%. Further studies will be conducted to test these new formulations in a vaccination trial on HuBL mice, in order to determine whether improved adsorption of P210 can lead to an improved immune response in the context of ApoB100-driven atherosclerosis.

To improve the binding of P210 to Adju-Phos is modified with a phosphate group. Phosphate ions and phosphorylated proteins have already been shown to bind to aluminium adjuvant particles through dative covalent bonds to the aluminium atom (Rinella). This is particularly effective for aluminium hydroxide particles (Alhydrogel), where hydroxide ions are replaced by phosphate ions. A phospho-tyrosine variant of P210 (pTyr-P210) was synthesized and its adsorption to Adju-Phos and Alhydrogel was performed in the same conditions as described above (see Figure 18) and was tested and quantified with fluorescamine. Taking the adsorption of P210 to Adju-Phos as reference, the adsorption of pTyr-P210 to Adju-Phos resulted in 69% increase in adsorbed peptide when expressed as weight of peptide per weight of Adju-Phos particles: 7.5 $\mu g.mg^{-1}$ and 12.7 $\mu g.mg^{-1}$, respectively (Figure 36). Although, it is very unlikely that the phosphoryl group introduced in pTyr-P210 will substitute phosphate ions in Adju-Phos, this material contains however a proportion of hydroxide ions, which could be substituted by the phosphoryl group of pTyr-P210, and might account for the increased amount of pTyr-P210 adsorbed to the particles. This improvement in adsorption is even more marked when Alhydrogel is used instead of Adju-Phos. Measurements with fluorescamine directly on the particles (Method 2) showed 27.3 µg pTyr-P210 per mg of Alhydrogel instead of 7.5 $\mu g.mg^{-1}$ for P210 to Adju-Phos (Figure 35), which corresponds to a 3.6-fold increase. Given that P210 is positively charged and so is Alhydrogel in the conditions of adsorption, electrostatic repulsion is expected to prevent P210 to adsorb to this material. However, this improvement in adsorption when a phosphoryl group is introduced in P210 structure (via phospho-tyrosine) is a clear indication that substitution of hydroxide ions by the phosphoryl group in Alhydrogel structure has occurred. One of the benefits of this result is the possibility to deliver P210 with Alhydrogel in addition to Adju-Phos, as Alhydrogel has different physico-chemical properties, such as a crystalline structure and opposite net charge to Adju-Phos.



Using ProBioGen human dendritic

Figure 35: *Adsorption of P210 and pTyr-P210 variant to aluminium adjuvants. Peptides were quantitatively assayed with fluorescamine: "Adsorbed" fraction was measured using "Method 1", whereas "Bound" fraction was measured after washes of the particles with "Method 2". The relative amount of "Adsorbed" peptide is expressed in % of the total amount of peptide loaded in the mixture. The amount of peptide bound to the adjuvants after washes is shown on the corresponding bars as µg of peptide per mg of adjuvant (w:w, dry weight)*

cell/T-cell (DC/TC) *in vitro* system to compare formulations of ApoB100 P210 antigen with aluminium salts adjuvants

Executive Summary

Based on the aforementioned approaches we manufactured a series of prototype vaccines, where either native P210 or phospho-Tyr-P210 were added to either Adju-Phos or Alhydrogel aluminium adjuvants.

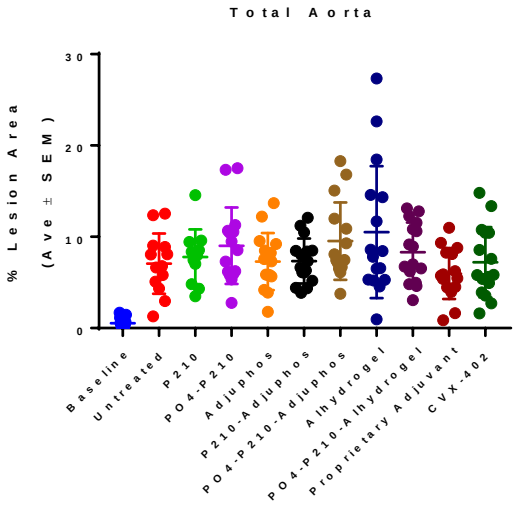
Immunization of *HuBL* mice with P210 adsorbed to aluminium-based adjuvants

A series of vaccine doses were prepared and characterized (P210 peptide adsorption) for triple sc injection in *HuBL* mice (every 3 weeks) on a western type diet and test the effect thereof on atherosclerotic plaque formation in the aorta. This animal study was conducted by P9 and results showed no significant differences between the control group and the various formulations of P210 or phospho-Tyr-P210, when atherosclerotic plaques or levels of cholesterol were quantified (Figure & Table 36) in *HuBL* mice aorta.

P210 has the following amino-acid sequence KTTKQSFDLSVKAQYKKNKH. PO4-P210 has the following sequence phospho-Tyr-PG-KTTKQSFDLSVKAQYKKNKH, with PG acting as a small flexible spacer between the reactive phospho-Tyr residue and P210 sequence. In conclusion of this study, no significant differences were observed between the diverse formulations of P210 or phospho-Tyr-P210, compared to the adjuvants or the peptides alone. This suggested no positive effect of the adjuvants in addition to peptides alone on atherosclerosis. Unfortunately no information on the amplitude of the innate or adaptive immune response.

Treatment	Mean (%)	% Change vs Untreated Control
Baseline	0.53	
Untreated	7.07	
P210	7.78	10%
PO4-P210	9.02	28%
Adjuphos	7.28	3%
P210-Adjuphos	7.35	4%
PO4-P210-Adjuphos	9.53	35%
Alhydrogel	10.52	49%
PO4-P210-Alhydrogel	8.32	18%
Proprietary Adjuvant	5.98	-15%
CVX-402	7.21	2%

Figure & Table 36. Effect of vaccine formulations with P210 and aluminium adjuvants on atherosclerotic plaque formation



Effect of vaccine formulations with P210 on DC/T cell assay

An identical series of vaccine prototypes was tested in the Dendritic Cell / T cell co-culture assay developed by ProBioGen (DC/T cell assay). No significant differences were observed between the various formulations of P210 and aluminium adjuvants. The quality of the dendritic cells and their differentiation or maturation process was donor dependently aberrant, which might have rendered them relatively insensitive to the adjuvants and the vaccines in this particular trial.We also studied the effects of these P210 prototype vaccines in the DC / TC assay developed by the team at P11 (Figure 37). An array of combinations using native P210 peptide or phospho-Tyr-P210 peptide, and either Adju-Phos or Alhydrogel as adjuvants, was tested (Table 3), which mirrored the array of vaccine doses for animal testing presented above. The amount of aluminium equivalents injected into the cell culture was kept constant to 25 µg/mL, whereas P210 concentration covered 2 orders of magnitude from 0.5 to 50.0 µg/mL (Table 1).

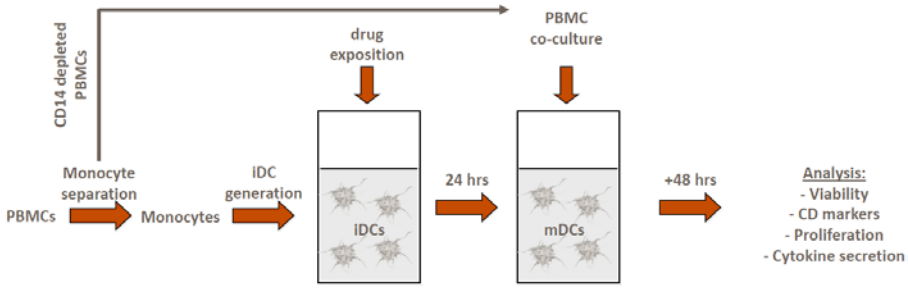


Figure & Table 37. Schematic overview of the DC/T cell assay performed by ProBioGen (top) and vaccine formulation (bottom). Immature dendritic cells (iDC) were exposed to the various formulations with P210 before being co-cultured with autologous CD14-depleted PBMCs.

Maturation of the DCs at the start of the co-culture is monitored by expression of specific PAN DC maturation markers CD86, CD83, CD80 and MHC Class I (HLA-ABC) and Class II (HLA-DR) cell receptors. The maturation of the DCs was inhibited by adjuvants, more so when Adju-Phos was used. No differences were observed with respect to the form (P210 or phospho-Tyr-P210) or the dose of peptide used. Frequencies of T-cell sub-populations were monitored by recording the fraction % of cells expressing specific PAN T-cell markers, CD3 (mature T-cell identity), CD25 (activated T-cell), CD69 (activation, maturation, proliferation T-cell), CD4 (T-helper cell identity) and CD8 (cytotoxic T-cell identity). None of the vaccine formulations had influence on the T-cell sub-populations frequencies, nor did any formulations induce T-cell proliferation.
Surprisingly, the only marked difference observed between the vaccine formulations is that Adju-Phos (both alone and with P210 peptides) induced IL-18 production. Aluminium adjuvants activate the NLRP3/Caspase inflammasome, which leads to IL-1ß and IL-18 secretion. It is puzzling that IL-33 is not co-detected with IL-18.

Final optimization of the HuALN model using model antigen

To improve cellular readouts the drug treatment and exposure time has been modified in the past. During the recent period an additional T cell stimulation was applied to the bioreactor in combination with a model antigen (KLH) in order to enhance overall T cell response. Monoclonal antibodies (anti-CD3/antiCD-28) have been added once-only to the system on day 5 of bioreactor run. Although these antibodies are known to induce polyclonal expansion of T cells, the application into the HuALN model seemed to enhance the antigen-specific immune response only. Exemplarily IFNy and TNFa are shown for this experiment (Figure 37). The T cell boost on day 5 only affected the antigen-pulsed bioreactors and the positive effect also lasted the restimulations with freshly antigen-matured DCs on day 7 and day 14. The T cell boost had no influence on control bioreactors.

WP4: Safety and regulatory issues

During the execution of the VIA project we came to the conclusion that the present data were not solid enough to starta clinical trial using the apoB100(peptide)-CTB or the apoB1100 (peptide)-PADRE approach. We came to the conclusion that most likely the choice of the peptide within the apoB100 protein (the antigen) was not secure enough to allow a First-In-Human clinical trial. Specifically partners and Leiden, Lund, Cardiovax and CHDR were involved in this discussion. To however proceed towards a vaccination protocol we choose to follow a backup plan and test the safety to address the best epitope so far, p45 using a monoclonal antibody. To that end we made the second amendment, which mainly involved the production of a monoclonal antibody targeting p45 that could be tested in subsequent clinical trials.
In short, the consortium partners selected a monoclonal antibody (mAb) against epitope p45 of oxidized low-density lipoprotein (oxLDL) to be evaluated in clinical trials as a potential drug candidate for the treatment of atherosclerotic cardiovascular disease (ACVD). The mAb was named AB-200 (also known as BI-204 and orticumab). As a first step towards producing orticumab for use in a clinical trial, a Chinese hamster ovary (CHO) clonal cell line that stably expressed orticumab was created. The cells were evaluated for critical attributes such as productivity and stability to ensure that the cell line is capable of production for clinical trials and meets the requirements of current Good Manufacturing Practices (cGMPs). A final clone was then selected and expanded into vials as a research cell bank (RCB). Additionally, a process was developed to grow the antibody in a bioreactor. A total of 8 bioreactor cultivations were completed, demonstrating the antibody could be produced at small scale. The same process will be used to produce the antibody at large scale to supply a clinical trial and this will be executed by partner Cardiovax.

The result is a is a stable CHO line that expresses orticumab and a bioreactor process that can be further used for GMP manufacturing of orticumab for clinical trials. Deliverables D4.4 and D4.5 have therefore successfully been completed and reported.

WP5:

The main objective of WP 5 was to execute a clinical trial on the possibility to use Prevenar as a vaccine to induce a protective humoral immune response against oxidized lipid components as present in oxidized low-density lipoprotein. Studies performed in WP2 and WP3 form the basis for this clinical study. The study was performed by P10 and the full details of the study have been presented in reporting of deliverable D5.3

***Proof-of-pharmacology clinical trial on a vaccine that elicits a* protective humoral immune response against oxidized low-density lipoprotein**

- Pneumococcal vaccine and humoral immunity against oxidized low density lipoprotein -

Background & Rationale

Atherosclerosis is the main cause of cardiovascular disease. Low density lipoprotein (LDL) plays an important role in atherosclerosis: after being oxidized in the vascular wall, it is phagocytosed by macrophages, forming foam cells and stimulating the overall inflammatory process. Animal research has demonstrated that the 13-valent pneumococcal polysaccharide conjugate vaccine that is currently used in clinical practice can induce immunoglobin M (IgM) antibodies against these oxidized LDL (oxLDL) particles, which resulted in a reduction of atherosclerotic lesion size. The aim of the present study is to determine the effect of vaccination on anti-oxLDL antibodies in man.

Main objective

The main objective of this work package is to determine specific immunoglobulin responses against oxLDL antigens after administration of a 13-valent pneumococcal conjugate vaccine. Secondary objectives include:

- To investigate if early revaccination with the 13-valent pneumococcal conjugate vaccine elicits an additional immune response against oxLDL
- To investigate if late revaccination with the 13-valent pneumococcal conjugate vaccine elicits an additional immune response against oxLDL
- The temporal resolution of immunoglobulin responses after vaccination after single, double and triple vaccination with the 13-valent pneumococcal conjugate vaccine.
- To determine the induction of immunoglobulin-oxLDL complexes after vaccination with the 13-valent pneumococcal conjugate vaccine;
- To determine the effect of vaccination with the 13-valent pneumococcal conjugate vaccine on total serum cholesterol, LDL, high density lipoprotein (HDL), triglycerides and Lp(a)

Project results

Twenty-four male subjects were included in the study; they were randomized to receive up to 3 vaccinations with the 13-valent pneumococcol vaccine. They received the first vaccination in April/May 2016, their second vaccination in May/June 2016 and the third and final vaccination in October/November 2016. Data on safety and tolerability is available up to December 2016. Data on efficacy is available up to 4 weeks after the first vaccination (prior to the second vaccination). The different vaccination schedules are displayed in table 1.

Group	Day 0	Week 4	Week 28	Total
1 (4 subjects)	**Active**	**Active**	**Active**	**3 vaccinations, 0 placebo**
2 (4 subjects)	**Active**	**Active**	**Placebo**	**2 vaccinations, 1 placebo**
3 (4 subjects)	**Active**	**Placebo**	**Active**	**2 vaccinations, 1 placebo**
4 (4 subjects)	**Active**	**Placebo**	**Placebo**	**1 vaccination, 2 placebo**
5 (8 subjects)	**Placebo**	**Placebo**	**Placebo**	**0 vaccinations, 3 placebo**

Table 1. *Vaccination schedule*

Safety/tolerability

The treatment was associated with mild injection site discomfort, which was temporary. No serious adverse events or severe adverse events have been reported; Blood panel, blood pressure and electrocardiographic results were normal or non-clinically significantly abnormal for all subjects for the duration of the study;

Results

All vaccination regimens that included at least on active treatment of Prevnar-13 (groups 1-4) resulted in significantly increased IgG titers (IgG2) to the vaccine preparation (Prevnar-13) after 40 weeks. IgM titers to Prevnar were also significantly increased compared to placebo treatment after 40 weeks, though only in groups 1 and 2 (i.e. following at least two initial vaccinations at day 0 and week 4). None of the vaccination regimens resulted in increased total IgG1, IgG2, IgG3, or IgG4 levels compared to placebo treatment. Moreover, cholesterol levels were not affected by any of the vaccination regimens.

IgM titers to oxLDL were significantly higher after 40 weeks compared to placebo treatment only in group 3 and a similar response was observed for anti-oxLDL IgG. A similar pattern could be observed for anti-PC IgM titers, consistent with fact that PC represents the target epitope in oxLDL. These data show that Prevnar-13 vaccination has the capacity to induce anti-oxLDL antibodies. However, in contrast to the anti-Prevnar-13 response a significant antibody response to oxLDL may require a different vaccination regimen that involves a longer period before the booster immunization. These findings are also consistent with the notion that anti-PC responses represent T cell-independent responses. Future vaccination protocols need to take this into account and should include repeated injections with larger time period between each boost.

The potential impact

IMPACT:

At the start of the project the following impact of the VIA project was expected:

1. Develop a vaccination protocol to protect against cardiovascular disease based on epitopes derived from Low-Density Lipoprotein that will finally reduce the mortality and morbidity associated with atherosclerotic cardiovascular disease.
2. Because of its specificity the atheroprotective vaccine will prove to be superior to other biological therapies under development.
3. The atheroprotective vaccine is expected to provide a highly favourable cost-medical benefits ratio and this will meet the enormous public health need to improve the treatment of cardiovascular disease
4. The VIA project is strongly focused on delivering a product: an atheroprotective vaccine to be tested in a First-In-Humans clinical trial
5. The industry and SMEs are involved in the essential parts of VIA project and their involvement will lead to the delivery of a product that will strengthen their position,

Obtained results with respect to the listed expected impact:

A vaccination protocol to protect against cardiovascular disease based on epitopes derived from Low-Density Lipoprotein.

The results obtained in the VIA project provide clear guidelines for the development of a vaccination protocol. Based on the experimental work performed by the various partners we expected that the primary vaccines would involve a protein based on the protein part of apoB100 (as present in low-density lipoprotein (LDL) or would involve a lipid part of the LDL particle.

ApoB100 based vaccines:

- To be able to identify so-called antigens in the apoB100 protein, several experiments were executed. Main findings were the identification of additional peptides in apoB100 (in addition to the well-known P2, P45 and P210 already identified at the start of the VIA project) that were not only effective in the experimental setting but also in the more humanized mouse models. The identification of these new human relevant peptides will provide new tools to develop atherosclerosis vaccines and the collaboration with industrial partners, such as Cardiovax (P9), will facilitate the development of these vaccines.
- The studies on peptides derived from apoB100 originally focused on inducing a tolerogenic response against peptides involved a characterization of the recognition of apoB100 peptides by T cells in such a way that tolerance was induced. The tested combinations of known peptides with CTB however were ineffective in reducing atherosclerosis, we therefore conclude that this vaccine has a low potential for further development. The most promising approach has come forward from a new formulation of an apoB100 peptide identified to bind to MHCII. The formulation of a peptide into a liposome to induce tolerance and reduce atherosclerosis is the best option for further research and translation into a human application, since liposomes are clinically approved in trials and existing vaccines and recent identification of apoB100 peptides binding to human HLA class II (Kuiper, unpublished data) pave the way for testing in patients, which will be followed up in combination with the CHDR.
- Since we were unable at the final stages of the VIA project to one hundred per cent identify epitopes within apoB100 that we can safely target using a classical or tolerizing vaccine, we undertook a major effort to produce a monoclonal antibody that can be readily used in human studies. The ultimate aim is to test whether targeting the peptide of apoB100 recognized by the monoclonal antibody is safe, improves CVD parameters and plaque stability. Subsequently as we

can use this peptide to construct a vaccine as a therapy to permanently address the desired peptide from apoB100.

Lipid-based vaccines

Vaccines based on the lipid moiety of LDL were the other goal of the project. Extensive in vivo work with atherosclerosis prone mice clearly indicated that mice vaccinated using Prevnar were protected against diet-induced atherosclerosis. The main mechanism behind the results was the formation of antibodies recognizing modified lipids as present in the vaccine and in modified LDL. Based on that we performed a First-in-Humans clinical trial using Prevnar. Although the results were encouraging we were not able to definitely find a positive effect of the vaccination possibly as a result of a lower lipid concentration in the batch of Prevnar we used in our clinical study. Still a large Prevnar vaccination study currently performed in Australia may shed more light on the effect of this vaccine on cardiovascular disease.

Overall impact of the VIA project for the treatment of cardiovascular disease:

- We clearly defined that tolerogenic liposomes loaded with a peptide that was binding to MHCII can form a good and readily applicable alternative for the use of other peptide-CTB complexes in combination with the use of an adjuvant. Recent literature has shown that a similar peptide in combination with an adjuvant can also be atheroprotective, which may lead to the conclusion that it is essential that the apoB100 peptide in the vaccine must bind with high affinity to the class II HLA of the patient. Recent, unpublished data show that these peptides can be identified and may form the basis for such a vaccine.
- We can clearly advise to use a pneumococcal based vaccine such as Prevnar ideally enriched with pneumococcal derived lipids to enhance the chance of raising antibodies that recognize the lipid moiety as present within modified LDL and as such lower the incidence of cardiovascular disease

- The VIA project therefore performed a First in Human vaccine based trial as the first vaccine approach described in the treatment of cardiovascular disease and subsequently identified clearly the requirements for a human vaccine although multiple steps need to be taken for the first performance of a peptide based vaccination.

Whether the atheroprotective vaccine will prove to be superior to other biological therapies under development will have to be determined in the future. At present a monoclonal antibody addressing Il-1beta has proven to be successful in the reduction of cardiovascular risk and complications (CANTOS trial), but it will not be used for treating CVD patients despite a maximal 25% beneficial effect, although there were serious side-effects such as life-threatening infections. Additionally a low dose methotrexate study did not prove any beneficial effect on CVD despite its beneficial effect in rheumatoid arthritis patients suffering from similar immunological disorders as CVD patients. Finally a low dose colchine (LODOCO) follow-up trial is on its way and this treatment was effective in an initial small trial. All together a atheroprotective vaccine may form a very welcome step in the anti-inflammatory treatment of CVD, since this approach has been shown to have a positive effect on CVD OUTCOME .

Impact of a effective treatment such as a tolerogenic or lipid based vaccine of cardiovascular disease: *public health need*

Also in 2019 cardiovascular disease (CVD) still remains a major public health problem in Europe. Within Europe 47% of all deaths (more than 4 million deaths) result from cardiovascular disease and it accounts for 40% of all deaths within the countries of the European Union (1.9 million deaths). Cardiovascular disease is not only deadly to the elderly but also to men and women younger than 65. Approximately 30% of all deaths before the age of 65 are caused by complications of cardiovascular disease. Furthermore, complications of cardiovascular disease are often associated with dramatic functional

impairment of heart and brain function. Thus, cardiovascular diseases lower the quality of life of cardiovascular patients for many years and lead to an immense loss in productivity.
Since the mid-1990's, major improvements in the treatment of cardiovascular disease have been achieved with the introduction of interventional procedures to restore blood flow in blocked arteries and with the introduction of medicines to improve management of cholesterol levels. This success notwithstanding, there is still a huge unmet medical need. Nearly 70% of patients do not adequately benefit from the current state-of-the-art treatments and most patients with established disease who develop a secondary event are already receiving state-of-the-art treatments. The focus of new drug development has been mainly on managing cholesterol homeostasis and the introductiomn of PCSK9 inhibitors has led to the possibility to optimally lower the LDL cholesterol levels, but at present it has not proven to lower the inflammatory component of CVD. During the execution of the VIA project positive news from the CANTOS trial indicated that the incidence of CVD was lowered by using the anti-IL-1ß monoclonal antibody. The trial was executed in a subgroup of CVD patients with a higher inflammatory status (high hsCRP) and lowering the inflammatory status indeed resulted in a lower incidence of cardiovascular complications. Although it is unlikely that Canakinumab will be used to treat CVD patient, it does underline the effectiveness of an anti-inflammatory treatment. Therefore we foresee that specific treatments designed to lower the inflammatory status of CVD have a great future and we foresee that the approaches we have designed such as liposomal vaccination using apoB100 peptides can have a great application from which CVD patients can benefit.
Therefore, the **urgent need to develop a novel approach to treat cardiovascular disease** we described at the start of the VIA consortium still exists and it is absolutely necessary to focus not only on cholesterol and triglyceride management but also on the autoimmune-like inflammatory response underlying CVD. The major impact that vaccination may have is **long-term protection** at an acceptable level of costs since vaccines form a class of affordable cost-effective drugs. Patients will not have to take a drug every day since compliance is a major problem with the use of drugs from which a patient does not experience an immediate perceptible effect, such as statins, which induce a lipid lowering but the beneficial effects on the quality of life and the health are not instantaneous and can only be expected after years of use. Vaccination will provide long-term protection via a limited number of dosages from the vaccine and patients will not have to comply daily with this anti-inflammatory treatment.
The final impact of a vaccine will be that the atheroprotective vaccination is primarily focusing at mechanisms other than those targeted by statins and PCSK9 inhibitors and **vaccination can be combined with a lipid lowering strategy**. Lipid-lowering strategies have contributed approximately 30% to the improved treatment of CVD and an atheroprotective vaccine, with a similar effect as the anti-IL-1ß treatment, is likely to result in a similar level of effect on the outcome of cardiovascular disease.

Impact on Society: Economic Burden
Overall, cardiovascular disease is estimated to cost the EU economy almost €200 billion a year. This immense economic burden can be differentiated into three separate figures:

- €109 billion (54%) is due to health care costs
- €47 billion (24%) due to productivity losses
- €43 billion (22%) due to the informal care of people with CARDIOVASCULAR DISEASE

The impact of the VIA project will be felt on all of these aspects of the economic burden of cardiovascular disease. By reducing the incidence of major adverse cardiac events and the associated morbidity, by 25% as shown in the CANTOS trial, one may calculate that an atheroprotective vaccine will primarily reduce the € 106 billion that is currently spent each year on caring for patients with complications of atherosclerotic cardiovascular disease. Similarly, with fewer major adverse cardiac

events, productivity losses will be reduced from the current level of €47 billion as will the €43 billion spent on ancillary healthcare providers. Vaccines are one of the greatest achievements of public health. In addition to the dramatic declines in morbidity and mortality associated with vaccination, significant savings have been documented as well. For example, it has been determined that for every dollar spent on vaccinating against measles resulted in savings of approximately $10.30 in direct medical costs and $3.20 in indirect societal benefit. **We expect that the atheroprotective vaccine also provides an excellent costs-medical benefits ratio**

With respect to other therapeutic under development, last year the CANTOS trial has finished and the outcome was that aa 25% disease reduction could be achieved on top of statin treatment. The estimated costs of anti-IL-1ß treatment are very high (up to €80,000 per year) and it is highly unlikely that it will be introduced in the wide population of patients with cardiovascular diseases. In addition, a drawback of anti-cytokine therapies such as the anti-IL-1β antibody is decreased host immune defense against infection leading to life-threatening infections.

Impact of the VIA program on SMEs

Within the VIA consortium, several SMEs and industries were involved. Probiogen and Brenntag were two industrial partners involved in the VIA project and both companies very actively participated in the project. Probiogen further developed their strategies to use the in vitro model for the human lymph node and tested several of the apoB100 peptides for their ability to bind to human HLA, which was a great addition to the project since most of the academic partners only used preclinical mouse data for their studies. So for the further development of an atheroprotective vaccine the information gathered in this type of experiments is highly relevant. The impact for Probiogen is that they can further extend their portfolio and use the data obtained in this project for extension of their industrial capacity.

Brenntag contributed by the very precise formulation of apoB100 peptides with adjuvants and description of the exact features of these possible vaccines. The proposed vaccines were also tested in collaboration with Probiogen in the human lymph node model. For Brenntag (now Croda) it is essential to extend their knowledge on the formulation of vaccines using their proprietary adjuvants. By this knowledge they can extend their portfolio from just delivering adjuvants to formulation of immunomodulatory vaccines.

CHDR and Cardiovax were the two SME's involved in the project. For CHDR the execution of testing a possible cardiovascular vaccine is a new focus point and by this clinical trial they can extend their portfolio in the direction of testing anti-inflammatory approaches in cardiovascular disease. The latter is an increasing market since the promising data published on the CANTOS trial. In addition CHDR has increase their knowledge on testing the effects of immunomodulatory treatments by setting up measurements for activation of human, patient-derived monocytes in culture using modified LDL and by measuring antibodies towards modified LDL.

Cardiovax was the main SME driving the development of an atheroprotective vaccine. They had an important Impact on both the management of the project and the execution of various experiments tot test the prototype vaccines, production of vaccines. The collaboration with the academic and SME partners was intense and greatly improved their development plans for such a vaccines. The last step in the execution of the VIA project was the production of a monoclonal antibody as an intermediate step towards an atheroprotective vaccine. This step will absolutely have a great impact on their capacity to finally reach the goal of testing a vaccine in patients.

The impact on the European approach:

It has been of major importance that the VIA consortium acted as a European-wide consortium. The academic partners had at the start of the project already contributed world-wide to the identification of the possibility to use the beneficial aspects of the immune response, regulatory T cells and protective antibodies against (ox)LDL, for the treatment of atherosclerosis. During the execution of the project, the academic partners have greatly improved the collaboration and extensively exchanged ideas on the development of a prototype atheroprotective vaccines which resulted in a First-In-Humans clinical trial using a possible atheroprotective vaccine.

Dissemination, exploitation of project results and of intellectual property

The *Executive Committee* has been responsible for coordination of the overall dissemination activities (WP 1). A detailed dissemination plan has been made and was used as a reference document and guideline for all VIA partners with respect to the VIA publication strategy.

During the project we have had two-weekly telco's with the partners involved in the management (Cardiovax and Jan Nilsson, Malmo) to discuss strategy, dissemination and future approaches towards the development of the vaccine and lastly development of a monoclonal antibody. The VIA consortium has arranged yearly two-day meetings in The Netherlands (2 times), Sweden, France and Austria. In these meetings results have been reported and discussed by all partners. This contributed largely to the intense collaborations between the partners and resulted in an optimal execution of the project.

All partners have or are in the process of disseminating their research results to the widest extent possible. Primary target for the academic partners has always Already now the consortium partners have impressive track records in scientific publications in high profile journals, with a large readership and visibility, and they will continue to do so. Publications will indicate the source of the work (VIA) and that the work has been funded by a grant from EU. In parallel, VIA results will be presented at scientific conferences, e.g. the annual European Society of Cardiology and European Atherosclerosis Society meetings, and other relevant national and international meetings.

All VIA partners are well connected to national professional organizations (such as the European Atherosclerosis Society and national Atherosclerosis Societies and other national societies on atherosclerosis and cardiovascular research). In addition, partners are well involved in the National Heart Foundations in for instance UK, Sweden and The Netherlands. Throughout the execution of the VIA project partners have used these important channels for dissemination of the results obtained and to disseminate their opinion to cardiovascular patients and medical practitioners (cardiologists). Dissemination to the general public is an important point, but also complicated since dissemination of preliminary data in the general public sometimes lead to false hope that a new treatment is already available for human application. Therefore we actively pursued presentation of research results at organized events when the results can be disseminated according to the Publication & IPR committee. Each of the universities, furthermore, used their own communication strategy for dissemination of research results.

To maximize the impact of the VIA proposal on the general public, dissemination to the non-specialist public has been optimized. A key criterion in all dissemination activities targeted to the non-scientific public will be the translation of the complex scientific work performed in the

VIA consortium into understandable messages. VIA partners disseminated novel developments in the discovery of an atheroprotective vaccine mostly on a national basis and illustrated the role in prevention and treatment of cardiovascular disease in order to improve the publics' appreciation of the general goals and objectives in the VIA research. In addition we have launched a website on the VIA project, called "vaccinationinatherosclerosis", but due to access problems with hosting the website we have not always been able to update the website.

During the project *the Publication & IPR Committee* will be responsible handling of matters dealing with IP. This committee has been formed to properly manage ownership issues arising within this project. In general, during the collaboration created intellectual property ("foreground") belongs to the beneficiary generating it. Intellectual Property Rights (IPR) are thus not owned by the consortium but rather by the individual researchers and/or partner organizations. However, different national regulations determine the ownership of foreground. In order to advise all partners and support the *Project Steering Board* in matters of IPR management in order to avoid conflicts, a *Publication & IPR Committee* will be implemented.

Exploitation:
The companies involved were all well-experienced in developing vaccines that will meet clinical requirements. During the course of WP4 and WP5 Cardiovax (P9) has been very instrumental in focusing on the marketing aspect of the results obtained, which for instance resulted in the . for industrial partners to bring the atheroprotective vaccine to the market. These actions will be discussed within the executive committee to determine the final approach for exploitation.