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Efficacy, safety and novel targets in cardiovascular disease : advanced applications in APOE*3-Leiden.CETP mice

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The AT04A vaccine against proprotein
convertase subtilisin/kexin type 9 reduces total
cholesterol, vascular inflammation,
and atherosclerosis in APOE*3-Leiden.CETP mice

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Abstract

Objectives: Proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged as a promising therapeutic target for the treatment of hypercholesterolaemia and atherosclerosis. PCSK9 binds to the low density lipoprotein receptor and enhances its degradation, which leads to the reduced clearance of low-density lipoprotein-cholesterol (LDL-C) and a higher risk of atherosclerosis. In this study, the AT04A anti-PCSK9 vaccine was evaluated for its therapeutic potential in ameliorating or even preventing coronary heart disease in the atherogenic APOE*3-Leiden.CETP mouse model.

Methods and results: Control and AT04A vaccine-treated mice were fed a Western-type diet for 18 weeks. Antibody titres, plasma lipids, and inflammatory markers were monitored by ELISA, FPLC, and multiplexed immunoassay, respectively. The progression of atherosclerosis was evaluated by histological analysis of serial cross-sections from the aortic sinus. The AT04A vaccine induced high and persistent antibody levels against PCSK9, causing a significant reduction in plasma total cholesterol (-53%, $p < 0.001$) and LDL-C compared with controls. Plasma inflammatory markers such as serum amyloid A (SAA), macrophage inflammatory protein-1 β (MIP-1 β /CCL4), macrophage-derived chemokine (MDC/CCL22), cytokine stem cell factor (SCF), and vascular endothelial growth factor A (VEGF-A) were significantly diminished in AT04A-treated mice. As a consequence, treatment with the AT04A vaccine resulted in a decrease in atherosclerotic lesion area (-64%, $p = 0.004$) and aortic inflammation as well as in more lesion-free aortic segments (+119%, $p = 0.026$), compared with control.

Conclusions: AT04A vaccine induces an effective immune response against PCSK9 in APOE*3-Leiden.CETP mice, leading to a significant reduction of plasma lipids, systemic and vascular inflammation, and atherosclerotic lesions in the aorta.

Introduction

Elevated circulating low density lipoprotein cholesterol (LDL-C) is one of the major risk factors positively correlated with premature development of cardiovascular disease (CVD) (1). The main pathway of LDL-C clearance from the blood circulation involves the low-density lipoprotein receptor (LDLR) on hepatocytes (2,3). The serine protease proprotein convertase subtilisin/kexin type 9 (PCSK9), mainly synthesized in the liver, binds to the LDLR and enhances its degradation, thereby modulating cholesterol levels of circulating apoB-containing lipoproteins (i.e., very-low density lipoprotein (VLDL) and LDL) (4,5). By inhibiting PCSK9, LDLR expression and its activity are increased, leading to plasma VLDL- and LDL-cholesterol lowering.

The current hypothesis relating to the role of cholesterol in cardiovascular disease (CVD) is that the effect of lowering LDL-C on CVD is independent of the means by which LDL-C is lowered (6). The most commonly-used drugs to treat hypercholesterolaemia are 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, known as statins. Statins in general have been shown to reduce LDL-C by 30–60%, providing an estimated 25–30% reduction in CVD (7). However, many patients are unable to achieve their optimal lipid levels despite intensive statin therapy (8,9), and high doses of statins may increase the incidence and severity of multiple adverse events such as myopathy and hepatotoxicity (10). Moreover, statins do not only induce a beneficial increase in LDLR, but also increase PCSK9, which leads to LDLR degradation, thus causing a negative feedback response that attenuates the statins' lipid effects (11).

The most advanced alternative approach for LDL-C lowering is the inhibition of PCSK9 action by monoclonal antibodies (mAbs). The novel anti-PCSK9 antibodies alirocumab and evolocumab, which were recently granted FDA approval, are safe and achieved a 50–70% reduction in LDL-C when used either as monotherapy or in combination with a statin (12,13). They are approved as second line treatment in patients at risk who are unable to reach the LDL-C goal despite maximally tolerated statin therapy or for patients who are statin-intolerant (14,15). Preclinical efficacy studies with the PCSK9 mAb alirocumab were performed in the APOE*3-Leiden.CETP mouse model. These double-transgenic mice represent a valuable model for the preclinical evaluation of interventions on atherosclerosis development, because of its humanized lipoprotein metabolism. The diet-induced development of atherosclerosis in these mice has a pro-inflammatory plaque phenotype, and shows responsiveness to all lipid-modulating interventions that are being used in the clinic (16–18). In this model, alirocumab was able to decrease plasma lipids and atherosclerosis development, and in combination with atorvastatin the beneficial effects of PCSK9 mAb treatment were enhanced (18,19). However, mAbs show relatively short *in vivo* half-lives, thus the long-term efficacy of mAb therapy is accompanied by frequent application and high costs. We developed an active immunization against the body's own PCSK9 for a widely-applicable and more cost-effective long-term LDL-C cholesterol

management (20). This so-called AT04A vaccine was tested for its efficacy in APOE*3-Leiden.CETP transgenic mice. AT04A was able to induce a high immune response against PCSK9 without any side-effects; leading to a significant reduction of plasma lipids over the whole intervention period. Consequently, a reduction of systemic and vascular inflammation and atherosclerotic lesions in the aorta at the end of the intervention period was observed.

Materials and methods

Animals

Female APOE*3-Leiden.CETP transgenic mice on a C57BL/6 background (7-9 weeks of age) received a Western-type diet (WTD) containing 15% cacao butter and 0.1% (w/w) cholesterol (AB Diets, Woerden, The Netherlands) for a time period of 3 weeks. Thereafter, the mice were matched by body weight, total plasma cholesterol (TC), and triglycerides (TG), and divided into control (n=15) and vaccine-treated (n=15) groups. The number of animals per group was calculated using a probability of 0.05. Based on our experience from previous studies, we expected to have a variance of 15% (sigma 40%) in plasma lipids and a minimal effect of treatment of 30%, resulting in 15 animals per group. Upon starting the immunization, the mice were fed a chow diet (ssniff Spezialdiäten GmbH, Soest, Germany) for 4 weeks, followed by WTD for 18 weeks, in order to induce atherosclerotic plaques. At the end of the experiment all animals were euthanized by CO₂ inhalation, and blood was collected via heart puncture. Hearts and livers were isolated to determine liver weight, atherosclerotic development, and lesion severity. Animal experiments were approved by the Animal Experiment Committee of The Netherlands Organization of Applied Scientific Research TNO under registration number 3655.

Vaccines

The proprietary AFFITOME® technology (21) was used to develop short immunogenic peptides, which mimic a N-terminal epitope of PCSK9 that is involved in the interaction with LDLR. The peptides were synthesized by Fmoc solid phase peptide synthesis and HPLC-purified (EMC microcollections GmbH, Germany). These peptides contain an additional C-terminal cysteine residue and were activated with the crosslinker N-γ-maleimidobutyl-oxysuccinimide ester (GMBS, Pierce, ThermoFisher Scientific) according to the manufacturer's protocol, and covalently linked to the carrier protein Keyhole Limpet Haemocyanin (KLH, Biosyn GmbH). The KLH-conjugated peptides were adsorbed to 0.2% Alhydrogel® (Brenntag Biosector, Denmark). Scrambled peptide conjugated to KLH was used as a negative control vaccine. Vaccines were stored at 4°C. Prior to injection, the vaccines were brought to room temperature (RT) and carefully mixed.

Treatment and immunization scheme

Mice were immunized 5 times (subcutaneous, 500 μ l in both the right and left flanks) either with AT04A or a control vaccine, in bi-weekly intervals. During the first two immunizations the mice were fed a chow diet ($t=-4$ to 0 weeks). The time point at which the food was switched to WTD 0.1% (w/w) cholesterol in order to induce atherosclerosis development refers to week 0. Three more immunizations were then performed at $t=0$, 2 and 4 weeks (**Figure 1**). Blood was withdrawn every four weeks after a 4 hour fasting period at the time points $t=-4$, 0, 4, 8, 12, and 16 weeks, and the final bleeding was performed at week 18 (**Figure 1**). Plasma samples were prepared and used for titre analysis, total cholesterol ($t=-4$, 0, 4, 8, 12 and 18 weeks) and lipoprotein profile analyses ($t=-4$, 0, 4, 8 and 18 weeks), which were determined from group pooled plasma samples.

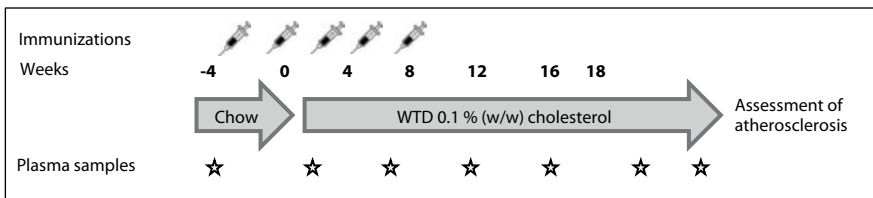


Figure 1 Study design. Mice were immunised 5 times either with AT04A or a control vaccine. Four weeks after prime immunization ($t=0$ weeks) normal chow was switched to WTD 0.1% (w/w) cholesterol. After 18 weeks mice were sacrificed to assess atherosclerosis development. Abbreviations: WTD, Western type diet.

ELISA analyses

To determine the titre of AT04A vaccine-induced antibodies, plasma samples were collected and analysed by ELISA. Briefly, 1 μ M of the antigenic peptides coupled to BSA were coated in 0.1 M NaHCO_3 (pH 9.2–9.4) to a 96-well Nunc-MaxiSorp plate. In order to test the reactivity against the target protein, a recombinant expressed human PCSK9 (“huPCSK9-V5-His”, AFFiRiS AG) was coated in 1 \times PBS. Free binding sites were blocked by the incubation with blocking buffer (1 \times PBS, 1% BSA) for 1 h at 37°C. Diluted plasma (1:400 and 1:100 in 1 \times PBS/0.1% BSA/0.1% Tween-20 for peptide and protein ELISA, respectively) were added, serially diluted 1:2, and incubated for 1 hour at 37°C. Each ELISA plate contained a standard antibody as internal control. For the detection, biotinylated anti-mu IgG (H + L) (Southern Biotech.; 1:2000) in 1 \times PBS/0.1% BSA/0.1% Tween-20 was applied and incubated for 1 h at 37°C. As a next step, horseradish peroxidase coupled to streptavidin (Roche) was added (30 min, 37°C) followed by the addition of the substrate 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (Bio-Chemica, AppliChem) (30 min, RT). The optical density (OD) at 405 nm was measured with a microwell plate

reader (Sunrise, Tecan, Switzerland) and the titres were defined as the dilution factor referring to 50% of the maximal optical density ($OD_{max}/2$). The mean titres \pm SEM of all animals per group are presented.

PCSK9 protein level in circulation

The plasma muPCSK9 concentration was determined by CircuLex muPCSK9 ELISA (CircuLex, Cy-8078, MBL), according to the manufacturer's protocol.

Plasma lipids and lipoprotein analysis

Plasma TC and TG were determined by the "Cholesterol CHOD-PAP" and "Triglycerides GPO-PAP" kit, respectively (Roche/Hitachi). Lipoprotein profiles for TC were measured after lipoprotein separation by fast protein liquid chromatography (FPLC) (16).

SAA measurements

Plasma SAA levels were measured at t=4, 8, 12, and 16 weeks after the onset of WTD, 0.1% cholesterol and 7 weeks before, as a reference value on normal chow diet. The ELISA kit of Tridelta Development Limited was used and analyses were performed according to manufacturer's protocol.

Immunological analyses

Myriad RBM's mouse inflammation multi analyte profile (Mouse Inflammation MAP® v.1.0) was used for quantitative analysis of multiple inflammatory analytes and pathways including cytokines, chemokines, and growth factors.

Histological assessment of atherosclerosis

Atherosclerotic lesion area and severity were assessed in the aortic root area, as reported previously (22). Briefly, the aortic root was identified by the appearance of aortic valve leaflets, and serial cross-sections of the entire aortic root area (5 μ m thick with intervals of 50 μ m) were mounted on 3-aminopropyl triethoxysilane-coated slides and stained with haematoxylin-phloxine-saffron (HPS). For each mouse, the lesion area was measured in 4 subsequent sections. Each section consisted of 3 segments (separated by the valves). For determination of atherosclerotic lesion size and severity, the lesions were classified into five categories according to the American Heart Association (AHA) criteria (23): type I (early fatty streak), type II (regular fatty streak), type III (mild plaque), type IV (moderate plaque), and type V (severe plaque). Images were taken with an Olympus BX 51 microscope, and areas were measured with Olympus analySIS image processing software cell^D. The total lesion area and number of lesions were calculated per cross-section. Lesion severity was calculated as relative amount of type I-V lesions in which the lesion-free segments are included. From this, the relative amounts of lesion-free segments and diseased segments were calculated, and the relative amount of diseased segments was further subdivided

into type I–V lesions, where types I–III refer to mild, and types IV–V to severe lesions. Lesion composition of type IV and V lesions was assessed after double immunostaining with anti- α smooth muscle actin (1:400; PROGEN Biotechnik GmbH, Germany) for α smooth muscle cells (α SMC), and anti-mouse MAC-3 (1:200; BD Pharmingen, the Netherlands) for macrophages. Sirius red staining was performed to assess collagen content. The necrotic area and cholesterol clefts were measured in the HPS-stained slides. Lesion stability index, as the ratio of collagen and α SMC area (i.e. stabilization factors) to macrophage and necrotic area (i.e. destabilisation factors) was calculated as described previously (18,22). In each segment used for lesion quantification, intracellular adhesion molecule 1 (ICAM-1) expression, NLR family pyrin domain containing 3 (NLRP3) expression, and the number of monocytes adhering to the endothelium were counted after immunostaining with mouse monoclonal antibody to ICAM-1 (1:400; Santa Cruz Biotechnology, Dallas, USA), rabbit polyclonal antibody to NLRP3 (1:400; Abcam, Cambridge, UK), and AIA 31240 antibody (1:500; Accurate Chemical and Scientific, New York, USA) respectively (24).

Statistical and correlation analysis

All values were evaluated for homoscedasticity and normality assumption using both Kolmogorov–Smirnov and Shapiro–Wilk normality tests. For the comparison of two groups, the unpaired two-tailed Student’s t-test was used, followed by the Mann–Whitney correction for non-parametric data as indicated in the respective figure legend. A Spearman’s rank-order correlation was used to determine the relation between two parameters. The software used for statistical analyses were IBM SPSS Statistics version 18. All data are presented as mean \pm SD unless stated otherwise. The p-values \leq 0.05 was considered statistically significant.

Results

AT04A induces a strong and persistent immune response and decreases plasma PCSK9 levels

APOE*3–Leiden.CETP mice were used as a model for atherosclerosis with a pro-inflammatory plaque phenotype that can be induced upon a high fat and cholesterol-containing diet. A peptide (AFFITOPE®) which was previously designed to mimic the N-terminal epitope of the mature human and homolog mouse PCSK9 protein (153aa–692aa) was formulated into the AT04A vaccine (20). The mice were immunized with AT04A and control vaccine five times in bi-weekly intervals, and an 18-week WTD was started 4 weeks after prime immunization, which refers to time point $t=0$ weeks (**Figure 1 and 2**). Titre analyses over time revealed that AT04A was able to induce a specific and long-lasting immune response against PCSK9, which reached a maximum mean titre of $1/12\,243$ ($OD_{\max}/2$) 12 weeks after prime immunization, which corresponds to $t=8$ weeks of WTD. The immune response to

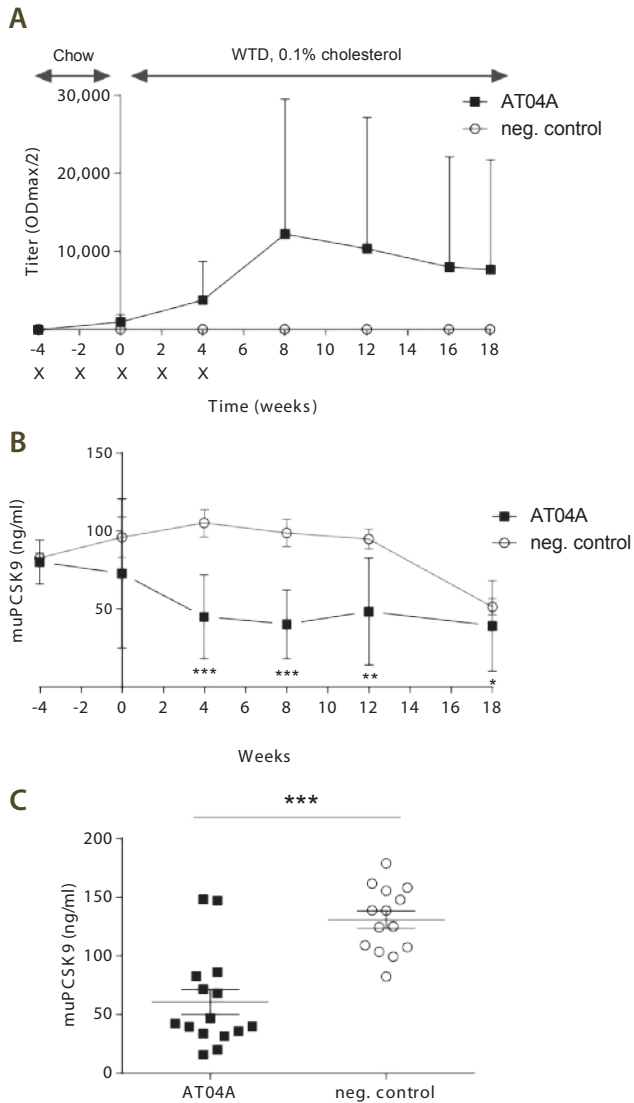


Figure 2 AT04A immunization induces a high and long-lasting immune response, and decreases plasma muPCSK9 levels. Antibody titres were assessed at different time points throughout the study. Crosses indicate the time points of the bi-weekly immunizations of control and AT04A vaccine (A). The muPCSK9 plasma concentration was determined at different time points throughout the study and compared to control (B–C). To compare AT04A and control treated group, the unpaired two-tailed Student’s t-test was used, followed by the Mann–Whitney correction for non-parametric data. Data are presented as group means \pm SD (n=15). * p <0.05, *** p <0.001.

PCSK9 highly varies between individual mice and no statistically significant differences in titres of t=4 to 18 weeks were found (**Figure 2A**). As expected, control immunized mice did not show any immune response against PCSK9 (**Figure 2A**). As evidence for a direct interaction between the induced anti-PCSK9 antibodies and the target protein, the plasma concentration of muPCSK9 in AT04A-treated vs. control immunized mice was determined over time (**Figure 2B and C**). At the immunization start (t=-4 weeks) the PCSK9 level in AT04A and control treated group was comparable. However, in t=4, 8, 12 and 18 AT04A vaccine treated mice showed a highly significant decrease in PCSK9 concentration of 57, 59, 49, and 24%, respectively ($p<0.001$, $p<0.001$, $p=0.001$, and $p=0.029$, respectively) (**Figure 2B**) demonstrating a consistent and long-lasting effectiveness of the AT04A vaccine (**Figure 2C**). Due to unknown reasons, the plasma PCSK9 level of control treated mice dropped in t=18 weeks, however, the difference between AT04A and control treated mice remained significant (**Figure 2B**).

AT04A decreases plasma TC and non-HDL-C levels in APOE*3-Leiden.CETP mice

Four weeks after the cholesterol-containing WTD was initiated, control immunized APOE*3-Leiden.CETP mice showed a strong increase of plasma TC level from 3.4 mmol/L (t=0 weeks) to 15.5 mmol/L (t=4 weeks), which remained elevated until t=18 weeks (12.1 mmol/L) (**Figure 3A**). In contrast, in the AT04A immunized mice a sustained reduction of plasma TC from t=0 weeks (2.8 mmol/L, $p=0.002$) to t=18 weeks (7.8 mmol/L, $p=0.002$) was observed (**Figure 3A**). Thus, cholesterol exposure over the whole time period of atherosclerosis inducing WTD (t=0 to 18 weeks) was decreased by 53% ($p<0.001$) in AT04A vs. control-treated mice (**Figure 3B**). The TC lipoprotein profile of pooled plasmas per group was determined by FPLC (**Figure 3C-F**). Plasma levels of HDL-C remained unaffected by AT04A vaccination, however the levels of non-HDL-C (VLDL and LDL) were clearly reduced (**Figure 3F-H**), indicating that the anti-PCSK9 vaccine may be a powerful therapeutic approach for long-term non-HDL-C/LDL-C management. A strong positive correlation between TC and plasma PCSK9 concentration was found ($R^2=0.75$; $p<0.001$) suggesting a specific and effective targeting of PCSK9 by AT04A vaccine.

AT04A reduces number, size, and severity of atherosclerotic lesions in the aorta

In order to assess the effect of AT04A on atherosclerotic development upon 18 weeks of WTD, the aortic root was isolated and the lesions as well as the lesion severity were determined. AT04A vaccine-treated mice showed a significantly reduced lesion area (-64%, $p=0.004$) (**Figure 4A**) and number of lesions per cross-section (-35%, $p=0.037$) (**Figure 4B**), compared with controls. Moreover, in AT04A-vaccinated mice a significant higher percentage of lesion-free area per section (+119%, $p=0.026$) was detected (**Figure 4C**). The lesion severity was categorized according to the American Heart Association guidelines

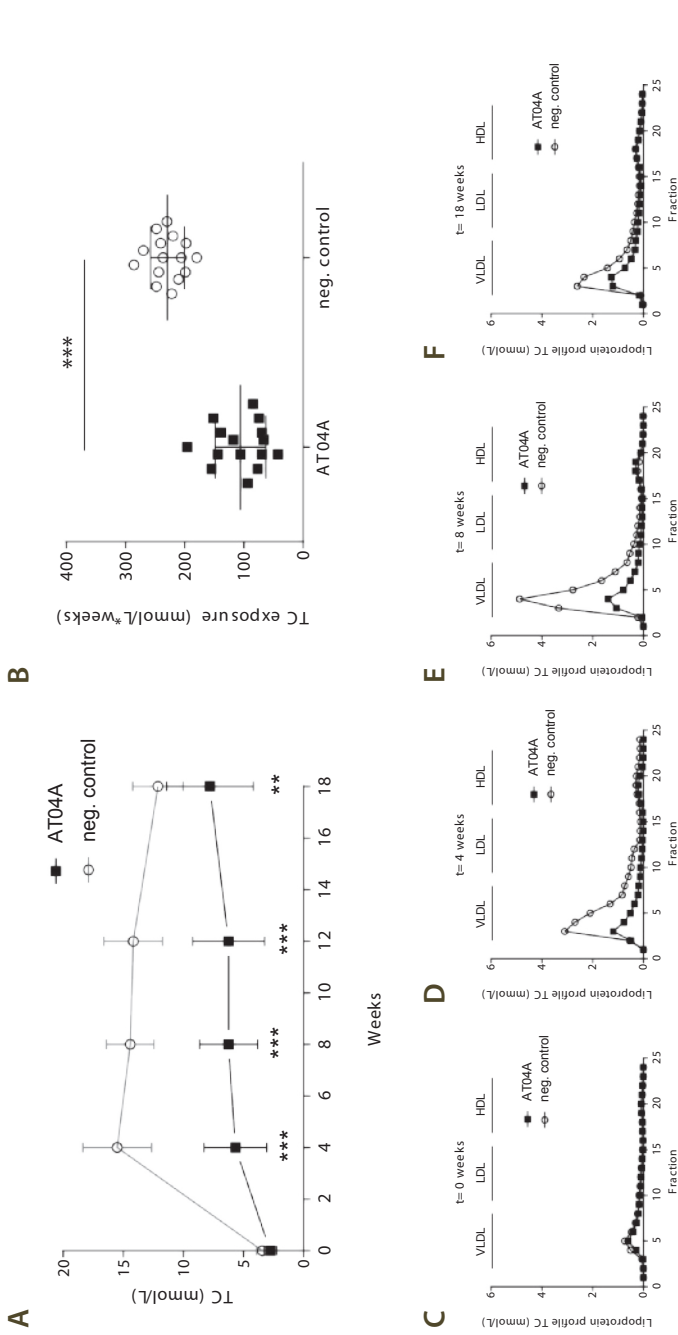


Figure 3 AT04A immunization decreases plasma TC and non-HDL-C. Plasma cholesterol levels were measured at various time points throughout the study (A) and TC exposure (mmol/L*weeks) was calculated (B). Plasma was pooled per group and the distribution of cholesterol over the individual lipoproteins was determined after separation by FPLC (C–F). To compare AT04A and control treated group, the unpaired two-tailed Student’s t-test was used, followed by the Mann–Whitney correction for non-parametric data. Data are presented as group means \pm SD (n=15). **p<0.01, ***p<0.001. Abbreviations: TC, total cholesterol, FPLC, fast protein liquid chromatography.

(23). Although the relative amount of mild lesions (type I–III) was similarly distributed between AT04A and control immunized mice, the more severe lesions (type IV–V) were decreased in AT04A treated mice (–64%, $p=0.051$) (**Figure 4C**). Representative images of the differently categorized atherosclerotic lesions in the aortic root of AT04A and control immunized mice are depicted in **Figure 4D**. A strong positive correlation between the atherosclerotic lesion area and plasma TC as well as plasma PCSK9 levels was observed ($R^2=0.75$, $p<0.001$ and $R^2=0.6$, $p=0.001$, respectively).

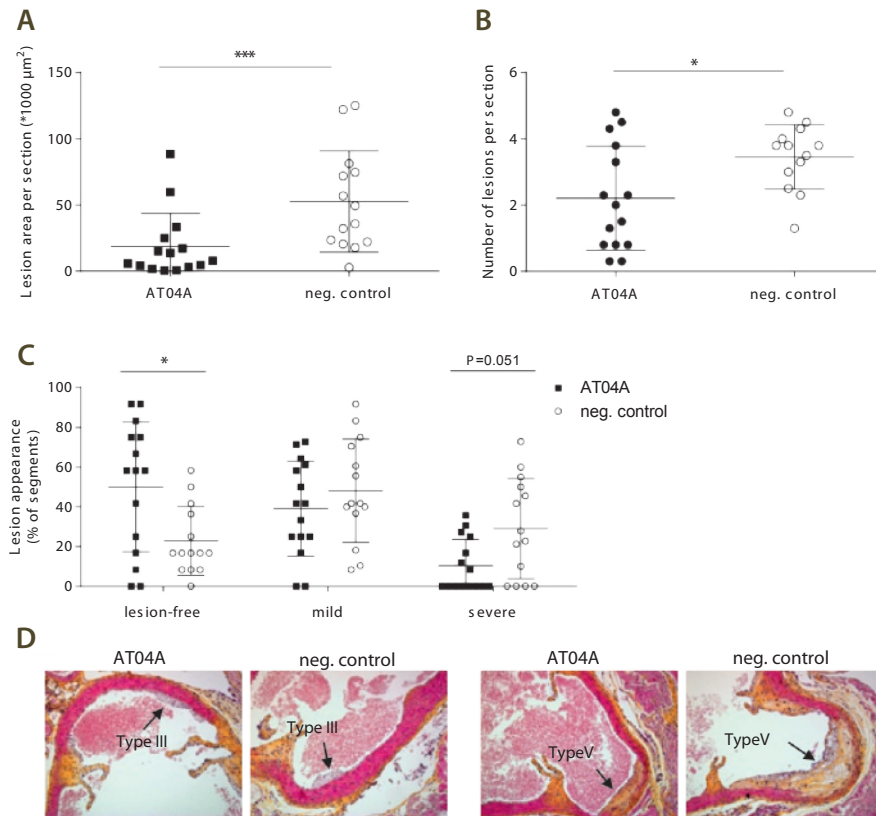


Figure 4 AT04A immunization decreases lesion size and severity. After 18 weeks of treatment, lesion area (A), number of lesions (B), and lesion severity (C) were determined per cross-section in the aortic sinus. Due to a technical error one mouse of the control group was excluded from analysis. Lesion severity was classified as mild (type I–III) or severe (type IV–V). Representative images of HPS-stained type III and V lesions in a cross-section (D). A Mann–Whitney U test was used for statistical analysis. Data are presented as group means \pm SD ($n=14$ –15). * $p<0.05$, ** $p<0.01$. Abbreviations: HPS, haematoxylin-phloxine-saffron.

AT04A immunization reduces necrotic core content

The effects of AT04A vaccination on the plaque composition of severe lesions (type IV–V) were assessed by immunohistochemistry. The necrotic core areas including cholesterol clefts and lesion macrophages were quantified as pro-inflammatory factors, and α SMCs in the fibrotic cap and collagen area as fortifying factors (**Figure 5A**). Representative images are shown in **Figure 5B**. All data are expressed as percentages of total plaque areas. In AT04A-treated mice the necrotic core area was 1.9 vs. 8.1% in the control-treated group, representing a reduction of 77% (**Figure 5A**) ($p=0.001$). Macrophage, α SMCs, and collagen content were not significantly altered by AT04A treatment. Despite the decrease in necrotic core content, the plaque stability index was not changed, and was 2.3 in control and 3.0 in AT04A treated mice.

AT04A reduces vascular inflammation

Since atherosclerosis is not only a lipid-driven disease, but also a chronic low-grade inflammatory disease of the vessel wall, the number of monocytes adhering to the activated endothelium, the expression of the adhesion molecule ICAM-1 in the endothelium as well as the expression of the caspase-1-activating inflammasome protein NLRP3 in macrophages was determined in the diseased aortic root area. In the control group 2.3 monocytes per cross-section were counted, compared with 1.4 in the AT04A-treated group (-38% , $p=0.014$) (**Figure 6A**). The reduction in monocyte adherence was reinforced by a similar significant decrease in ICAM-1 expression in endothelial cells upon AT04A vaccination (-37% , $p=0.018$) (**Figure 6B and C**). Since NLRP3 is predominantly expressed by myeloid cells, we determined the amount of NLRP3 expression in the macrophage area and a decrease by 68% in AT04A compared with control treated mice was observed (0.3 ± 0.2 vs. 0.9 ± 0.8 , $p=0.06$) (**Figure 6D and E**). Together, these findings suggest that AT04A immunization results in a reduction of activated endothelial cells and pro-inflammatory macrophages.

AT04A decreases inflammatory biomarkers

In order to assess system inflammation upon high-fat diet-induced atherosclerosis in APOE*3-Leiden.CETP mice, a panel of plasma inflammatory markers was analysed. The liver-derived inflammation marker SAA, reported to be elevated in diet-induced atherosclerosis in mice (25), showed a significant reduction in anti-PCSK9 AT04A treated mice at $t=4$ weeks (-21% ; $p=0.007$) and $t=8$ weeks (-28% ; $p<0.001$) relative to the negative control, but was not significantly affected later in the study (**Figure 7**). Myriad RBM's mouse inflammation multi-analyte profile represents a comprehensive and quantitative analysis of multiple inflammatory biomarkers and pathways including cytokines, chemokines, and growth factors. In plasma obtained 18 weeks after the start of WTD, 4 markers, namely macrophage inflammatory protein-1 β (MIP-1 β /CCL4), macrophage-derived chemokine (MDC/CCL22), the cytokine stem cell factor (SCF), and vascular endothelial growth factor

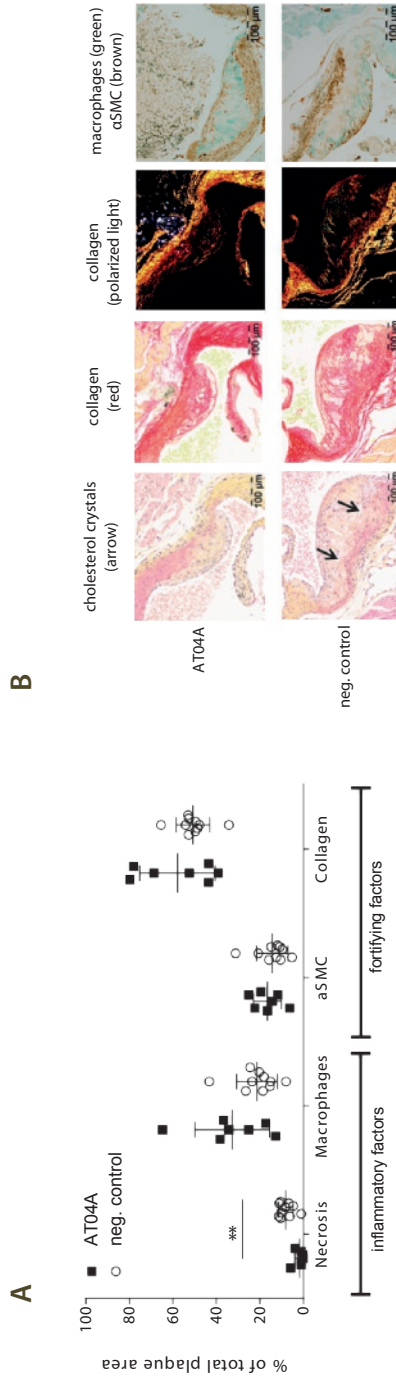


Figure 5 AT04A immunization reduces necrotic content and cholesterol crystals in plaques. Necrotic and macrophage content as unstable factors, and aSMCs and collagen as stable factors, were determined in the severe (type IV–V) lesions, and expressed as percentage of total plaque area. 10 mice in the control and 7 in vaccine group had severe lesions and were included in this analysis (A). Representative images of HPS staining, Sirius red staining for collagen, collagen expression under polarized light and double immunostaining with α -actin for aSMCs (brown) and MAC-3 for macrophages (green) (B). The arrows depict necrotic areas, including cholesterol clefts. A Mann–Whitney U test was used for statistical analysis. Data are presented as group means \pm SD (n=7–10). **p<0.01. Abbreviations: aSMCs, α -smooth-muscle-cells; MAC-3, purified anti-mouse CD107b.

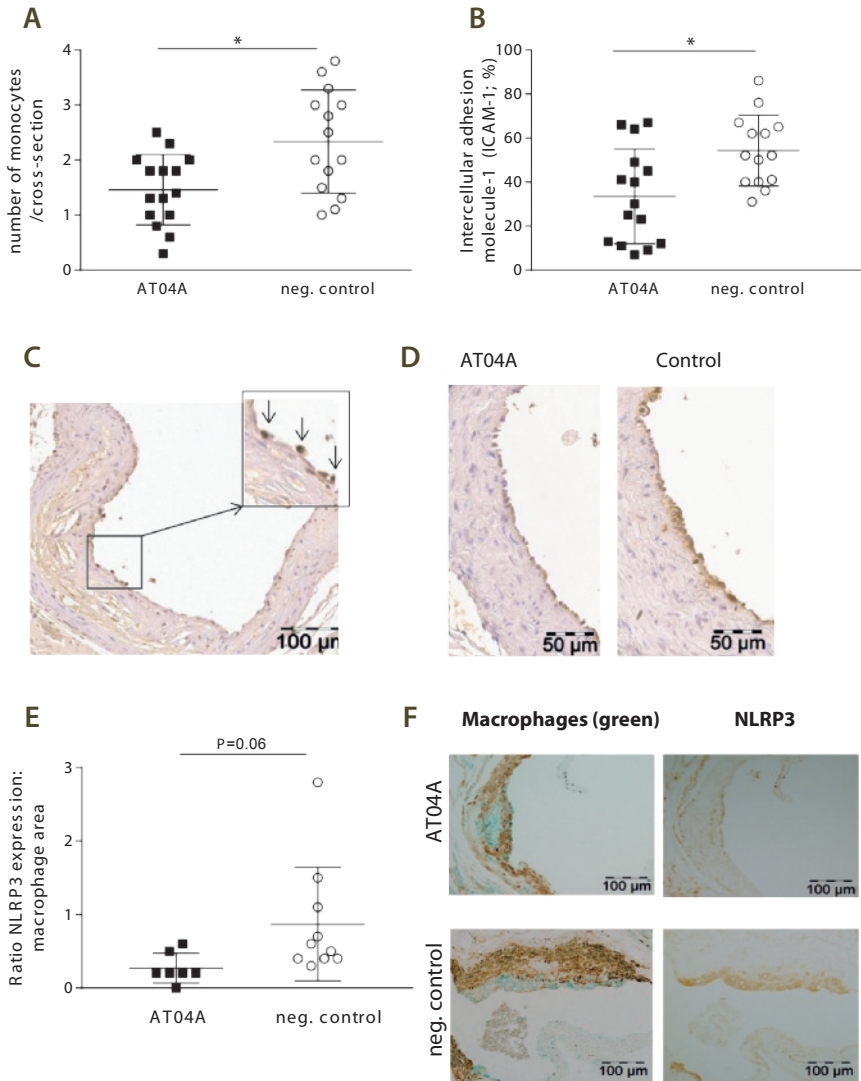


Figure 6 AT04A immunization reduces the number of monocytes adhering to the endothelium, ICAM-1 expression and NLRP3 expression in macrophages. (A) The number of monocytes was counted per cross-section after staining with AIA 31240 and (B) endothelial ICAM-1 was determined as percentage of the endothelial surface in the cross-sections and (C-D) a representative image is shown. (E) NLRP3 expression was determined in the severe (type IV–V) lesions after immune staining with anti-NLRP3 antibodies and the amount of NLRP3 expression in the macrophage area was calculated. (F) Photographs of atherosclerotic plaques stained with α SMC (brown), macrophages (green) and NLRP3 (brown). Data are presented as means \pm SD ($n=14-15$ in A–B, $n=7-10$ in D). * $p<0.05$.

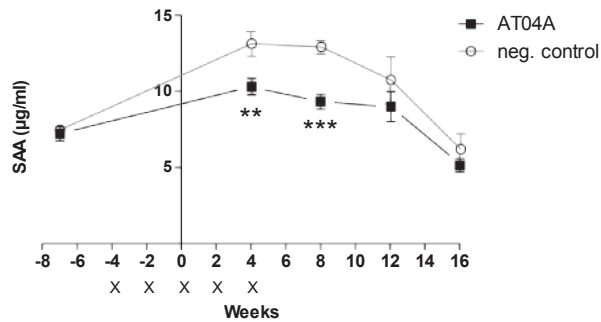


Figure 7 The liver-derived inflammation marker SAA was measured over time. SAA concentrations were assessed at t=-7, 4, 8, 12 and 16 weeks after the initiation of WTD, 0.1% cholesterol. A Mann-Whitney U-test was used for statistical analysis. Data are presented as group means \pm SD (n=15). **p<0.01, ***p<0.001. Abbreviations: SAA serum A amyloid; WTD, Western type diet.

A (VEGF-A), showed a significant reduction in AT04A compared with control-immunized mice whereas the decreases in SCF was most pronounced (**Table 1**). Furthermore, the macrophage colony stimulating factor 1 (M-CSF-1) and monocyte chemotactic protein 5 (MCP-5/CCL12) tended to be decreased (**Table 1**). A Spearman's test showed a strong, positive correlation between TC and SCF ($R^2=0.65$; $p<0.001$) and VEGF-A ($R^2=0.58$; $p=0.001$), but not for TC and MIP-1 β /CCL4 ($R^2=0.02$; $p=0.93$) and MDC/CCL22 ($R^2=0.26$; $p=0.18$).

Safety aspects

No effects on viability, body weight, or food intake were found in the AT04A-compared with control-immunized mice (**Table 2**). Liver weight, which is considered as a sensitive indicator for toxicity, was not affected by AT04A vaccine-treated mice (**Table 2**). Moreover, plasma pooled per group showed no apparent differences in AST and ALT values as markers of hepatocellular damage (**Table 2**). Other safety aspects such as absence of target-specific T-cell response and absence of cross-reactivity of induced antibodies with other endogenous proteins have been previously reported by Galabova et al (20).

Table 1 Quantitative analyses of multiple inflammatory biomarkers using the Myriad RBM mouse inflammation multi-analyte profile

Marker*1	Neg. control		AT04A		AT04A vs control
	Mean (n=15)	SD	Mean (n=15)	SD	p-value
SCF (pg/mL)	1097.0	235.1	760.6	196.9	0.0002
VEGF-A (pg/mL)	549.1	66.5	246.8	75.5	0,0304
MIP-1 beta (pg/mL)	229.6	60.6	174.0	70.9	0.0319
MDC (pg/mL)	395.5	132.9	308.5	56.5	0.0397
M-CSF-1 (ng/mL)	9.3	1.6	8.2	1.0	0.0533
MCP-5 (pg/mL)	19.9	5.3	16.4	5.1	0.0552
IP-10 (ng/mL)	105.9	22.7	92.0	26.7	0.0848
MIP-3 beta (pg/mL)	3.6	1.3	3.0	0.8	0.1190
TIMP-1 Mouse (ng/mL)	1.7	0.8	1.4	0.3	0.2101
IL-18 (ng/mL)	34.5	12.4	35.1	7.9	0.2576
EGF Mouse (pg/mL)	189.7	30.1	179.7	17.2	0.3605
MCP-1 (pg/mL)	117.7	40.3	112.4	32.3	0.4427
MIP-1 alpha (pg/mL)	7.5	0.9	7.7	2.7	0.5066
MCP-3 (pg/mL)	215.7	83.7	207.2	37.1	0.6186
Thrombopoietin (ng/mL)	48.2	6.8	43.4	12.2	0,7400
IL-1 beta (ng/mL)	12.7	2.4	12.0	2.1	0.7996
Eotaxin (pg/mL)	434.0	90.8	450.7	138.0	0.8034

*1 the inflammatory markers FGF-9, FGF-basic, GM-CSF, KC/GRO, INF-gamma, IL-1 alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-11, IL-12, IL-17A, LIF, MIP-2, OSM, TNF-alpha were measured, but were either below the limit of detection or the limit of quantification.

Table 2 Safety aspects of PCSK9 immunisation

	Body weight	Food intake	Liver weight	ALT	AST
	gram	gram/mouse/day	gram	U/L	U/L
Baseline	22.5 ± 1.2	3.4 ± 0.1	NA	80	141
Control	25.2 ± 2.7	2.7 ± 0.2	1.5 ± 0.4	69	251
AT04A	25.6 ± 2.8	2.5 ± 0.1	1.4 ± 0.3	74	289

No differences in body weight, food intake (per cage), liver weight, plasma ALT (pooled per group), and plasma AST (pooled per group) between the control and AT04A group at sacrifice (t=18 weeks). Data are presented as group means ± SD (n=14-15). Abbreviations: alanine transaminase (ALT), aspartate transaminase (AST).

Discussion

The present study shows that active immunization against PCSK9 with the so-called AT04A peptide-based vaccine elicits antibodies that effectively bind and remove PCSK9 from the circulation and reduce circulating TC, (V)LDL-C, TG, and biomarkers of inflammation, which is accompanied by reduction of vascular inflammation and atherosclerotic lesions and plaques in the aortas of a mouse model of atherosclerosis.

PCSK9 plays a fundamental role in LDL metabolism through the binding and degradation of LDLR. With respect to LDL-C as one of the major risk factors positively correlated with premature development of CVD (1), PCSK9 inhibition is supposed to reduce atherosclerotic events. Recently, the human PCSK9 mAbs evolocumab and alirocumab reached FDA approval and were recommended in the 2016 ESC/EAS Guidelines (14) as second line treatment for high LDL-C for adults whose cholesterol is not adequately controlled by diet or statin treatment. However, whereas both mAbs reduced the incidence of cardiovascular events in a post-hoc exploratory analysis, trial results whether anti-PCSK9 mAb therapy actually reduces atherosclerotic burden and CVD events is still under investigation (13).

The main limitation for mAb therapies with broader applications certainly is that their use for chronic disorders is cost-prohibitive. Moreover, it has been reported that a substantial proportion of patients undergoing a sustained treatment with mAbs lose responsiveness over time due to the induction of anti-drug antibodies (26). Thus, an appealing new alternative to mAb-based therapies is the active immunization against self-antigens involved in chronic disorders (27,28).

Previously, we showed that our AFFITOPE®-based anti-PCSK9 active immunization approach is able to induce a strong humoral immune response against PCSK9, which persisted for up to one year in vaccinated mice (20). Furthermore, TC concentration was reduced by up to 30% and LDL-C up to 50% in anti-PCSK9 immunized compared with control mice. Immunization against PCSK9 has also been tested by others using different approaches (29). In the first approach, human recombinant PCSK9, along with a DNA oligonucleotide as an adjuvant, was tested and found to induce an approximately 40% reduction of LDL-C in mice (30). Crossey et al.(29) designed a human PCSK9 vaccine where PCSK9 peptides are displayed at high valency on the surface of a bacteriophage virus-like particle, resulting in a ~55% reduction of TC relative to controls.

However, the present study is the first to show that active immunization against PCSK9 does not only reduce TC and (V)LDL-C, but also has an impact on systemic and vascular inflammation and atherosclerotic development in a mouse model of diet-induced atherosclerosis. Vaccination with AT04A, which led to a reduction of TC exposure by 53%, had a strong impact on atherosclerotic development. The AT04A vaccine was able to induce a specific immune response against PCSK9. In contrast to previously reported studies where an anti-PCSK9 peptide vaccine (20) as well as monoclonal anti-PCSK9 antibodies (19) rather stabilized the target protein and increased the murine plasma PCSK9

level, AT04A-treated APOE*3-Leiden.CETP mice showed a reduction of the target protein exposure during the study by 48%. Already after the second immunization with AT04A vaccine the induced anti-PCSK9 antibody concentration was sufficient to significantly decrease TC in APOE*3-Leiden.CETP mice on normal chow (t=0 weeks). Four weeks after the onset of WTD the anti-PCSK9 titre in AT04A-vaccinated mice rose to approximately 1/4000, corresponding to an antibody concentration of ~200 µg/mL, and resulted in a TC decrease of 63% compared with control ($p < 0.001$). Although the mean anti-PCSK9 titre reached its maximum in week 8, no additional TC lowering was found at this timepoint, indicating a plateau effect after 4 weeks with maximally retained efficacy during the study. Due to this sustained TC reduction, anti-PCSK9 vaccine-treated mice showed significantly less lesion areas (-64%, $p = 0.004$) and severity (-64%, $p = 0.051$) in the aortic root compared with control. Consistent with our data, Kühnast et al. (18) reported a dose-dependent decrease in atherosclerosis development in APOE*3-Leiden.CETP mice treated with the human PCSK9 monoclonal antibody alirocumab. Notably, the AT04A vaccination was applied as a stand-alone therapy and thus, future evaluations are required to test whether these effects are still ensured on a statin background treatment.

For many years, atherosclerosis was considered to be mainly a lipid-driven disease caused by the continuous accumulation of cholesterol in the arterial intima. However, it is increasingly recognized that atherosclerosis is predominately a chronic low-grade inflammatory disease of the vessel wall with an interplay of humoral, cellular, and locally produced pro-inflammatory factors (31,32). One of the initial stages of atherosclerotic disease is endothelial cell activation and the recruitment of inflammatory cells to the vessel wall (31,32). Multi-analyte profiling of inflammatory markers showed a reduction of several cytokines with chemotactic activity for monocytes by AT04A vaccine (**Table 1**) which is in line with the reduced expression of endothelial ICAM-1 and adherence of monocytes to the vessel wall. The decreased levels of M-CSF-1 and VEGF-A, which activate endothelial cells and stimulate monocyte/macrophage migration, may have additionally contributed to the reduction in lesion number and size.

Interestingly, despite the reduced monocyte adherence, the macrophage content was not affected by AT04A immunization. A potential explanation is that the amount of macrophages is similar but that the macrophages in the control group are more activated and overloaded with cholesterol which may result in damage and necrosis. The necrotic core is a major characteristic of advanced atherosclerotic lesions and plays an important role in both their progression to a hazardous state and their vulnerability (33). To test this hypothesis, we measured the necrotic core and NLRP3 as marker of macrophage activation. Morphological analysis indicated that vaccination with AT04A strongly decreased the necrotic core content, including cholesterol clefts, by 77%. Overload of lipid material in the vessel wall leads to apoptosis and necrosis of foam cells (34), and to deposition of cholesterol crystals (35,36), which stimulate caspase-1-activating NLRP3 inflammasomes and aggravate the inflammatory response (35,37). Indeed NLRP3

expression in the macrophage area was three-fold increased in the control mice as compared to the AT04-treated mice, which substantiates this hypothesis. Collectively, these data indicate that immunization with AT04A diminishes the formation of a necrotic core and macrophage inflammation, which may have contributed to the reduced inflammatory response in the vessel wall and attenuation of atherosclerotic development.

Little is known about the pro-inflammatory role of PCSK9. Inflammatory cytokines were measured in the plasma from septic shock patients carrying either a PCSK9 LOF allele or a PCSK9 GOF allele. Significantly lower plasma cytokine concentrations in patients carrying a LOF allele were found (38). These results mirrored the data obtained from PCSK9 knockout mice, which displayed decreases in inflammatory cytokine production in response to LPS (38). It was also reported that PCSK9 of macrophage origin directly promotes lesion inflammation in mice, independently of systemic lipid changes. PCSK9 accumulated in the artery wall induced infiltration of pro-inflammatory Ly6C^{High} monocytes into the atherosclerotic lesion, indicating a local effect of human PCSK9 on atherosclerotic lesion composition (39). Thus, lowering of plasma PCSK9 levels per se may also have direct local effects on vascular inflammation and atherosclerotic plaque formation. Unfortunately, we were not able to examine the local effects of PCSK9 in aortic plaques due to known technical limitations (40).

The current challenge for the prevention of atherosclerotic events is certainly the side-effect free lowering of lifetime LDL-C exposure, thus preventing chronic low-grade inflammatory disease of the vessel walls. The association between the change in relative risk for CHD and the absolute change in LDL-C levels over lifetime due to genetic variation has recently been emphasized (41).

The AT04A anti-PCSK9 vaccine would be an ideal therapeutic agent to fulfill these requirements for long-term LDL-C management, because of its sustained efficacy and cost-effective application, accompanied by anti-inflammatory effects. AT04A is currently being tested in a phase I clinical trial.

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Disclosures

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References

1. Yusuf S, Hawken S, Ounpuu S, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* (London, England). 2004 Sep;364(9438):937–52.
2. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science*. 1986 Apr;232(4746):34–47.
3. Ishibashi S, Brown MS, Goldstein JL, et al. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest*. 1993 Aug;92(2):883–93.
4. Poirier S, Mayer G, Benjannet S, et al. The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. *J Biol Chem*. 2008 Jan;283(4):2363–72.
5. Zhang D-W, Lagace TA, Garuti R, et al. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *J Biol Chem*. 2007 Jun;282(25):18602–12.
6. Ference BA, Majeed F, Penumetcha R, et al. Effect of naturally random allocation to lower low-density lipoprotein cholesterol on the risk of coronary heart disease mediated by polymorphisms in NPC1L1, HMGCR, or both: a 2 x 2 factorial Mendelian randomization study. *J Am Coll Cardiol*. 2015 Apr;65(15):1552–61.
7. Law MR, Wald NJ, Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. *BMJ*. 2003 Jun;326(7404):1423.
8. Chong PH, Bachenheimer BS. Current, new and future treatments in dyslipidaemia and atherosclerosis. *Drugs*. 2000 Jul;60(1):55–93.
9. Waters DD, Brotons C, Chiang C-W, et al. Lipid treatment assessment project 2: a multinational survey to evaluate the proportion of patients achieving low-density lipoprotein cholesterol goals. *Circulation*. 2009 Jul;120(1):28–34.
10. Beltowski J, Wojcicka G, Jamroz-Wisniewska A. Adverse effects of statins - mechanisms and consequences. *Curr Drug Saf*. 2009 Sep;4(3):209–28.
11. Taylor BA, Thompson PD. Statins and Their Effect on PCSK9-Impact and Clinical Relevance. *Curr Atheroscler Rep*. 2016 Aug;18(8):46.
12. Robinson JG, Farnier M, Krempf M, et al. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. *N Engl J Med*. 2015 Apr;372(16):1489–99.
13. Sabatine MS, Giugliano RP, Wiviott SD, et al. Efficacy and safety of evolocumab in reducing lipids and cardiovascular events. *N Engl J Med*. 2015 Apr;372(16):1500–9.
14. Catapano AL, Graham I, De Backer G, et al. 2016 ESC/EAS Guidelines for the Management of Dyslipidaemias. *Eur Heart J*. 2016 Oct;37(39):2999–3058.
15. Lloyd-Jones DM, Morris PB, Ballantyne CM, et al. 2016 ACC Expert Consensus Decision Pathway on the Role of Non-Statins Therapies for LDL-Cholesterol Lowering in the Management of Atherosclerotic Cardiovascular Disease Risk: A Report of the American College of Cardiology Task Force on Clinical Expert Cons. *J Am Coll Cardiol*. 2016 Jul;68(1):92–125.
16. Westerterp M, van der Hoogt CC, de Haan W, et al. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arterioscler Thromb Vasc Biol*. 2006 Nov;26(11):2552–9.
17. de Haan W, van der Hoogt CC, Westerterp M, et al. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008 Mar;197(1):57–63.
18. Kuhnast S, van der Hoorn JWA, Pieterman EJ, et al. Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin. *J Lipid Res*. 2014 Oct;55(10):2103–12.
19. Ason B, van der Hoorn JWA, Chan J, et al. PCSK9 inhibition fails to alter hepatic LDLR, circulating cholesterol, and atherosclerosis in the absence of ApoE. *J Lipid Res*. 2014 Nov;55(11):2370–9.
20. Galabova G, Brunner S, Winsauer G, et al. Peptide-based anti-PCSK9 vaccines - an approach for long-term LDLc management. *PLoS One*. 2014;9(12):e114469.
21. Schneeberger A, Mandler M, Mattner F, et al. AFFITOME(R) technology in neurodegenerative diseases: the doubling advantage. *Hum Vaccin*. 2010 Nov;6(11):948–52.

22. Kuhnast S, van der Hoorn JWA, van den Hoek AM, et al. Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden.CETP transgenic mice with or without treatment with atorvastatin. *J Hypertens*. 2012 Jan;30(1):107–16.
23. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*. 1995 Sep;92(5):1355–74.
24. Kooistra T, Verschuren L, de Vries-van der Weij J, et al. Fenofibrate reduces atherogenesis in ApoE*3Leiden mice: evidence for multiple antiatherogenic effects besides lowering plasma cholesterol. *Arterioscler Thromb Vasc Biol*. 2006 Oct;26(10):2322–30.
25. Lewis KE, Kirk EA, McDonald TO, et al. Increase in serum amyloid A evoked by dietary cholesterol is associated with increased atherosclerosis in mice. *Circulation*. 2004 Aug;110(5):540–5.
26. Bartelds GM, Krieckaert CLM, Nurmohamed MT, et al. Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up. *JAMA*. 2011 Apr;305(14):1460–8.
27. Bachmann MF, Dyer MR. Therapeutic vaccination for chronic diseases: a new class of drugs in sight. *Nat Rev Drug Discov*. 2004 Jan;3(1):81–8.
28. Bachmann MF, Whitehead P. Active immunotherapy for chronic diseases. *Vaccine*. 2013 Apr;31(14):1777–84.
29. Crossley E, Amar MJA, Sampson M, et al. A cholesterol-lowering VLP vaccine that targets PCSK9. *Vaccine*. 2015 Oct;33(43):5747–55.
30. Fattori E, Cappelletti M, Lo Surdo P, et al. Immunization against proprotein convertase subtilisin-like/kexin type 9 lowers plasma LDL-cholesterol levels in mice. *J Lipid Res*. 2012 Aug;53(8):1654–61.
31. Libby P. Inflammation in atherosclerosis. *Nature*. 2002 Dec;420(6917):868–74.
32. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999 Jan;340(2):115–26.
33. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. *N Engl J Med*. 2013 May;368(21):2004–13.
34. Tabas I. Free cholesterol-induced cytotoxicity a possible contributing factor to macrophage foam cell necrosis in advanced atherosclerotic lesions. *Trends Cardiovasc Med*. 1997 Oct;7(7):256–63.
35. Abela GS. Cholesterol crystals piercing the arterial plaque and intima trigger local and systemic inflammation. *J Clin Lipidol*. 2010;4(3):156–64.
36. van De Poll SW, Romer TJ, Volger OL, et al. Raman spectroscopic evaluation of the effects of diet and lipid-lowering therapy on atherosclerotic plaque development in mice. *Arterioscler Thromb Vasc Biol*. 2001 Oct;21(10):1630–5.
37. Duewell P, Kono H, Rayner KJ, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. 2010 Apr;464(7293):1357–61.
38. Walley KR, Thain KR, Russell JA, et al. PCSK9 is a critical regulator of the innate immune response and septic shock outcome. *Sci Transl Med*. 2014 Oct;6(258):258ra143.
39. Giunzioni I, Tavori H, Covarrubias R, et al. Local effects of human PCSK9 on the atherosclerotic lesion. *J Pathol*. 2016 Jan;238(1):52–62.
40. Liu M, Wu G, Baysarowich J, et al. PCSK9 is not involved in the degradation of LDL receptors and BACE1 in the adult mouse brain. *J Lipid Res*. 2010 Sep;51(9):2611–8.
41. Wadhera RK, Steen DL, Khan I, et al. A review of low-density lipoprotein cholesterol, treatment strategies, and its impact on cardiovascular disease morbidity and mortality. *J Clin Lipidol*. 2016;10(3):472–89.