Immunomodulation of atherosclerosis

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Section II

Vaccination against Atherosclerosis

Chapter 4

Vaccination against interleukin 17 attenuates atherosclerosis in LDLr$^{-/-}$ mice

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Abstract

Interleukin 17 (IL-17) is a T cell-derived pro-inflammatory cytokine that has been linked to several autoimmune diseases. IL-17 exhibits pleiotropic effects on atheroma-associated cell types and induces the secretion of pro-inflammatory cytokines, chemokines and matrix metalloproteinases, which enhances the inflammatory response in atherosclerotic lesions. In the present study we aimed to study the effect of IL-17 blockade by vaccination on the initiation of atherosclerosis. A plasmid (pcDNA3.1) encoding IL-17 and the dominant T helper cell epitope HEL was used to vaccinate LDL receptor deficient mice prior to induction of atherosclerosis by feeding a Western type diet and collar placement. Successful functional blockade of IL-17 upon vaccination was shown by reduced induction of serum IL-6 levels after administration of IL-17. DNA vaccination by intramuscular injection of the HEL-IL-17 plasmid or by oral administration of *S. typhimurium* transformed with this plasmid resulted in a decrease in atherosclerosis of 90% and 75%, respectively. Oral vaccination of LDLr$^{-/-}$ mice with *S. typhimurium* transformed with an IL-17 plasmid without the HEL sequence did not affect atherosclerosis. We conclude that effective interruption of the IL-17 pathway by DNA vaccination depended on the presence of the HEL epitope in the IL-17 DNA vaccine. Effective blockade of IL-17 by DNA vaccination resulted in a vast decrease in atherosclerosis and it may be speculated that IL-17 production by the newly identified IL-17 memory cells form a major target for IL-17 vaccination.

Introduction

Atherosclerosis is the leading cause of mortality in the Western world and atherosclerotic lesion initiation and progression has been shown to depend on persistent and chronic inflammation of the arterial wall.[1,2] Already in the early stages of atherogenesis macrophages and T cells are present in the intima and play a crucial role in the development and progression of the atherosclerotic plaque.[1,3,4] Atherosclerosis is considered to be a T helper (Th) cell driven immunopathological condition in which a disturbed balance of differentiation of naive $CD4^+$ T cells into either Th1 or Th2 cells plays a prominent role. Th1 cells predominate in atherosclerosis and create a pro-inflammatory environment within the plaque by secreting cytokines and chemokines, resulting in enhanced recruitment and activation of other inflammatory cells.[5,6]

An important pro-inflammatory cytokine is interleukin (IL)-17, which already has been shown to be involved in (auto)immune diseases.[7-9] IL-17 is a mainly T cell-derived pro-inflammatory cytokine and it exhibits pleiotropic biological actions on the various atheroma-associated cell types, such as endothelial cells, vascular smooth muscle cells, and macrophages.[10-12] IL-17 activates these cells and induces the secretion of pro-inflammatory cytokines and chemokines including IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1).[10] IL-17 also acts synergistically with other cytokines including tumor necrosis factor (TNF)-α and IL-1, creating an even more pro-inflammatory microenvironment.[10-12] Furthermore, the IL-17 pathway is also involved in the production of matrix metalloproteinases (MMPs) 3, 9 and 13, thereby possibly interfering with plaque stability.[13]

Although IL-17 is produced by activated T cells, previous reports have not provided clear classification of IL-17 within the paradigm of Th1 and Th2 polarized cytokine profiles.[14]

However, recently a new subset of T helper cells with a T memory cell phenotype has been identified and these cells may form a major source of IL-17 in several (auto)immune diseases.[15]

The development of these IL-17 expressing memory T cells from naïve $CD4^+$ T cells is mainly driven by IL-23 in the presence of a specific antigen and is distinct from Th1/Th2 development.[16] Th1 and Th2 cytokines, such as IFN-γ and IL-4 respectively, inhibit the IL-17 expressing memory T cell development, but fully differentiated IL-17 expressing memory T cells show a stable phenotype and are not responsive anymore to IFN-γ and IL-4.[17] These IL-23 dependent IL-17 producing T memory cells have already been identified in several autoimmune diseases, such as Experimental Autoimmune Encephalomyelitis (EAE) and rheumatoid arthritis and a possible role may be anticipated in atherosclerosis.[7,18] In this study we vaccinated against IL-17 using a novel DNA vaccination technique to block endogenous IL-17 and determine the effect of IL-17 in the process of atherogenesis. To break T cell tolerance for IL-17 a specific immunodominant T helper cell epitope was used.[19] This epitope, which is derived from the hen egg-white lysozyme (HEL), enables a polyclonal antibody response against the self-antigen. This approach has been proven successful with protein vaccination against TNF-α.[20] We show in this study that two different vaccination protocols, the first using an i.m. injection of the naked HEL-IL-17 plasmid and the second using an oral administration of attenuated *Salmonella typhimurium* (*S. typhimurium*), transformed with the HEL-IL-17 plasmid both establish a strong protection against atherosclerosis, indicating that vaccination against IL-17 may contribute to the development of novel therapies against atherosclerosis.

Methods

Vaccine preparation

Plasmids for vaccination were cloned into pcDNA3.1(-) (Invitrogen, The Netherlands) with a CMV promoter for expression in eukaryotic cells. cDNA coding for murine IL-17 was obtained by PCR on stimulated murine spleen cells. The following primers were used: forward primer 5’- GAT CAG GAC GCG CAA ACA- 3’, reverse primer 5’- GGGTTTCTTAGGGGTCAG- 3’. The IL-17 PCR product was cloned into pcDNA3.1(-). The HEL sequence was derived from the amino acid sequence 81-95 of hen egg lysozyme and was cloned upstream of IL-17.[21] The following sequences were used: upper strand, 5'- CTA GAA TGT CAG CCC TGC TGA GCT CAG ACA TAA CAG CGA GCG TGA ACT GCG CGC CT- 3' lower strand 5'- CTA GAG GCG CGC AGT TCA CGC TCG CTG TTA TGT CTG AGC TCA GCA GGG CTG ACA TT- 3'. Expression of constructs was tested by transient transfection of COS7 cells with the plasmid.

Transient transfection

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).

Vaccination

All animal work was carried out in compliance with the guidelines issued by the Dutch government. For intramuscular vaccination male low-density lipoprotein receptor deficient mice (LDLr$^{-/-}$), 10-12 weeks old, were used. Three days prior to first vaccination mice received a bilateral Cardiotoxin I (Sigma, USA) i.m. injection, 10 μM, 50 μl per muscle. Mice were vaccinated by three bilateral i.m. injections of 100 μg plasmid in 100 µl PBS, 50 µl per muscle, with two-week intervals.

For the *S. typhimurium* vaccination, LDLr$^{-/-}$ mice were pretreated 10 minutes before each vaccination with 100 μl of 7.5% sodium bicarbonate ($CHNaO_3$) by oral gavage. Hereafter mice were immunized three times at two-week intervals with 100 µl PBS containing 1.10^8 attenuated *S. typhimurium* harboring empty plasmid or HEL-IL-17 plasmid, by oral gavage. In a second experiment mice were treated in the same manner but in this experiment mice received attenuated *S. typhimurium*, transformed with the IL-17 plasmid or with empty plasmid without the HEL sequence. In these two groups of mice atherosclerotic lesions were analyzed at the site of the semilunar valves in the aortic root.

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 in carotid arteries by bilateral perivascular collar placement, as described previously.[22] During the experiment plasma samples were obtained by tail vein bleeding.

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 injection of the vaccine 200 ng mouse IL-17 (Biosource, The Netherlands) in 200 µl PBS was injected intravenously. Four hours after the injection of IL-17 blood was collected for quantification of serum IL-6 levels by a mouse IL-6 ELISA (eBioscience, The Netherlands).

Tissue harvesting

Six weeks after collar placement, carotid arteries were obtained after in situ perfusion for 15 minutes using PBS. 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.[22,23]

Histological analysis and morphometry

Cryosections were routinely stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). Corresponding sections were stained for lipids by Oil red O staining. Hematoxylin-eosin stained sections of right 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.[23]

Data analysis

Values are expressed as mean±SEM. A Mann-Whitney test was performed to compare individual groups of mice. A level of $p<0.05$ was considered significant.

Results

In vitro expression of HEL-IL-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. Twenty-four 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 (Fig.1). Cells transfected with the empty pcDNA3.1 plasmid or control treated cells showed no expression of IL-17 (Fig.1).

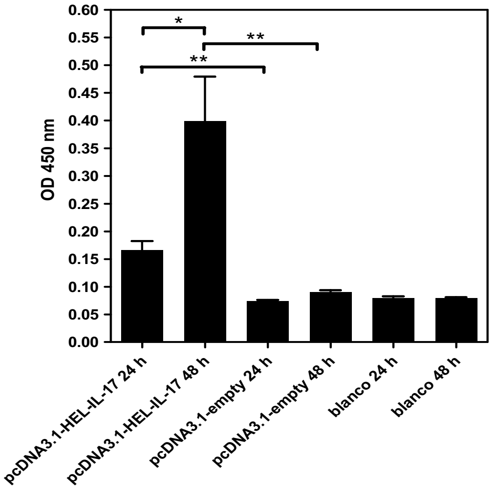
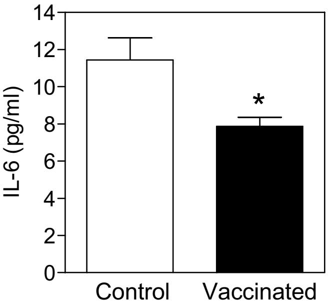


Figure 1. Relative expression of HEL-IL-17 of transfected COS7 cells. Culture supernatant was colleted 24 or 48 hours after transfection with either HEL-IL-17 construct, an empty plasmid or PBS. IL-17 was determined using an mouse IL-17 specific ELISA. The optical density was measured with a wavelength of 450 nm. *$p< 0.05$; **$p<0.01$.

Effect of vaccination against IL-17 on IL-17 induced IL-6 levels

To determine whether functional blockade of IL-17 was established by vaccination, male LDLr$^{-/-}$ mice were vaccinated against IL-17 or control vaccinated. Three days after the last i.m. injection of the vaccine mouse IL-17 was administered intravenously and 4 hours thereafter blood was collected for quantification of serum IL-6 levels. Upon administration of IL-17 the serum concentration of IL-6 was significantly reduced by 31% in the anti-IL-17 vaccinated mice as compared to the control vaccinated mice, indicating the impaired functional activity of IL-17 by vaccination (Fig.2).

Figure 2. Serum IL-6 concentration 4 hours after administration of IL-17 to mice that were either vaccinated against IL-17 or control vaccinated. Induction of IL-6 by IL-17 was significantly impaired by 31% after vaccination against IL-17 as compared to IL-6 levels in control vaccinated mice. *p<0.05.



Effect of IL-17 vaccination on atherogenesis

To study the effect of IL-17 vaccination on *de novo* plaque formation we used LDLr$^{-/-}$ mice fed a Western type diet. The first group of mice was vaccinated three times intramuscularly at a two-week time interval with the naked HEL-IL-17 plasmid after pre-treatment of the mice with cardiotoxin. The second group of mice was vaccinated three times orally at a two-week time interval with attenuated *S. typhimurium*, transformed with the HEL-IL-17 plasmid. For both vaccination protocols control groups of mice were vaccinated with the same plasmid without the HEL-IL-17 encoding region. After the last DNA vaccination the LDLr$^{-/-}$ mice were fed a Western type diet (0.25% cholesterol) followed two weeks later by perivascular carotid collar placement to induce atherosclerosis. Both types of anti-IL-17 vaccinations did not affect serum cholesterol levels and similar cholesterol levels were observed in anti-IL-17 vaccinated and control vaccinated mice (Fig.3).

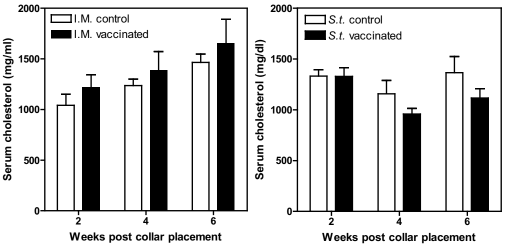


Figure 3. Mice were vaccinated by i.m. injection of HEL-IL-17 plasmid or empty plasmid (left panel) or by administration of attenuated *S. typhimurium* (*S.t.*) transformed with HEL-IL-17 plasmid or empty plasmid (right panel). Subsequently mice were put on a Western type diet and two weeks later equipped with a perivascular collar. At the indicated time points cholesterol levels were determined in the various groups of mice.

Six weeks after collar placement mice were sacrificed and the plaque size proximal of the collar was quantified in HE stained sections. Intramuscular vaccination with naked HEL-IL-17 plasmid significantly reduced the formation of atherosclerotic lesions by 90.2%, indicating that atherosclerosis in the IL-17 vaccinated group was almost completely blocked (Fig.4).

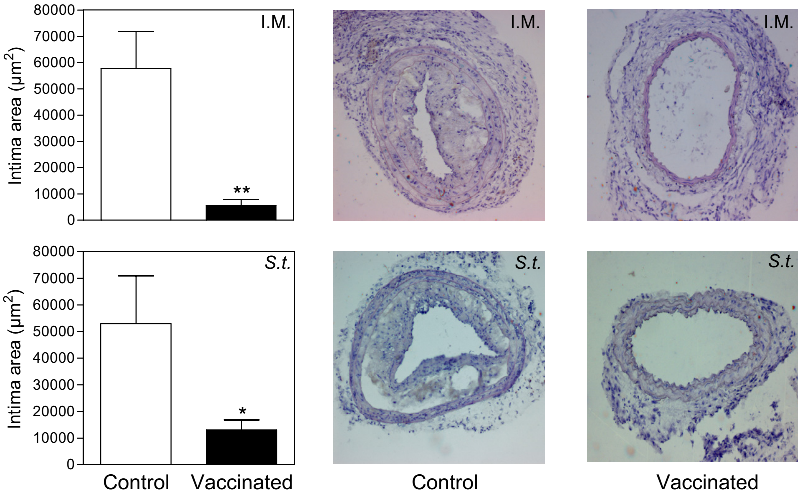


Figure 4. Mice were vaccinated by intramuscular injection (I.M.) of HEL-IL-17 plasmid (n=9) or empty plasmid (n=8) (upper panel) or by administration of attenuated *S. typhimurium* (*S.t.*) transformed with HEL-IL-17 plasmid (n=8) or empty plasmid (n=7) (lower panel). Subsequently mice were put on a Western type diet and two weeks later equipped with a perivascular collar. Six weeks after collar placement mice were sacrificed and the degree of atherosclerosis was determined. *p<0.05, **p<0.01.

Oral vaccination of LDLr$^{-/-}$ mice with the HEL-IL-17 plasmid carried by the attenuated *S. typhimurium* strain also resulted in a significant 75.3% decrease in atherosclerosis in the carotid artery, but the inhibition of atherosclerosis was less than that obtained with the intramuscular vaccination (Fig.4).

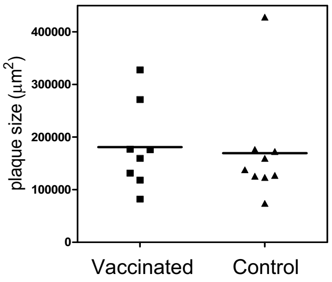


Figure 5. Mice were vaccinated by administration of attenuated *S. typhimurium* transformed with IL-17 plasmid (without HEL sequence) or empty plasmid (n=9). Subsequently mice were put on a Western type diet and eight weeks later mice were sacrificed and the degree of atherosclerosis was determined at the site of the semilunar aortic valves.

In contrast, vaccination with attenuated *S. typhimurium* transformed with the IL-17 plasmid without the HEL sequence did not result in a significant difference in the degree of atherosclerosis as determined in the aortic valve region (Fig.5).

Discussion

Atherogenesis is an autoimmune-like inflammatory process in the context of high plasma cholesterol levels and a high expression of pro-inflammatory cytokines plays an important role in the initiation and progression of atherosclerosis. This disturbed cytokine profile contributes largely to the pathological condition that is observed in a number of autoimmune diseases. CD4 positive T helper cells play an essential role in the development of immune responses and the two types of helper cells, Th1 and Th2 cells, secrete pro-inflammatory and anti-inflammatory, respectively. Atherogenesis results from an exaggerated pro-inflammatory response due to an enhanced development of Th1 cells and blockade of pro-inflammatory cytokines by vaccination may form a new therapy for atherosclerosis. We showed recently that vaccination against the prominent Th1 cytokine IL-12 reduces atherosclerosis in LDLr$^{-/-}$ mice.[24] Along this line we argued that the blockade of IL-17 may improve atherosclerosis and developed a DNA vaccine against IL-17 and studied the effect of anti-IL-17 vaccination on atherogenesis. One of the sources of IL-17 is a recently described T cell subset, the IL-17 producing T memory cell that has been identified in various autoimmune diseases.[15] These IL-17 producing cells are regulated independently of Th1/Th2 cytokines but are dependent on the pro-inflammatory cytokines IL-23 and IL-15 and the presence of a specific antigen for their maturation.[15] IL-17 produced by these cells may have pleiotropic effects on several cell types within the atherosclerotic lesion and may stimulate a pro-inflammatory environment.

For the vaccination against IL-17 we cloned murine IL-17 into a pcDNA3.1 preceded by a specific immunodominant T-helper epitope to break T cell tolerance. To determine the expression of IL-17 upon transfection with the HEL-IL-17 plasmid we transiently transfected COS cells. We detected relatively high concentrations of IL-17 in the supernatant of the transfected cells using an ELISA specific for murine IL-17, indicating that the HEL peptide did not interfere with the excretion of the protein into the medium, which is important for the vaccination procedure.

Subsequently we vaccinated LDLr$^{-/-}$ mice three times at a two-week interval by an intramuscular injection of the HEL-IL-17 plasmid or by an oral administration of attenuated *S.typhimurium* transfected with the same plasmid. The intramuscular vaccination of naked DNA was preceded by treatment of the hind leg muscles with cardiotoxin I, which induces a mild skeletal muscle necrosis and subsequently an inflammatory environment that improves the outcome of DNA vaccinations.[25] In the present study we show that the function of IL-17 is blocked by vaccination against IL-17. Administration of IL-17 led to significant reduced induction of serum IL-6 levels in the anti-IL-17 vaccinated group as compared to the control group. The *S. typhimurium* approach has so far been successfully applied in our laboratory to generate a cytotoxic response mediated by CD8 positive T cells that recognize cells overexpressing the gene encoded by the plasmid with which the *S.typhimurium* was transformed.[26] So far this route of vaccination has not been used to vaccinate against soluble factors, although unpublished results from our laboratory show that using the same vaccination protocol and anti-IL-15 vaccination resulted in a mild increase in the level of anti-IL-15 antibodies.[27]

After three vaccinations and six weeks after induction of atherosclerosis by collar placement the mice were sacrificed and the carotid artery was removed and analyzed. During the experiment no effect of either vaccination protocol on plasma cholesterol levels was observed. Within the intramuscular vaccinated group a highly significant reduction in atherosclerosis of 90% was observed as compared to the control group (p=0.0055), while a reduction of 75 % in plaque size observed within the *S.typhimurium* group (p=0.014). Interestingly we observed that vaccination using the attenuated *S. typhimurium* transformed with an IL-17 plasmid lacking the HEL sequence did not result in an attenuation of atherosclerosis, which indicates that the introduction of the HEL sequence is essential for breaking the self tolerance against IL-17 and therefore essential for the success of the anti-IL-17 vaccination. Previous studies on the vaccination against TNF-alpha, IL-9 and IL-12 indicated that the presence of a HEL sequence or a comparable T cell epitope is necessary for the induction of antibodies against self cytokines.

Interruption of the IL-17 pathway resulted in an even larger reduction in atherosclerosis than after blockade of IL-12/IL-23 or IL-15 by vaccination.[24,27] It is interesting that these two cytokines are involved in the induction of the so-called IL-17 memory cells and we may speculate that the production of Il-17 by these memory cells may be of great importance in the development of atherosclerosis.

Next to the initiation of atherosclerosis, IL-17 may induce a more vulnerable plaque phenotype since IL-17 induces the expression of matrix metalloproteinases in macrophages and smooth muscle cells and it may be expected that anti-IL-17 vaccination may improve the stability of the plaque. The plaques we observed after vaccination were however too small to reliably determine the effect of anti-IL-17 vaccination on plaque stability. Future experiment on anti-IL-17 vaccination in mice with pre-existing lesions will therefore be needed to determine the effect of interruption of the IL-17 pathway on plaque stability. The fact that a bone marrow transplantation from IL-17 receptor deficient mice into LDLr$^{-/-}$ mice inhibited atherosclerosis by 50% indicates that the response of macrophages to IL-17 contributes significantly to the initiation of atherosclerosis.[28] Besides the induction of MMP's in macrophages the induction of IL-8 and TNF-α in macrophages by IL-17 may contribute to the development of the plaque.

Since IL-17 independent of IL-12 and INF-gamma plays a role in the early host defense against different gram-negative bacteria and fungi, we have to determine whether the anti-IL-17 vaccination results in an increased risk of infection.[29,30]

We conclude that anti-IL-17 vaccination using the HEL-IL-17 plasmid results in a significant inhibition of the initiation of atherosclerosis and future experiments will have to be performed whether the anti-IL-17 resulted in lower numbers of IL-17 producing T helper memory cells or the effect of IL-17 was directly blocked within the atherosclerotic lesions.

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