Immunomodulation of atherosclerosis

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Chapter 8

Summary and perspectives

Introduction

Despite extensive attempts to prevent or treat atherosclerosis by lipid lowering or anti-thrombotic drugs in combination with life style instructions, atherosclerosis remains the major underlying cause of death world-wide, indicating the need for the development of novel strategies that attenuate atherosclerosis and its consequences.[1] Both further unraveling of the mechanisms that are involved in the pathogenesis of atherosclerosis and perpetual search for innovative methods to interfere in these mechanisms will contribute to the development of an effective therapy for atherosclerosis. This thesis payed attention to both of these aspects. In chapter 2 and 3, the potential role of *C. pneumoniae* in the pathogenesis of atherosclerosis was studied. In chapter 4 to 7, several vaccination strategies were evaluated as potential novel methods of interference in mechanisms involved in lesion formation, which may contribute to new therapies for atherosclerosis.

C. pneumoniae in atherosclerosis

Since atherosclerosis has been considered as an inflammatory response to injury many studies have focused on the role of infectious agents in atherosclerosis.[2-4] Identification of pathogens that stimulate the inflammatory response and hereby promote atherosclerosis may lead to novel ways of inhibiting atherosclerosis, such as the use of specific antibiotics or vaccines that protect against these pathogens. Although many infectious agents have been implicated to contribute to atherosclerotic lesion formation, most evidence has accumulated for a role of *C. pneumoniae* in atherosclerosis. Epidemiological studies showed associations between serum anti-*C. pneumoniae* antibobies and cardiovascular disease.[5] Furthermore, *C. pneumoniae* was detected in half of the atherosclerotic lesions.[6] These data linked *C. pneumoniae* to atherosclerotic disease, but were not suitable to show a contributary role for *C. pneumoniae* in this disease. Therefore, animal studies were performed to indicate a potential contributory role for these bacteria in atherosclerosis. In several murine models for atherosclerosis lung infection with *C. pneumoniae* did enhance lesion formation.[7] *In vitro* studies further elaborated on the potential mechanisms through which *C. pneumoniae* might promote atherosclerosis by showing atherogenic effects on atheroma-associated cell types, such as increased expression of pro-inflammatory cytokines or adhesion molecules by endothelial cells, or accelerated foam cell formation by macrophages.[8-15] Use of antibiotics against *C. pneumoniae* as a treatment for atherosclerosis was evaluated in both animals and humans and resulted in several studies in reduced atherosclerosis or its clinical consequences.[16-19]

However, except for the *in vitro* studies, all lines of research led to conflicting results, which can at least partly be explained by the different experimental conditions.[7,20] For example, in the animal experiments different animal strains, *Chlamydia* strains, or high fat diets were used leading to different results. Recently, the initial promising results with antibiotics were contradicted by two large clinical trials, in which the anti-*C. pneumoniae* antibiotics gatifloxacin or azithromycin were used for secondary prevention of

coronary events, but did not result in clinical benefits.[21,22] Initial data, in which antibiotics did affect atherosclerosis in rabbits, were attributed to the fact that protection against *C. pneumoniae* only leads to reduced levels of atherosclerosis when interference takes place at an early stage of atherosclerosis.[20] At the other hand, due to the persistent state, in which these bacteria can reside in cells, *C. pneumoniae* can escape from antibiotic treatment, leading to no benefit of this treatment.[23] Therefore, the lack of clinical benefits from these antibiotic treatments does not exclude a contributary role for *C. pneumoniae* in atherosclerosis. These examples of contradictory results in animal and antibiotic studies illustrate the need for further evaluation of the role of *C. pneumoniae* in atherosclerosis.

In this thesis we hypothesized in **chapter 2** that one of the crucial conditions that caused the different results in animal studies, may be the time point of infection in relation to the onset of atherosclerosis. Therefore, we performed a variety of experiments in LDL receptor deficient mice, in which the time point of induction of a lung infection with *C. pneumoniae* in relation to the induction of atherosclerosis by perivascular collar placement was varied. Interestingly, only if mice were infected by *C. pneumoniae* simultaneously with the induction of atherosclerosis by collar placement, infection did promote atherosclerosis, whereas no effect on carotid atherosclerosis was observed after infection on other time points. From these observations we concluded that the time point of infection with *C. pneumoniae* in relation to the onset of atherosclerosis is crucial for the exerted effect on lesion formation. These results suggest that some of the conflicting results, obtained in the past with animal studies, may be based on the different time points of infection in relation to the onset of atherosclerosis. Furthermore, this study suggests that potential anti-*C. pneumoniae* treatments, for example by antibiotics or vaccination, will only be useful in a selection of patients in which atherosclerosis is present in a specific stage. Although further research has to be performed to substantiate the underlying mechanisms that cause the different results at different time points, one of the explanations is based on the fact that the time point of infection may be crucial for the amount of *C. pneumoniae* that enters the vascular wall or atherosclerotic plaque.

Therefore, in **chapter 3** we focused on the effect of the presence of *C. pneumoniae* in the vascular wall on atherosclerotic lesion formation. In this study, a novel model was established in which *C. pneumoniae* was locally delivered from the luminal site to the arterial wall of the carotid artery. Positive infection of the arterial wall was confirmed by detecting *C. pneumoniae* specific DNA (the *OMP A* gene) 7 days after local incubation with these bacteria. We also observed an inflammatory response against these bacteria, reflected by increased chemokine and adhesion molecule expression. Combined with collar induced atherosclerosis, the presence of *C. pneumoniae* promoted the development of lesion formation in the carotid artery. Since *C. pneumoniae* DNA was not detected in other organs we concluded that the presence of *C. pneumoniae* in the atherosclerotic lesion is sufficient for the exerted effect on atherosclerosis and that a lung infection with *C. pneumoniae* or systemic inflammation is not a necessary stimulus for

the exerted effect of these bacteria on atherosclerosis. The fact that a lung infection with *C. pneumoniae* does not per se lead to positive infection of the vessel wall may explain some of the conflicting effects of *C. pneumoniae* in atherosclerosis prone mice. In addition, this novel model can be used to further disect the mechanisms through which the presence of *C. pneumoniae* in the vascular wall enhances atherosclerosis.
The data obtained in **chapter 2** and **3** support the hypothesis that *C. pneumoniae* is able to play a contributory role in atherosclerosis, and that *C. pneumoniae* is often not just an innocent by-stander. However, the exerted effects of *C. pneumoniae* on atherosclerosis is dependent on the conditions, under which the infection takes place. **Chapter 3** suggests that if a lung infection leads to positive infection of the vascular wall atherosclerosis is enhanced.

Vaccination against atherosclerosis
A variety of vaccination techniques is available, which makes it possible to block the function of proteins or remove specific cells. These vaccination methods are therefore suitable to study the contribution of certain proteins or cell types in atherosclerosis. In addition, vaccination may be used as a potential therapy for atherosclerosis. In **chapter 4-7**, we used several vaccination methods to determine the involvement of two interleukins (IL-17 and IL-12) and two specific cell types (highly expressing VEGFR2 or TIE2) in atherosclerosis. Furthermore, we aimed to evaluate vaccination as a useful strategy to therapeutically interfere in the development of atherosclerosis. By inducing humoral immunity we aimed to block interleukin IL-17 and IL-12, whereas removal of VEGFR2 or TIE2 overexpressing cells was established by induction of a cellular immune response against these cells.

Vaccination against IL-17 or IL-12
Although the role of IL-17 in atherosclerosis has not been elucidated yet, this cytokine has several characteristics that make it a potential pro-atherogenic candidate. Exposure of macrophages, which play a central role in atherosclerosis, to IL-17 induces the expression of several proinflammatory cytokines, that have been shown to promote atherosclerosis, such as IL-1, IL-6, and TNF-α.[24,25] Furthermore binding of IL-17 to its receptor activates NF-κB, which is a nuclear factor that regulates the expression of many interleukins (IL-1, IL-2, IL-3, IL-6, IL-8, and IL-12), enzymes (inducible nitric oxide synthase and cyclooxygenase-2), and adhesion molecules involved in chronic inflammation and atherosclerosis.[26-28] Due to these potential pro-atherogenic properties we aimed in **chapter 4** to clarify the role of IL-17 in atherosclerosis by blocking the function of this cytokine with two vaccination techniques. Both oral delivery of the vaccine, which comprised *Salmonella typhimurium* containing pcDNA3.1 that encoded IL-17 and HEL (T cel epitope), as well as intramuscular injection of naked pcDNA3.1 encoding IL-17 and HEL dramatically reduced atherosclerosis in LDL receptor deficient mice. These results indicate that vaccination against pro-inflammatory

cytokines may be of potential use in the development of novel therapies against atherosclerosis.

The role of IL-12 in atherosclerosis has already been implicated by several studies. By immunohistochemistry an increased presence of IL-12 was shown in human atherosclerotic plaques as compared to normal arteries.[29] In aortas of apoE deficient mice, IL-12 appears to be upregulated in an early stage of atherosclerosis at mRNA and protein level, which was accompanied by increased IFN-γ expression.[30] IFN-γ is a pro-inflammatory cytokine, which has been shown to promote atherosclerosis in a variety of studies.[31,32] Furthermore, two experiments were performed that indicated the contributory role of IL-12 in the development of atherosclerosis. ApoE/IL-12 double knockout mice develop less atherosclerosis in the aortic root as compared to mice that are only deficient in apoE.[33] In addition, daily administration of IL-12 promotes atherosclerosis in young apoE deficient mice.[29] These observations nicely fit with the major action of IL-12, which comprises the induction of inflammation by stimulating the differentiation of T helper 1 cells, which produce pro-inflammatory cytokines that stimulate atheroma-associated cell types atherosclerosis.[34-36,20] In the present study we aimed to confirm the involvement of IL-12 in atherosclerosis by blocking the function of IL-12 by vaccination, but more importantly we aimed to establish a novel strategy to inhibit the inflammatory response and to diminish atherosclerosis.

In this study we used a protein vaccination technique which was developed by the group of van Snick.[37] They showed that this vaccine blocked the function of IL-12 and used this vaccine to attenuate experimental autoimmune encephalomyelitis in mice. The vaccine comprises the mouse IL-12 peptide sequence coupled to a highly immunogenic T cell epitope peptide sequence (PADRE), which induces the necessary T cell help for the B cells to produce antibodies against IL-12. In our study, which is described in **chapter 5**, we confirmed that this vaccine, when injected in combination with an adjuvant, induces high levels of antibodies, which blocked the function of IL-12 in LDL receptor deficient mice. This blockade of IL-12 function resulted in a 68% reduction in atherosclerosis and induced a more stable phenotype of the atherosclerotic plaque, reflected by increased smooth muscle cell and collagen content after vaccination against IL-12. Furthermore, immunohistochemical analysis revealed reduced IFN-γ levels within the atherosclerotic plaques upon vaccination against IL-12, suggesting a reduction in inflammatory response. Several factors make this vaccination strategy potentially useful in the development of novel treatments against atherosclerosis. Apart from the reduction in plaque size and risk of rupture, this vaccination strategy induces long-standing protection (antibodies are elevated at least 24 weeks in mice), avoids the potential immune response against non-self antibodies, as can be the case with passive immunization, and furthermore this approach is relatively cheap. The possible disadvantage of this technique is based on the potential increased sensitivity against certain infections upon blocking IL-12. Initial studies on 3 IL-12Rβ1-deficient patients showed an increased risk of idiopathic *Mycobacteria* and *Salmonella* infections.[38] However, a larger, more recent study on 41 IL-12R$^{\beta}$1 deficient

adult individuals showed a relative resistance to infection, suggesting that human IL-12 is redundant in the protective immunity against most micro-organisms other than *Mycobacteria* and *Salmonella*. IL-12 is also redundant for primary immunity to *Mycobacteria* and *Salmonella* in many individuals and for secondary immunity to *Mycobacteria* but not to *Salmonella* in most individuals.[39] In addition, a recent study, in which IL-12 function was blocked by administration of anti-IL-12 antibodies to treat active Crohn's disease in humans, did not reveal an increased incidence of infections.[40] However, the effects of long-term blockade of IL-12 function by vaccination on the incidence of specific infections deserves further investigation, as blocking IL-12 function forms an attractive strategy for the treatment of atherosclerosis.

Vaccination against VEGFR2 or TIE2

Induction of cellular immunity against self cells that have changed their characteristics and are involved in disease processes has been extensively studied in relation to cancer.[41,42] These studies generally aimed to induce an immune response against tumor cells by a variety of vaccination techniques. Although none of these vaccination strategies has been licensed yet, there are about a dozen cancer vaccines in advanced clinical trials.[43-45] However, apart from targeting tumor cells, other cell types that contribute to processes involved in tumor growth can be the target, such as cells that contribute to tumor angiogenesis. Recently, the group of Reisfeld elegantly showed in mice how tumor growth can be inhibited by inducing cellular immunity against endothelial cells that overexpress VEGFR2 and are involved in tumor angiogenesis.[46] In order to establish cellular immunity, they used a novel oral DNA vaccination strategy, in which plasmids (pcDNA3.1), encoding the murine VEGFR2 are carried by the live attenuated bacterium *Salmonella typhimurium*. Upon administration of the vaccine specific cytotoxicity against VEGFR2 overexpressing cells was shown, indicating the successful induction of cell-mediated immunity. Since VEGFR2 overexpressing cells are also involved in processes that potentially contribute to the development of atherosclerosis, such as angiogenesis, we hypothesized in chapter 6 that this vaccination strategy may also interfere in the development of atherosclerosis.[47-50] Vaccination of hypercholesterolemic mice against VEGFR2 resulted in cytotoxic $CD8^+$ T cells that specifically killed cells that overexpressed VEGFR2. In **chapter 6** we showed that vaccination indeed inhibited angiogenesis in a hind limb ischemia model. Furthermore, vaccination against VEGFR2 resulted in a reduction of both the initiation and the progression of atherosclerosis, indicating the involvement of VEGFR2 overexpressing cells in atherosclerosis. However, induction of cell-mediated immunity against these cells led to increased post-interventional neointima formation, which was most likely caused by delayed reendothelialization after denudation of the endothelium. Although further studies are needed to determine the exact mechanisms, we can conclude that cells that overexpress VEGFR2 contribute to atherosclerosis. The fact that post-interventional neointima formation was enhanced by vaccination indicates that potential use of this vaccine in future therapies for atherosclerosis may

be limited to a selection of patients that does not qualify for percutaneous transluminal angioplasty.
In **chapter 7**, we used a similar approach to induce cellular immunity against cells that overexpress TIE2, which are also involved in angiogenesis, a process that potentially contributes to atherogenesis.[51] Vaccination against TIE2 also resulted in specific killing of cells that overexpress TIE2, indicated by the reduced amount of TIE2 positive cells that were present in the circulation after vaccination. Furthermore, vaccination against TIE2 resulted in reduced collar induced carotid atherosclerosis, but also inhibited the degree of plaque formation in the aortic root. In addition, plaques developed after vaccination against TIE2 contained relatively more collagen, suggesting a more stable plaque phenotype. In conclusion, the observed effects of vaccination against TIE2 confirm the hypothesis that induction of cellular immunity against self cells can reduce atherosclerosis.

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