Vaccination against atherosclerosis

Es, T. van

Chapter 3

Vaccination against interleukin-17 attenuates atherosclerosis in LDL receptor deficient mice

Thomas van Es, Arnaud D. Hauer, Gijs H.M. van Puijvelde, Eva J.A. van Wanrooij, Paula de Vos, Niels Peterse, Theo J.C. van Berkel and Johan Kuiper

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ABSTRACT

Interleukin-17 (IL-17) is a T cell-derived pro-inflammatory cytokine that is linked to autoimmune diseases. IL-17 exhibits pleiotropic effects on atheroma-associated cell types and induces the secretion of pro-inflammatory cytokines and chemokines.

In this study, we investigated the effect of IL-17 blockade on the initiation of atherosclerosis by vaccination against IL-17. A plasmid (pcDNA3.1) encoding IL-17 and the dominant T helper cell HEL epitope was used to vaccinate LDL receptor deficient ($LDLr^{-/-}$) mice prior to induction of atherosclerosis by feeding a Western-type diet and collar placement.

Functional blockade of IL-17 upon vaccination was demonstrated by a reduced induction of serum IL-6 levels after administration of IL-17. DNA vaccination with the HEL-IL-17 plasmid resulted in a 90.2% reduction in lesion size in the carotid artery ($P<0.01$) and 59% reduction in the aortic root ($P<0.05$). This reduction was dependent on the HEL sequence to break tolerance against endogenous IL-17 during vaccination.

Neutralizing the IL-17 production by vaccination forms a promising approach to inhibit atherosclerotic lesion development.

INTRODUCTION

Cardiovascular disease (CVD), with atherosclerosis as the main underlying pathology is the leading cause of death in the Western world and atherosclerotic lesion initiation and progression has been shown to be associated with a chronic inflammatory response.[1] Macrophages and T cells are present within the intima in early stages of atherogenesis and play a crucial role in the initiation and subsequently in the progression of the atherosclerotic plaque.[1, 2] $CD4^+$ effector T cells are the major T cell subset in atherosclerotic lesions and T cells are involved in atherosclerotic lesion formation.[3]

Traditionally, it was postulated that activated T cells differentiate into either T helper 1 (Th1) or Th2 cells. Th1 cells produce mainly pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-α, whereas Th2 cells predominantly produce anti-inflammatory cytokines, such as IL-4, IL-5 and IL-10.[4] A disturbed balance between Th1 and Th2 cells is thought to be responsible for the immunopathological conditions in several autoimmune diseases, such as atherosclerosis.[5]

More recently, a new subset of T helper cells with a T memory cell phenotype has been identified, the T helper 17 (Th17) cell and this cell type is associated with several (auto)immune diseases.[6] Although the loss of IFN-γ receptor appears to be protective in atherosclerosis[7], this newly identified Th cell may also be involved in the development of atherosclerosis.

The differentiation of Th17 cells is subject to debate. TGF-β, IL-1β and IL-6 in the presence of the appropriate antigen are ascribed to participate in the differentiation and initiation of Th17 cells.[8, 9] Th1 and Th2 cytokines, such as IFN-γ and IL-4 may antagonize the Th17 development, but fully differentiated Th17 are unresponsive to these cytokines.[10] IL-17 production is the hallmark of the Th17 cell and IL-17 has been identified in several autoimmune diseases, such as Experimental Autoimmune Encephalomyelitis (EAE) and rheumatoid arthritis to have a negative effect on disease progression.[11, 12] IL-17 exhibits pleiotropic biological effects on various cell types , such as endothelial cells, vascular smooth muscle cells, and macrophages that are associated with atherosclerosis.[13, 14] In these cells IL-17 can induce a number of pro-inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs), including IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1) and MMP-9.[15, 16]

However, the role of IL-17 is not yet established in atherosclerosis. Therefore, we vaccinated mice against endogenous IL-17 via DNA vaccination in order to neutralize endogenous IL-17. To break T cell tolerance for IL-17, a specific T helper cell epitope (HEL) was coupled to IL-17.[17] Vaccination against HEL-IL-17 established a strong protection against atherosclerosis, which may indicate that IL-

17 is important in atherosclerosis and therapies to neutralize the action of IL-17 may provide a new approach to treat atherosclerosis.

METHODS

ANIMALS

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. LDLr$^{-/-}$ mice on a C57/Bl6 background were from Jacksons Laboratory and were kept under standard laboratory conditions and food and water were administered *ad libitum*.

VACCINE PREPARATION

Genes coding for the antigen were cloned into pcDNA3.1(-) (Invitrogen, The Netherlands). cDNA coding for murine IL-17 was obtained by PCR on stimulated murine spleen cells. The following primers were used: IL-17, 5'- GAT CAG GAC GCG CAA ACA- 3' (forward) and IL-17, 5'- GGG TTT CTT AGG GGT CAG- 3' (reverse). The IL-17 PCR product was cloned into a pcDNA3.1(-) plasmid. The HEL sequence was derived from the amino acid sequence 81-95 of hen egg-white lysozyme and was cloned upstream of IL-17 with *XbaI*.[21] The following HEL sequence was used: 5'- CTA GAA TGT CAG CCC TGC TGA GCT CAG ACA TAA CAG CGA GCG TGA ACT GCG CGC CT- 3'.

TESTING THE CONSTRUCT

Expression of constructs was tested by transient transfection of COS7 cells with the plasmid. COS7 cells were transfected with pcDNA3.1-HEL-IL-17 and pcDNA3.1 using Exgen500 according to manufacturer's protocol (Fermentas, Germany). Supernatant was collected 24 and 48 hours after transfection. Expression of IL-17 was determined with a specific murine IL-17 ELISA according to manufacturer's protocol (BD Bioscience, The Netherlands). To confirm the functional blockade of IL-17 after vaccination we performed an experiment in which we made use of the fact that IL-17 is able to induce IL-6 production. In a separate experiment LDLr$^{-/-}$ mice were vaccinated against IL-17 or control vaccinated. Three days after the last vaccination, 0.2 µg of murine IL-17 (1 µg/ml) was injected intravenously. Four hours after the injection of IL-17, blood was collected and serum IL-6 levels were quantified with a mouse IL-6 ELISA (eBioscience, Belgium).

CELL CULTURE

The murine monocyte/macrophage cell line (RAW 246.7) and an endothelial cell line (H5V) were cultured in DMEM supplemented with 10% FCS, 2 mmol/L glutamin, 0.1 U/L penicillin, and 100 mg/L streptomycin. Primary vascular smooth muscle cells were isolated from murine aorta and cultured as described previously.[22]

IN VITRO ASSESSMENT OF IL-17

0.5 ml of cells were added to a 24-wells plate ($2.5x10^5$ RAW cells/ml, $1.0x10^5$ cells for H5V and vSMC). Unless stated otherwise, 100 ng/ml recombinant IL-17 was added to the culture medium and culture medium alone served as a control. Cells were incubated for 24 hours, and thereafter the supernatant was used for ELISA. All experiments were performed in triplicate. Where indicated cells were used for qPCR.

QUANTATIVE PCR ASSAYS

Total RNA was isolated using guanidium isothiocyanate (GTC) method and reverse transcribed to cDNA (RevertAid™ M-MuLV reverse transcriptase, Fermentas). Gene expression was analyzed with an ABI PRISM 7700 (Applied Biosystems, Foster city, CA) using SYBR Green technology. Primer pairs as described in table 3.1 were used to quantify IL-17, ADAM-15 and MMP-9 gene expression. As a reference gene hypoxanthine phosphoribosyl transferase (HPRT) was used. The relative gene expression was calculated by subtracting the threshold of the target gene from the reference gene and raising 2 to the power of this difference.

TABLE 3.1: SEQUENCE OF PRIMERS. Primer sequences for the genes analyzed with qPCR.

GENE	FORWARD PRIMER	REVERSE PRIMER
IL-17	5′-CCAGGGAGAGCTTCATCTGTGT-3′	5′-AAGTCCTTGGCCTCAGTGTTTG-3′
ADAM-15	5′-TGTGGCTTCCCAGATGAATG-3′	5′-GTTTTGACAACAGGGTCCATCA-3′
MMP-9	5′-CTGGCGTGTGAGTTTCCAAAAT-3′	5′-TGCACGGTTGAAGCAAAGAA-3′
HPRT	5′-TTG CTCGAGATGTCATGAAGGA-3′	5′-AGCAGGTCAGCAAAGAACTTATAG-3′

VACCINATION AND INDUCTION OF ATHEROSCLEROSIS

The DNA vaccine was isolated with an Endofree plasmid Giga kit (Qiagen, The Netherlands). Male low-density lipoprotein receptor deficient (LDLr$^{-/-}$) mice, 10-12 weeks old, were used for the *i.m.* vaccination. Three days prior to the first vaccination, mice received an *i.m.* bilateral Cardiotoxin I (Sigma, USA) injection, 10 µM, 50 µl per muscle. Mice were vaccinated by a total of three bilateral *i.m.* injections of 100 µg plasmid in 100 µl PBS, 50 µl per muscle, with two-week intervals (N=15 each group). Immediately after the last vaccination, mice were put on a Western type diet, containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, UK). After two weeks of Western type diet feeding, atherosclerosis was induced within the carotid arteries by bilateral perivascular collar placement, as described previously.[23] During the experiment, plasma samples were obtained by tail vein bleeding. Total cholesterol levels were quantified during the experiment using an enzymatic procedure (Roche Diagnostics, Germany) using Precipath as an internal standard.

TISSUE HARVESTING

Six weeks after collar placement, carotid arteries were obtained after *in situ* perfusion for 15 minutes with Formalfixx. Carotids were embedded in OCT compound (Sakura Finetek, The Netherlands), snap-frozen in liquid nitrogen and stored at -20°C until further use. Transverse 5 µm cryosections were prepared in a proximal direction from the carotid bifurcation and were mounted on a parallel series of slides. For analysis of atherosclerosis at the site of the aortic semilunar valves, 10 µm transverse cryosections were made of the aortic root as previously described.[23, 24]

HISTOLOGICAL ANALYSIS AND MORPHOMETRY

Cryosections were routinely stained with hematoxylin (Sigma Aldrich, Zwijndrecht, The Netherlands) and eosin (Merck Diagnostica, Germany). Corresponding sections were stained for lipids by Oil red O staining. Hematoxylin-eosin stained sections of carotid arteries were used for morphometric analysis of atherosclerotic lesions. Each vessel was assessed ~0.5 mm proximal to the collar, and the site of maximal stenosis was used for morphometric assessment. Atherosclerosis in the aortic root was quantified with Oil red O stained sections of plaques developed in the region of the aortic semilunar valves, as previously described.[24]

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. The two-tailed student's t-test was used to compare individual groups of mice or cells. Mann-Witney test was applied to compare not normally distributed values. *P* values of <0.05 were considered significant.

RESULTS

EVALUATING THE EXPRESSION OF IL-17 IN ATHEROSCLEROSIS

To define the role of IL-17 in atherosclerosis we determined the expression of IL-17 in atherosclerosis prone mice, fed a Western type diet (WTD, 0.25% cholesterol). Starting from three weeks after the start of the Western type diet we observed a significant 2-fold increase in the IL-17 gene expression in the spleen (Figure 3.1; 1 ± 0.22 versus 2.20 ± 0.32, *P*<0.05). Subsequently, the expression of IL-17 steadily increased, leading to a more than 3-fold increase at week 6 of Western type diet feeding (Figure 3.1; 3.18 ± 0.30, *P*<0.01).

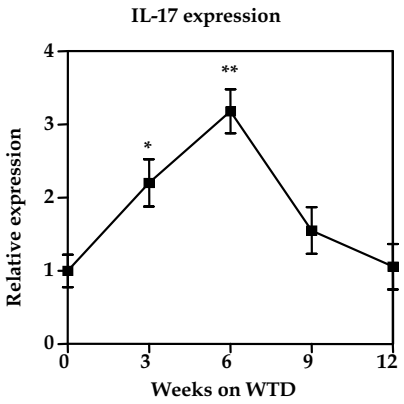

FIGURE 3.1: MRNA EXPRESSION OF IL-17 IN RESPONSE TO A WESTERN-TYPE DIET.
At different time points spleen samples were obtained and mRNA was isolated using the guanidium-isothiocyanate method and IL-17 expression was determined. Expression of IL-17 is expressed relative to HPRT and subsequently related to the expression of mice on chow diet. * *P*< 0.05, ** *P*<0.001

Thereafter, the expression of IL-17 rapidly decreased and declined to basal levels at 12 weeks of Western-type diet, indicating specifically the induction of IL-17 during the initiation of atherosclerosis. Next, we studied the effect of IL-17 on different athero-associated cell types in vitro. A macrophage cell line (RAW264.7), an endothelial cell line (H5V) and primary mouse vascular smooth muscle cells were stimulated with IL-17 and the production of IL-6 and TNF-α was determined.

RAW cells responded with a strong increase in TNF-α production (Figure 3.2A; 81 ± 10 pg/ml versus 372 ± 49 pg/ml, $P<0.01$), whereas H5V did not respond to IL-17 (data not shown). Isolated VSMC responded to IL-17 with a significant increase in IL-6 production (Figure 3.2B; 65.6 ± 4.1 pg/ml versus 293.4 ± 7.9 pg/ml, $P<0.001$).

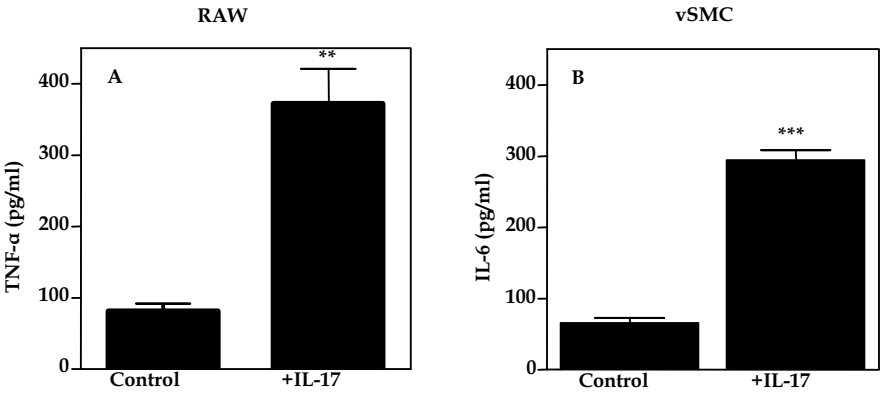

FIGURE 3.2: STIMULATION OF RAW AND VSMCS WITH IL-17.
RAW and vSMCs were stimulated with 100 ng/ml IL-17 and after 24 hours the supernatant was collected and analyzed with a TNF-α (A) or IL-6 (B) ELISA. $^{**}P<0.01$, $^{***}P<0.001$

Since, VSMC play an important role in the production of MMPs,[25, 26] thereby contributing to remodeling and stability of the plaque, we measured the expression of MMP-9 and ADAM metallopeptidase domain 15 (ADAM-15) by VSMCs. IL-17 specifically enhanced MMP-9 expression in vSMCs significantly but did not affect ADAM15 expression (Figure 3.3).

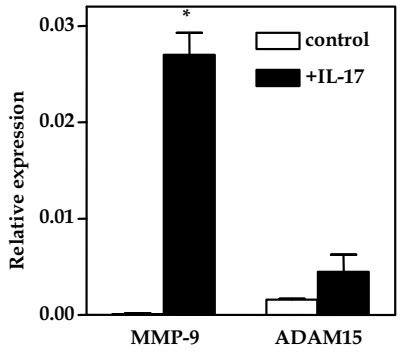

FIGURE 3.3: EFFECT OF IL-17 ON METALLOPROTEINASE EXPRESSION IN VSMCS.
vSMCs were stimulated with 100 ng/ml IL-17 and incubated for 24 hours. mRNA was isolated using the guanidium-isothiocyanate method. Expression of MMP-9 and ADAM15 are shown relative to HPRT. * $P< 0.05$

CONSTRUCTION OF A HEL-IL-17 DNA VACCINE

We cloned the IL-17 coding sequence of murine IL-17A into the eukaryotic expression vector, pcDNA3.1. The coding sequence is preceded by the Th cell epitope HEL, to enhance the breaking of tolerance against endogenous IL-17 as has been shown before for vaccination against several self antigens such as TNF-alpha.[17] *In vitro* experiments were performed to determine the expression and functionality of the used construct. COS7 cells were transfected with the HEL-IL-17 plasmid and the supernatant was collected after 24 and 48 hours of incubation. We assessed the supernatant for IL-17 expression with a mouse specific IL-17 ELISA. 24 hours after transfection the HEL-IL-17 protein was detectable in the supernatant of the COS7 cells using an IL-17 ELISA. At 48 hours the concentration of HEL-IL-17 protein in the supernatant was almost doubled (Figure 3.4), indicating that IL-17 together with the HEL epitope is translated and excreted into the medium. Cells transfected with the empty pcDNA3.1 plasmid or non-transfected cells showed no expression of IL-17 (Figure 3.4).

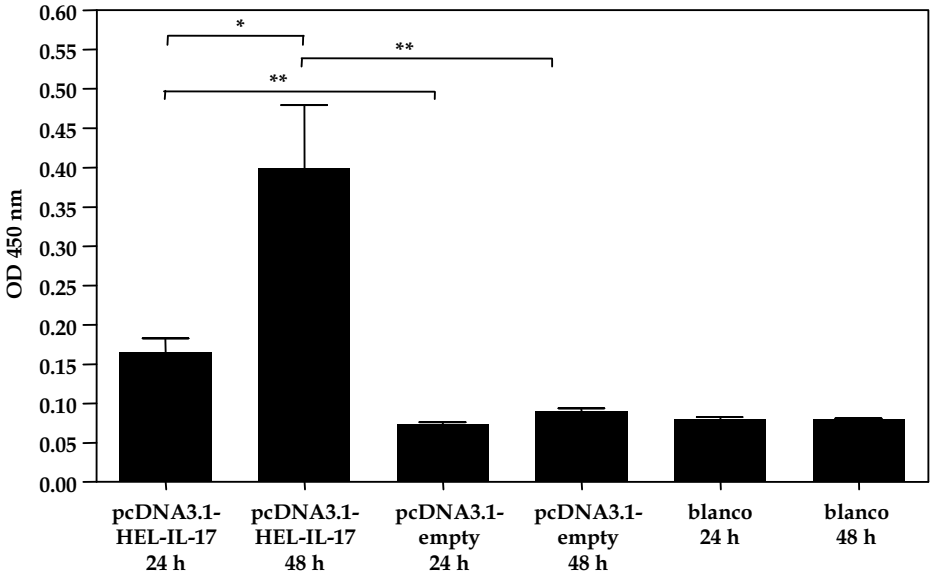

FIGURE 3.4: EXPRESSION OF THE VACCINE IN AN EUKARYOTIC EXPRESSION SYSTEM.
Expression of IL-17 was determined by transfection the plasmid to COS7 cells. Culture supernatant was collected 24 or 48 hours after transfecting with either HEL-IL-17 construct, an empty plasmid or PBS. IL-17 production was determined using a mouse IL-17 specific ELISA. The optical density was measured at a wavelength of 450 nm. *P< 0.05; **P<0.01

IMPAIRED IL-17 SIGNALING IN IL-17 VACCINATED MICE

To determine whether a functional blockade of IL-17 was established by vaccination, male LDLr$^{-/-}$ mice received a triple vaccination either with a plasmid encoding HEL-IL-17 or with an empty plasmid, both after pre-treatment of the mice with cardiotoxin I (CTX-I) at the site of vaccination to enhance the vaccination efficacy. Three days after the last *i.m.* vaccination, IL-17 was injected intravenously and 4 hours thereafter blood was collected to quantify the IL-17 response in terms of IL-6 production. Injection of IL-17 led to a significant 31% reduction in serum concentration of IL-6 in mice vaccinated against IL-17 as compared to control vaccinated mice (Figure 3.5; 7.87 pg/ml versus 11.44 pg/ml, $P<0.05$), indicating that the anti-IL-17 vaccination induced at least a partially blockade of the function of IL-17.

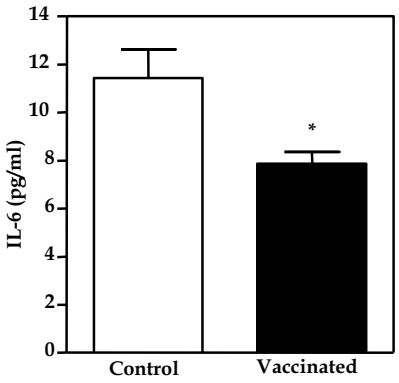

FIGURE 3.5: DETERMINATION OF NEUTRALIZING ACTIVITY OF THE VACCINE. Serum IL-6 concentration was determined with an ELISA 4 hours after administration of IL-17 to mice that were either vaccinated against IL-17 or control vaccinated. *$P<0.05$

EFFECT OF IL-17 VACCINATION ON ATHEROGENESIS

As the vaccine proved to effectively reduce IL-17 signaling, we studied the effect of anti-IL-17 vaccination on *de novo* plaque formation in male LDLr$^{-/-}$ mice using the *i.m.* IL-17-HEL vaccine. Following the last DNA vaccination LDLr$^{-/-}$ mice were put on a Western-type diet to induce hypercholesterolemia. Two weeks later, perivascular carotid collars were placed to induce atherosclerosis within the carotid arteries. During the experiment, we did not detect any difference in cholesterol levels between the anti-IL-17 vaccinated group and the control vaccinated group (Figure 3.6A). Six weeks after collar placement mice were sacrificed and the plaque size proximal to the collar was quantified. Vaccination with the HEL-IL-17 plasmid significantly reduced the formation of atherosclerotic lesions by 90.2% (Figure 3.6 B-D; 5654 ± 2099 μm^2 versus 57702 ± 14120 μm^2,

P<0.01), indicating that the initiation of atherosclerosis was largely blocked in the IL-17 vaccinated group. Furthermore, a beneficial 87.5% reduction in intima/media ratio (Figure 3.6E; 0.13 ± 0.033 versus 1.00 ± 0.27, *P*<0.01) and a 79.6% reduction in intima/lumen ratio (Figure 3.6F; 0.14 ± 0.043 versus 0.70 ± 0.087, *P*<0.05) were observed, which indicates reduced stenosis in mice vaccinated with HEL-IL-17. We determined plaque development also at another site in the vascular bed, the aortic valve region. In mice vaccinated with HEL-IL-17 (Figure 3.7A) a significant 59% reduction in plaque size (Figure 3.7C; 126691 ± 20739 μm2 versus 310170 ± 78706 μm2 *P*<0.05) was observed compared to the control group (Figure 3.7B). We assessed plaque composition in the lesions in terms of collagen and macrophage composition but detected no differences between the control group and vaccinated groups (data not shown).

DISCUSSION

Already in 1986 Mosmann *et al.* described two distinct Th subsets, Th1 and Th2 cells, with their own specific production of cytokines.[4] An exaggerated pro-inflammatory response due to an overexpression of Th1 associated cytokines such as IL-12 and IFN-γ enhances atherosclerosis.[24] Blockade of pro-inflammatory cytokines or cytokines involved in Th1 differentiation may introduce a new therapy for atherosclerosis. We previously demonstrated that vaccination against IL-12, a prominent Th1 cytokine, reduces atherosclerosis in LDLr$^{-/-}$ mice.[25] However, some experimental results with respect to the role of IL-4 in atherosclerosis can not be explained by the classical Th1/Th2 model.[26-28] These observations may indicate that other T helper subsets are involved in atherosclerosis and we focused in our present work on the role of IL-17 a main product of the pro-inflammatory T helper cell subset, the Th17 cells.[6, 10, 27, 29, 30]

Th17 cells and IL-17 have been identified in various autoimmune disease[29, 31] and may also play a role in atherosclerosis, which is also recognized as an autoimmune disease.[32-34] IL-17 may have pleiotropic effects on the various cell types within the atherosclerotic lesion and may thereby stimulate a pro-inflammatory environment, which aggravates atherosclerosis.[14, 15, 29, 35-37]

To address the role of IL-17 in atherosclerosis we first examined the effect of hypercholesterolemia on the expression of IL-17 within the spleen by feeding LDLr$^{-/-}$ mice a Western-type diet. 6 weeks after starting Western-type diet feeding, the IL-17 expression was more than 3-fold increased compared to the level before the diet. This may indicate a relation between the initiation of the inflammatory response during atherosclerosis and IL-17 expression.

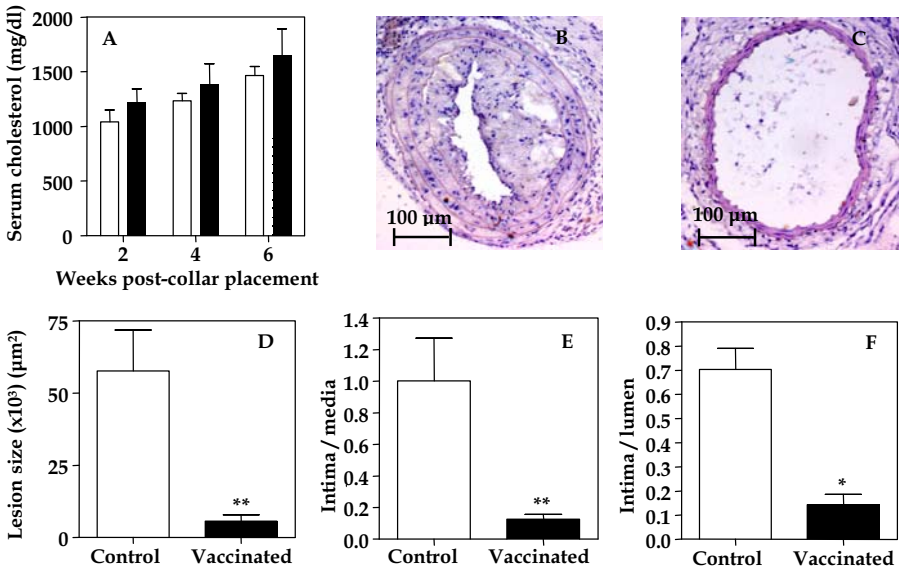

FIGURE 3.6: INITIAL ATHEROSCLEROTIC LESION FORMATION IN VACCINATED MICE. Mice were vaccinated by intramuscular (i.m.) injection of HEL-IL-17 plasmid or empty plasmid. Subsequently mice were put on a Western type diet and two weeks later equipped with a perivascular collar. At the indicated time points, cholesterol levels were determined in the various groups of mice (A). Six weeks after collar placement mice were sacrificed and the carotid arteries of control mice (B) and vaccinated mice (C) were sectioned and stained with hematoxylin-eaosin. Lesion size from all mice(D) as well as intima/media ratio (E) and intima/lumen ratio (F) were determined by computer-assisted morphometric analysis to asses the degree of atherosclerosis. *P<0.05, **P<0.01

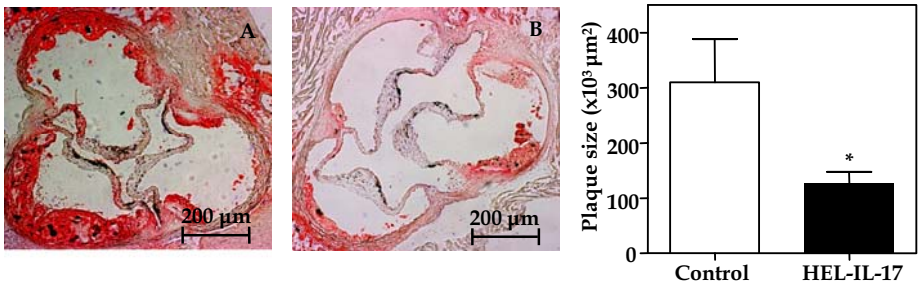

FIGURE 3.7: EFFECT OF IL-17 VACCINATION ON PLAQUE FORMATION IN THE AORTIC ROOT. Mice were *i.m.* vaccinated with an empty plasmid (A) or with a HEL-IL-17 plasmid (B). Subsequently mice were put on a Western type diet and 8 weeks later the mice were scarified and sections of the aortic root were stained with Oil-red-O and hematoxylin. Plaque size was determined by computer assisted analysis (C). *P<0.05

For the vaccination against IL-17, murine IL-17 was cloned into a pcDNA3.1 plasmid preceded by the specific immunodominant T-helper epitope HEL to break T cell tolerance. In this study we used a specific part of the HEL sequence which binds with high affinity to MHC class II and is able to activate T cells, which can subsequently provide the necessary stimuli to break the tolerance against self-antigens and help B cells to produce antibodies against IL-17.[17, 38] This approach is in line with data from Dalum *et al.* who used a HEL-TNF-α to vaccinate atherosclerosis prone apoE$^{-/-}$ mice against TNF-α.[17, 38] We detected high concentrations of IL-17 in the supernatant of cells transfected with HEL-IL-17, which indicates that the HEL peptide did not alter the conformation of IL-17 and did not interfere with the excretion of the protein into the medium, which is important for successful vaccination. To study the role of IL-17 in the process of atherogenesis we vaccinated LDLr$^{-/-}$ mice against HEL-IL-17 and vaccination was preceded by treatment of the hind leg muscles with Cardiotoxin I, which improves the outcome of DNA vaccinations.[39]

In the present study, we show that the function of IL-17 is partially blocked by vaccination against IL-17. Intravenous administration of recombinant IL-17 after anti-IL-17 vaccination resulted in a significant reduction in serum IL-6 levels. This indicates that vaccination against IL-17 induced a partial and functional blockade of IL-17 *in vivo*. In the HEL-IL-17 vaccinated mice we observed a highly significant reduction of 90.2% in plaque size in the carotid artery and a 59.0% reduction in the aortic valve region. Furthermore, the beneficial effect on lesion initiation is illustrated by an 87.5% reduction in intima/media ratio and a 79.6% reduction in intima/lumen ratio. Interestingly, unpublished data from our lab show that *i.m.* vaccination with an IL-17 plasmid lacking the HEL sequence did not have any effect on atherosclerosis, which clearly indicates that the HEL sequence in the vaccine is essential for a functional blockade of IL-17 (data not shown). In line with the findings on vaccination against Il-17, we have observed that transplantation of IL-17 receptor deficient bone marrow into LDLr$^{-/-}$ mice inhibits atherosclerosis by almost 50% (Van Es *et al.* unpublished results, chapter 4). It should be kept in mind that Th17 cells do not form the only source of IL-17, since more cell types such as γδT cells and NKT-like cells are able to produce IL-17.[40] Additional research is therefore needed to assess the role of these different cell types to IL-17 production and their individual role in atherosclerosis.

In conclusion, in this study we describe a prominent role for IL-17 in atherosclerosis: IL-17 is upregulated in hypercholesterolemic LDLr$^{-/-}$ mice upon Western-type diet feeding and neutralization of IL-17 by vaccination, using a novel DNA vaccination strategy, attenuates atherosclerotic lesion formation. Therefore interfering in the IL-17 pathway will be an interesting target for therapeutic intervention in cardiovascular disease.

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