Vaccination Against IL-16 Accelerates Atherosclerosis in LDL Receptor Deficient Mice

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Abstract

Atherosclerosis is an auto-immune disease characterized by the influx of leukocytes into the vessel wall and the deposition of lipids. Interruption of the migration of specific subsets of leukocytes into the lesion can exert protective effects on lesion formation. IL-16 is a chemotactic factor that specifically induces the migration of CD4+ cells to sites of inflammation. It has been shown to have pro-inflammatory effects in several auto-immune diseases and we therefore proposed that blockade of IL-16 would be protective in atherogenesis. We constructed an orally administrated DNA IL-16 vaccine and confirmed the induction of anti-IL16 specific antibodies. LDL receptor deficient mice were vaccinated against IL-16 and we determined the effect on lesion formation in the aortic leaflet area and in collar induced atherogenesis after 8 weeks of diet feeding. No effects were observed in the aortic leaflets, but lesion formation was significantly induced in the carotid artery. At this time point, the number of circulating T cells, both CD4+ and CD8+, was significantly increased in IL-16 vaccinated mice compared to control. Vaccinated mice on a diet for 20 weeks showed an even more profound 2-fold induction of lesion formation in the total aorta. Vaccination did not affect serum cholesterol and triglyceride levels. We propose that IL-16 has a protective role, especially in late stage atherogenesis. More research will be necessary to address the mechanism behind the observed anti-atherogenic effect.
Chapter 7

Introduction

Accumulation of leukocytes in the vessel wall is a hallmark of atherosclerosis. Both macrophages and CD4+ T cells are attracted to the activated endothelium and contribute to the growing atherosclerotic plaque. IL-16 is one of the first described proteins that displays chemoattractant properties for lymphocytes and was therefore originally designated as Lymphocyte Chemoattractant Factor (LCF)\(^1\,^2\). It selectively mediates the migration of CD4+ T cells to sites of inflammation and has been extensively studied since it was cloned in 1994\(^3\). IL-16 is produced by a variety of cells, including leukocytes, fibroblasts and epithelial cells and is synthesized as a precursor that has to be cleaved by caspase-3 to become biologically active\(^4\,^5\). Secreted IL-16 forms homo-tetramers that are able to induce cross linking of its receptor CD4. CD4 is expressed by a subset of T cells and to a lesser extent on most monocytes/macrophages. IL-16 induces cell activation and migration, and therefore a prominent role for this molecule can be suggested in (auto-)immune diseases. IL-16 seems to exert a pro-inflammatory stimulus in animal models for experimental autoimmune encephalomyelitis (EAE)\(^6\,^7\), rheumatoid arthritis (RA)\(^8\,^9\) and asthma\(^10\,^11\). Leukocyte accumulation in the arterial wall initiates the formation of atherosclerotic plaques and inhibition of this migration, for example by selective blockade of chemokines or pro-inflammatory mediators, has resulted in the successful attenuation of lesion formation. It can be speculated that blockade of IL-16 will result in decreased migration of CD4 positive cells, and thus will result in attenuated plaque formation. This hypothesis was further underlined by the finding that CD4 deficient mice show reduced lesion formation when backcrossed to several atherosclerosis prone animal models and adoptive transfer of CD4+ cells led to accelerated atherosclerosis\(^13\,^15\).

Lynch et al have identified CCR5 as a co-receptor for IL-16\(^16\). As blockade of CCR5 results in significant attenuation of atherosclerotic lesion formation\(^17\,^18\), this could point to a pro-atherogenic effect of IL-16 as well.

In this study, we used oral DNA vaccination to induce IL-16 specific antibodies that selectively block IL-16. To break IL-16 tolerance, we used a PAN-DR epitope that enables a MHC-II mediated antibody response against the self-peptide IL-16. We show that vaccination against IL-16 results in increased lesion formation in the carotid artery in LDL receptor deficient (LDLr\(^{-}\)) mice. This effect was more prominent in later stages of plaque formation in the total aorta. We therefore propose an anti-inflammatory role for IL-16 in atherosclerotic lesion formation.
Material and methods

Vaccination strategy
The sequence of murine IL-16 was fused with the PADRE sequence and cloned into pcDNA3.1 plasmid (Invitrogen, CA) and electroporated into attenuated *Salmonella typhimurium* (Aro/A (strain SL7207). HEK 293-T cells were transfected with the plasmids and IL-16 protein expression was determined by Western blotting. Vaccination was performed by 3 times oral administration of 1x10^8 cfu of *S. typhimurium* transformed with pcDNA3.1-PADRE-IL-16 or pcDNA3.1-PADRE (control) in 100 μl of PBS at 2 week intervals. All animal experiments were in accordance with guidelines issued by the Dutch law and approved by the ethics committee for animal Experiments of Leiden University (DEC).

Antibody titer determination
96-wells plates were coated with 250 ng/mL IL-16 in PBS and incubated overnight at 4°C. After washing, the plates were blocked with FCS for 1 hour at room temperature. Plasma of IL-15 or control vaccinated mice was added in serial dilutions with PBS 5% Triton-X100 and incubated for 1 hour at room temperature. Plates were washed with PBS + 0.1% Tween-20, and horseradish peroxidase conjugated Goat-anti Mouse antibody (Dako) was used as detection antibody. After washing, the anti-IL-16 antibody titer was determined using a TMB kit (Pierce).

Atherosclerosis experiments
Female LDLr^-/- mice were vaccinated with control (pcDNA3.1-PADRE) or pcDNA3.1-PADRE-IL-16 as described (n=12/13 per group). After the last vaccination, mice were fed an atherogenic diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Service, UK). After 2 weeks of diet, atherosclerosis was induced by placing silastic collars around both common carotid arteries as previously described. After 8 and 20 weeks of Western type diet, mice were sacrificed and lesion size (8 weeks aortic root, 20 weeks en face aorta) was analyzed using a Leica-DM-RE microscope and Leica Qwin software. Concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

Histological analysis
Cryostat sections of the aortic root (10 μm) and carotid artery (5 μm) were collected and stained with Oil-red-O and H/E respectively. Lesion size was determined in 5 sections of the aortic valve leaflet area and at the site of maximal stenosis in the carotid artery. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:50).
Goat anti-rat IgG-AP (dilution 1:100) was used as secondary antibody and NBT-BCIP as enzyme substrates.

**Real time PCR assays**
Total RNA was isolated from aortic arch and was DNase treated. Quantitative gene expression analysis was performed on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (Appendix I) were designed using Primer Express 1.7 software with the manufacturer’s default settings (Applied Biosystems). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

**Flow cytometry**
Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada). Cell suspensions from spleen, blood, lymph nodes draining from the aortic arch and peritoneal cavity were stained for surface markers (0.2 μg Ab/300,000 cells) and subsequently subjected to flow cytometric analysis (FACS). Antibodies were purchased from Immunoscource (Belgium). All data were acquired on a FACSCalibur and were analyzed with CELLQuest software (BD Biosciences).

**Statistical analysis**
Values are expressed as mean ± SEM unless indicated otherwise. Two-tailed student’s T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was used to compare not normally distributed data. A probability value of P<0.05 was considered to be significant for both tests.

**Results**

**IL-16 mRNA is increased during initial lesion formation**
IL-16 functions as a chemotactic factor for CD4+ cells and we speculated that IL-16 could be involved in the attraction of leukocytes during atherosclerotic plaque formation.
We used LDL receptor deficient mice on a Western type diet as a model for atherosclerosis and the mRNA expression of IL-16 during lesion formation was monitored in the aortic arch at different stages of western type diet feeding. A vast and significant increase was observed in IL-16 mRNA expression after 9 weeks of diet (20-fold, p=0.001), indicating a role for IL-16 in diet-induced atherosclerotic lesion formation (Figure 1). At this time point a moderate influx of CD4+ and CD68+ cells was observed in the vessel wall (data not shown), indicating that lesion formation has started.
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**Figure 1:** mRNA expression of IL-16 is significantly upregulated in the aortic arch of LDLr<sup>-/-</sup> mice on Western typed diet feeding. mRNA was isolated from the aortic arch of LDLr<sup>-/-</sup> mice and expression of IL-16 is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student t test was applied to test whether IL-16 mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (*p < 0.05, n= 6 per time point).

**PADRE-IL-16 vaccination**

Once we found a diet induced regulation of IL-16, we constructed a DNA vaccine against IL-16 to address the functional role of this interleukin during lesion formation. The vaccination strategy is based on the generation of IL-16 specific antibodies that will functionally block IL-16 signaling. We used a PAN-DR epitope (PADRE) to overcome tolerance against the self peptide IL-16 by facilitating MHC-II mediated T cell activation. We cloned a fusion construct of PADRE-IL-16 into pcDNA 3.1 and in vitro experiments were used to determine the expression of the used construct in eukaryotic cells. HEK-293T cells were transfected with the PADRE-IL-16 plasmid and the presence of pro-IL-16 protein was confirmed by Western blot (Figure 2A). Lane 4 and 5 clearly show a band of approximately 70 kD that indicated pro-IL-16 protein (68 kD). Non-transfected control cells and transfection with pcDNA 3.1 empty did not show the expression of pro-IL-16 (Lane 2 and 3).

To test the induction of anti-IL-16 antibodies after vaccination, we titrated serum from control and IL-16 vaccinated mice to determine the concentration of anti-IL-16 antibodies by ELISA. Serial dilutions of serum from IL-16 vaccinated mice resulted in a sigmoidal decrease in the binding of antibodies to the IL-16 coated wells as shown in figure 2B. Binding of antibodies in serum of control vaccinated mice to IL-16 was not observed in control vaccinated mice (open dots).

**Vaccination against IL-16 aggravates atherogenesis in LDLr<sup>-/-</sup> mice**

Subsequently, we used the plasmid in a vaccination protocol to determine the effect of the induction of anti-IL-16 antibodies on lesion formation. Mice were vaccinated 3 times by oral administration of attenuated *S. typhimurium* transformed with pcDNA 3.1PADRE-IL16 or control (pcDNA 3.1PADRE). After vaccination, mice were fed a Western type diet for 2 weeks after which collars were placed around the carotid artery to induce atherosclerosis in the carotid arteries. Six weeks after collar placement, animals were sacrificed and initial atherosclerotic lesion formation was assessed. Vaccination did not affect cholesterol (2753 ± 432 vs. 2733 ± 544 mg/dl) and triglyceride levels (475 ± 134 vs. 533 ± 205 mg/dl) during the experiment.
Representative sections of the aortic valve leaflet area of control (A) and IL-16 vaccinated mice (B) are shown in figure 3. Lesion formation at this site was not affected by IL-16 vaccination (2.85 ± 0.26 *10^5 μm^2 vs. 2.56 ± 0.56 *10^5 μm^2, p=0.55).

We also assessed the degree of atherosclerosis in a model for flow-induced lesion formation. Representative slides of collar induced atherosclerosis in the carotid artery are shown for control (A) and PADRE-IL-16 (B) vaccinated mice in figure 4. Lesion formation at this site was significantly increased by 60% upon IL-16 vaccination. (44,850 ± 7,036 μm^2 vs. 71,739 ± 10,383 μm^2, p=0.04) (figure 4C). This was reflected in a 73% increase in intima media ratio (0.86±0.12 vs. 1.50 ± 0.20, p=0.01, 4E) and also the relevant clinical parameter, intima lumen ratio, increased by 41% (0.46 ± 0.06 vs. 0.65 ± 0.06, p=0.05, 4D).
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In addition to the effect of IL-16 vaccination on initial lesion formation after 8 weeks of Western type diet, we assessed the result of IL-16 vaccination on advanced lesion formation. Again, animals were vaccinated with control or PADRE-IL-16 3 times at a two-week interval and were fed a Western type diet for 20 weeks. The degree of plaque formation was determined along the aorta by en face staining of the lipid rich areas using Oil red O. Representative examples of control (A) and IL-16 vaccinated (B) aortas are shown in figure 5. The lesion burden in IL-16 vaccinated mice is 1.9-fold higher in comparison to control vaccinated mice (13.8 ± 1.3 % vs. 26.2 ± 2.3 %, p=0.0004).

Circulating T cell numbers are increased after IL-16 vaccination
IL-16 functions as a chemotactic factor for CD4 positive cells and we were interested in the effect of IL-16 vaccination on the leukocyte population during atherogenesis. To investigate this we harvested leukocytes from blood, spleen and lymph nodes, which drain from the aortic arch from control and IL-16 vaccinated mice after 6 weeks of Western type diet. We stained the cell suspensions for CD4, CD8 and F4/80 and the percentage of positive cells was determined by FACS.

Figure 6A shows the relative percentage of CD4+ T cells in blood, spleen and lymph nodes. A significant 50 % increase is observed in the percentage of these cells in the circulation of IL-16 vaccinated mice compared to PADRE vaccinated mice (17.2 ± 1.5 vs. 24.9 ± 2.1, p=0.01). No differential effects of IL-16 vaccination were observed on the CD4 population in spleen and lymph nodes. A similar pattern, however more modest, can be observed in the CD8+ population (6B). The percentage CD8+ cells is significantly increased in blood after IL-16 vaccination (10.8 ± 1.6 vs. 12.8 ± 0.8, p=0.03), and no such effects were observed in spleen or lymph nodes. As macrophages and monocytes can express (low amounts of) CD4, we also analyzed the F4/80 positive population. No differential effects were observed on this cell type after vaccination against IL-16 (figure 6C). No significant differences in total white blood cell counts were observed between control and treated mice.
Discussion

In this study we showed anti-atherogenic effects of IL-16 in atherosclerosis. We used a DNA vaccination strategy to block IL-16 function in vivo. IL-16 is an endogenous protein and antibody formation against an endogenous protein is inhibited due to the lack of T cell help resulting in tolerance. A vaccination strategy that will result in antibodies against a self protein is therefore depending on overcoming this T cell unresponsiveness. Vaccination against IL-12 by using the fusion protein of IL-12 and the T helper cell restricted epitope PADRE has been shown to be successful in preventing both experimental autoimmune encephalomyelitis (EAE) and atherosclerosis by induction of IL-12 autoantibodies. In this study, we use a DNA vaccination strategy where we overcome tolerance by linking the PADRE sequence to murine pro-IL-16 and cloned the fusion construct into pcDNA 3.1.

As protein expression of the construct is essential for further immunological processing, we show that transformation of eukaryotic cells with this plasmid resulted in protein production of the fusion construct. We then transformed Salmonella typhimurium with the plasmid and vaccinated mice by multiple oral administrations of the transformed bacteria. Niethammer et al. have nicely shown that oral vaccination results in the uptake of the transformed Salmonella by M cells and subsequent processing in the Peyers patch leads to translation of the proteins encoded by the plasmid. In our case, by using the PADRE epitope, the fusion protein will be recognized by IL-16 specific B cells.
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Figure 5: Lesion formation after 20 weeks of diet is significantly induced after IL-16 vaccination. Relative plaque area was quantified in en face pinned aortas after vaccination and 20 weeks of diet feeding. Representative pictures are shown for control (A) and IL-16 vaccinated mice (B) and lesion formation is significantly induced by 100% (n=8 per group, p=0.0004).

T cell help will be facilitated via the specific MHC class II presentation of PADRE by the antigen presenting cells (ie the B cell), which results in T cell help for the IL-16 specific B cell. We confirmed the resulting IL-16 specific antibody titers by ELISA.

Once we successfully validated the vaccination protocol, we vaccinated LDLr<sup>-/-</sup> mice against IL-16 to determine the role of this molecule in atherogenesis. No effect of IL-16 was observed on serum cholesterol and triglyceride levels and IL-16 vaccination did not affect initial lesion formation in the aortic valves. However, an increase in lesion formation was determined in the carotid artery and after 20 weeks of diet feeding, lesion formation in the total aorta was almost 100% increased compared to control vaccinated mice. This indicated that there is a discrepancy in the effects of IL-16 vaccination between different sites and time points, and this phenomenon is observed by more authors<sup>25,26</sup>. For example Frederickson et al. observed a significant reduction in lesion size in the descending aorta and not in the aortic valve leaflets in apoE deficient mice after immunization with apoB-100<sup>25</sup>. Furthermore, Hauer et al. showed that DNA vaccination against vascular endothelial growth factor receptor 2 very significantly reduced lesion formation in the carotid artery and in contrast, no effect was observed in the valve leaflet<sup>26</sup>.

The observed profound effects of IL-16 vaccination on advanced lesion formation and to a lesser extend on initial lesion formation may be due to the processing route of IL-16. It is synthesized as a pro-form and only actively secreted after cleavage by caspase-3<sup>5,27</sup>. This enzyme is highly expressed in lipid rich plaques and associated with macrophage apoptosis due to oxLDL loading<sup>28</sup>. It was also shown that IL-16 is spontaneously released from monocytes cells that undergo apoptosis<sup>29</sup>. In general, during plaque growth and differentiation the lipid rich core associated with necrosis and apoptosis will increase. This indicates that the amount of caspase-3 will increase with plaque progression, and therefore the active form of IL-16 will probably be more present in advanced atherosclerosis. Blocking the function of this molecule will thus have a more
profound effect in advanced plaque progression than in the development of (initial) fatty streaks. This mechanism is not in contrast with the observed elevation of IL-16 mRNA in the vessel wall already after 9 weeks of diet feeding. Pro-IL-16 mRNA is constitutively expressed in leukocytes, and cellular activation or apoptosis induction is required to release the active form of IL-16.

**Figure 6: Relative number of circulating T cells is increased upon IL-16 vaccination.** Mononuclear cell suspensions of draining lymph nodes, spleen and blood were isolated from control mice and mice following control or IL-16 vaccination. Cells were stained for CD4 (A), and CD8 (B) and F4/80 (C). Results represent the mean percentage of positive cells ± SEM from 6 individual mice (*=P<0.05, Students T test). An increase is seen in the percentage CD4 and CD8 positive T cells in blood in the IL-16 vaccinated group (White bars) compared to control (Black bars).

Although IL-16 is mainly presented in literature as a pro-inflammatory mediator, we propose that IL-16 has a protective effect during diet induced lesion formation. This rather unexpected finding can have several reasons. IL-16 is able to induce migration of CD4+ cells and vaccination against IL-16 will thus result in the reduction of IL-16 mediated migration. Not all attracted cells during the formation of an atherosclerotic plaque contribute to its growth. Specific attraction of cell subsets such as naturally occurring regulatory T cells or other suppressive subsets have profound protective effects on lesion formation. It was shown that IL-16 can induce expression of CD25, and is a possible stimulator of regulatory T cells in this way. Blocking this signalling route can locally modify the balance between regulatory and effector T cells and lead to accelerated atherosclerosis. The effects of IL-16 are not restricted to the T cell population. Macrophages and dendritic cells are known to express low to moderate amounts of CD4. When these cells are exposed to IL-16, the production of several pro-inflammatory and pro-atherogenic mediators is increased. However, IL-16 is also able to induce the secretion of IL-10 in these
cells, indicating a possible protective role via the induction of this potent anti-atherogenic molecule.

In conclusion we propose that IL-16 has protective effects in advanced lesion formation, possibly by the attenuation of anti-atherogenic T cell subsets or by reducing the amount of protective cytokine production. Further research will be necessary to determine the specific mechanism by which IL-16 exerts these beneficial effects on plaque formation.
Chapter 7

References


