Regulation of T cell responses in atherosclerosis

Puijvelde, G.H.M. van

Chapter 8

Summary and perspectives

During the last decades, more and more effort is put into the quest for a treatment of atherosclerosis, which is the major underlying pathology of cardiovascular diseases. Lipid lowering statins, anti-thrombotic drugs, and life style advice contributed to the decline in the incidence of atherosclerosis, but despite all the efforts, atherosclerosis remains the leading cause of death in the Western society. Atherosclerosis is a chronic inflammatory disease of the vasculature and is driven by a disturbed lipid metabolism and an autoimmune immune response against several autoantigens. The main aim of this thesis was to modulate the various autoimmune responses as a way to develop experimental treatments for atherosclerosis. In chapter 2 and 3, the protective role of regulatory T cells was studied. In chapter 4 and 6, dendritic cells were used in a vaccination protocol and in chapter 5, 6 and 7, the role of NKT cells in atherosclerosis was evaluated.

The most important modulators of immune responses and thus of atherosclerosis are cytokines. These cytokines can be roughly divided in two groups, the Th1 and the Th2 cytokines. The most important Th1 cytokines produced by Th1 cells are IL-12, IFN-γ, and TNF-α and these cytokines are mainly pro-atherogenic. On the other hand, most of the Th2-cytokines produced by Th2 cells (IL-5, IL10 and IL-13) are anti-atherogenic. It was long accepted that a disturbed balance between these Th1 and Th2 cells underlies the immune response in atherosclerosis. T cell responses in atherosclerosis are directed against several autoantigens. In figure 8.1 (processes I and II), the immune response to several possible antigens is shown. HSPs, oxidatively modified apoB100 and modified lipids such as oxLDL and MDA-LDL may elicit an immune response in which several cell types are important, especially the T cells. The antigens are ingested and processed by antigen presenting cells (DCs and macrophages) both within the atherosclerotic lesion and in secondary lymphoid organs such as the spleen and draining lymph nodes, which are lymph nodes near the atherosclerotic lesion (Figure 8.1, process I). The uptake of oxLDL by APCs can result in foam cell formation, both in macrophages and DCs. Normally, a DC, which has ingested an antigen maturates and migrates from the inflamed tissue towards the secondary lymphoid organs where they may induce a specific T cell or B cell response (Figure 8.1, process II). Subsequently, these activated T cells may migrate back to the inflamed tissue and to draining lymph nodes and elicit an immune response against the specific antigen, presented by the APCs in the lesion or lymph nodes (Figure 8.1, process II). In atherosclerosis, the DCs that ingest (modified) lipids and become foam cells show impaired migration to the

secondary lymph nodes.[1–3] There is however still activation of T cells specific for atherosclerotic antigens and this is due to the presence of the specific antigens through the whole body and not only within the lesion. The T cell response in atherosclerosis is mainly driven by Th1 cells, which produce IFN-γ, TNF-α and IL-12 within the lesion (Figure 8.1, process III). These cytokines are responsible for the ongoing inflammation in atherosclerosis.

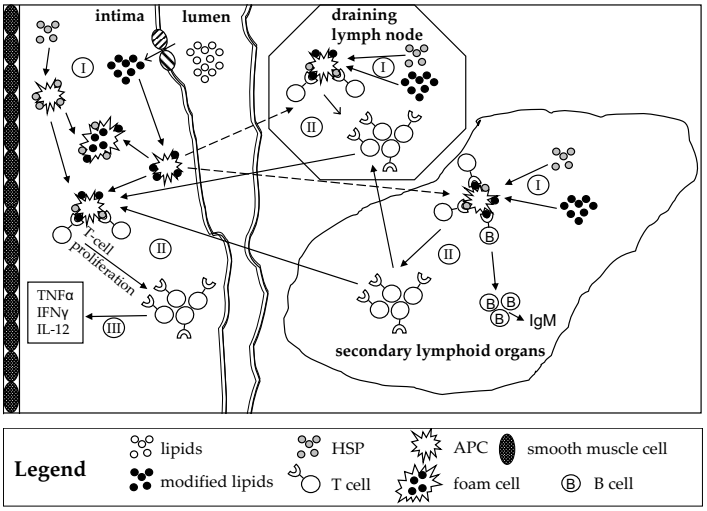

Figure 8.1: Potential sites for antigen-specific activation of T and B cells in atherosclerosis. Several critical steps are indicated by numbers: I=antigen uptake by APCs II=T and B cell activation and proliferation III=production of pro-atherogenic cytokines by Th1 cells within the atherosclerotic plaque.

Oral tolerance induction as treatment in atherosclerosis

Reducing the expression of Th1 cytokines or stimulating the production of Th2 cytokines are excessively used techniques to reduce atherosclerosis in mouse models. IL-4, considered to be a Th2 cytokine, has however pro-atherogenic properties.[4–6] Recently, the view on the disturbed balance between Th1 and Th2 cells has changed. Mallat et al. postulated that in atherosclerosis a disturbed balance exists between pathogenic Th1 and Th2 antigen-specific T cells and regulatory T cells (Tregs).[7] The Tregs mainly produce IL-10 and TGF-β, both strong anti-atherogenic cytokines. Reducing the number of Tregs accelerates atherosclerosis, whereas a stimulation and a transfer of Tregs ameliorates atherosclerosis.[7–9] Recently, Yang et al. showed that *in vitro* induced HSP60-specific Tregs prevent atherosclerotic lesion development.[10] The different types of Tregs develop in the thymus from a common ancestor. In the thymus both Foxp3$^+$ and Foxp3- Tregs T cells exist. The Foxp3$^+$ or natural Tregs migrate to the periphery and can exert their immune-suppressing properties via cell-cell contact and cytokine secretion (TGF-β and IL-10). The exact fate of natural Tregs, whether they can be antigen-specific and whether they can be activated and expand in the periphery is still not clear. In contrast with the natural Tregs, the adaptive Tregs are known for their ability to become activated in the periphery

such as in the Peyer's patches. These adaptive Tregs, which are Foxp3$^-$ when they leave the thymus, can be subdivided in Tr1 cells producing IL-10 and Th3 cells producing TGF-β. Tr1 cells do not express Foxp3, but Th3 cells may express Foxp3 upon activation[11] (Figure 8.2, process I). Adoptive Tregs may be induced via mucosal tolerance induction and may be antigen specific. In the Peyer's patches, located around the small intestines, tolerogenic DCs are present. These DCs develop especially in an environment rich in IL-10. Induction of Tregs specific for antigens in atherosclerosis was the aim of **chapters 2 and 3**. Oral tolerance is a technique that can be used to induce a Treg-response against harmful autoantigens. The fed antigens are transported into the Peyer's patches by M cells where they can be ingested by the tolerogenic DCs. This may result in the activation and proliferation of adaptive Tregs (Figure 8.2, process II). Whether natural Tregs are important in this process is not clear. In **chapter 2**, oral administration of low doses of oxLDL resulted in an increase in oxLDL-specific CD4$^+$CD25$^+$Foxp3$^+$ Tregs in both the spleen and the mesenteric lymph nodes. Tregs isolated from these lymph nodes produced more TGF-β upon *in vitro* re-stimulation with oxLDL, when compared with Tregs from control-treated mice. This indicates that the activated Tregs are most likely adaptive Th3 cells expressing Foxp3. In addition they may be natural Tregs that expand in the Peyer's patches, but this needs further investigation. The induced Tregs showed to be effective in reducing atherosclerotic plaque formation in an early and an advanced stage (Figure 8.2, processes IV and V). However, tolerance induction to MDA-LDL was not effective in inducing Tregs and consequently no effect on lesion formation was observed. The exact reason for this observation is not clear but oral tolerance induction to MDA-LDL did not induce an increase in Tregs specific for this antigen. We suggest that after uptake, MDA-LDL is not presented by the tolerogenic DCs in such a way that this leads to a proper activation of Tregs.

In the study described in **chapter 3**, HSP60 and a small peptide of HSP60 (aa 253-268) were administered orally and this resulted in an increase in CD4$^+$CD25$^+$Foxp3$^+$ Tregs in blood, Peyer's patches, mesenteric lymph nodes and spleen. The presence in blood and the decrease in Peyer's patches after two weeks indicate a migration of Tregs from the gut to the secondary lymphoid organs (Figure 8.2, process III). After re-stimulation with HSP60 *in vitro*, the splenocytes from HSP60-treated mice produced increased levels of TGF-β and IL-10 and they showed a lower HSP60-specific proliferative response when compared with splenocytes from control-treated mice. The Tregs induced after oral treatment with HSP60 ameliorated atherosclerosis in an early stage. The observation of an increased level of IL-10 upon re-stimulation may indicate that in addition to Th3 cells also Tr1 cells are induced, but these cells do not contribute to the increase in Foxp3$^+$ cells. In both studies on oral tolerance induction, an increased expression of Foxp3, CD25 and CTLA-4 in the atherosclerotic lesion indicated that the Tregs induced in the Peyer's patches may migrate to the site of inflammation i.e. the atherosclerotic lesion (Figure 8.2, process III). These data are in line with a recently published study in which oral tolerance was induced with an antigen conjugated to cholera toxin B subunit. They found several different types of Tregs that are important in oral tolerance induction i.e.

the natural Tregs but also Foxp3$^+$ and Foxp3$^-$CD25$^-$CD4$^+$ Tregs.[12] The data obtained in **chapter 2 and 3** show for the first time that the protective effect of oral tolerance induction in atherosclerosis may be caused by an activation of specific Tregs. These data support the hypothesis of Mallat et al.[7] that Tregs can counteract the pathogenic Th1 and Th2 cells specific for the same and different antigens. Within the lesion the Tregs may suppress the Th1 and Th2 response directed against the same or different antigens. This suppression may be indirect via the production of TGF-β and IL-10 (adaptive Tregs), which may result in an inhibition of the immune response against several antigens (Figure 8.2, process IV) or the suppression may be direct via cell-cell contact (natural Tregs) (Figure 8.2, process V). Normal CD4$^+$ T cells (Th1 or Th2) may upregulate the expression of the TGF-β receptor II (TβRII) after recognition of the antigen presented by APCs. Tregs specific for the same antigen also recognize the presented antigen on the APCs and start expressing surface-bound TGF-β. The subsequent interaction between TGF-β on natural Tregs and the TβRII on pathogenic T cells may result in a reduced proliferation of the pathogenic T cells (Figure 8.2, enlarged box, process V). In the future, selective elimination or inducible knockouts of Foxp3 will clarify the exact role of Tregs in atherosclerosis and tolerance induction. Furthermore, oral tolerance is a rather easy way of treatment for atherosclerosis and we will investigate whether a clinical trial is feasible.

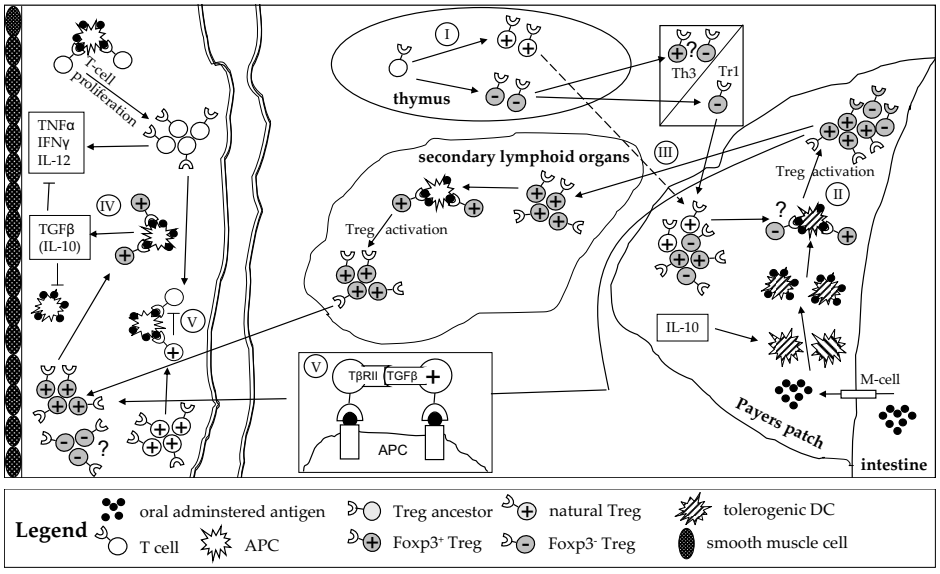

Figure 8.2: The effect of oral tolerance induction on atherosclerosis; an important role for regulatory T cells. Several critical steps are indicated by numbers: I=development of Tregs in the thymus II=activation and proliferation of adaptive Tregs III=migration of Tregs to secondary lymphoid organs and the atherosclerotic plaque IV=production of atherosclerosis-protective cytokines and the inhibition of the Th1 response within the plaque V=inhibitory effect of natural Tregs via cell-cell contact.

Dendritic cells as vaccination units in atherosclerosis

Antigen presenting cells (APCs) play a central role in immune responses and are necessary for the activation of adaptive Tregs in the gut whereas in the atherosclerotic plaque and lymphoid organs they present antigens to activate effector T cells. Dendritic cells (DCs) are the most powerful APCs. In healthy vessels only small numbers of DCs are present in the intima, immediately beneath the endothelial layer and in the adventitia.[13,14] At atherosclerosis-prone sites these DCs can start clustering in a very early stage and can elicit the immune response against autoantigens such as oxLDL and HSPs. In advanced stages, more DCs enter the lesion and clusters of DCs with T cells and NKT cells are formed in the rupture-prone shoulder regions.[1,15,16] Because of their capacity to activate T cells and NKT cells, DCs are widely used as immuno-modulating units in vaccination studies. In **chapter 4 and 6**, DCs were used to modulate two different immune responses in atherosclerosis. The DCs were isolated from the bone-marrow of C57BL/6 mice and maturated by the addition of LPS. In **chapter 4** the DCs were loaded *ex vivo* with oxLDL. This oxLDL is taken up by the DCs, shown by an Oil-red-O positive staining. OxLDL will be processed and small peptidic epitopes of oxLDL may be presented on the surface of the DCs loaded on MHC class I and II molecules. To modulate the *in vivo* immune response to oxLDL these pulsed DCs were injected in $LDLr^{-/-}$ mice (Figure 8.3, process I). After 72 hours, the injected DCs can be found especially in the lungs and the liver but also in the spleen and lymph nodes. Injections with the oxLDL-pulsed DCs resulted in an attenuation of atherosclerotic plaque formation. In addition, a decreased number of macrophages and an increase in collagen fibers was observed, contributing to a more stable lesion. OxLDL pulsed DCs also induced a significant decrease in cholesterol after 10 weeks of Western-type diet feeding. This lowering in cholesterol was accompanied by an increase in oxLDL-specific IgG antibodies which indicates that there was an activation of oxLDL specific T cells (Figure 8.3, process II). We now hypothesize that the injected DCs that homed to the spleen induced an oxLDL-specific T cell response which subsequently resulted in an activation of B cells producing oxLDL-specific IgG antibodies (Figure 8.3, process III). These antibodies may form immune-complexes with oxLDL in the circulation and this may be responsible for the reduced foam cell formation observed when macrophages were incubated with oxLDL together with serum of the DC-treated mice. A possible explanation is that the IgG antibodies form immune-complexes with oxLDL which may reduce the uptake of oxLDL via scavenger receptors on macrophages within the atherosclerotic lesion (Figure 8.3, process IV). Secondly, immune-complex formation may result in a better opsonization of oxLDL, resulting in a fast Fc-dependent removal of oxLDL from the circulation, or in neutralization of the effects of oxLDL systemically and locally which may prevent oxLDL from exerting pro-inflammatory and toxic effects in the vascular wall (Figure 8.3, process IV). The immuno-protective role of IgG antibodies was already shown in several studies in which mice were immunized with several forms of modified lipids and peptides.[17–20] The protective function is especially important because the DCs were injected before the mice were fed a Western-

type diet, and thus before oxLDL was generated. The injected oxLDL-pulsed DCs may restore the impaired migration of oxLDL-loaded DCs from the plaque to the secondary lymphoid organs, even before oxLDL was taken up by DCs within the lesion. Therefore T cells and IgG antibodies are generated that quickly respond when oxLDL is generated after high fat diet feeding. This process is like normal vaccination. Several studies already described that immunization with oxLDL, MDA-LDL or modified apoB100 peptides results in a reduction in atherosclerosis.[20–22] Major difference between this "vaccination" technique and ours is that in DC-vaccinations no adjuvant is needed. The clinical application is therefore wider than with other immunization studies. The only drawback could be that DCs from the patient itself are needed for vaccination, although these DCs can easily be generated from peripheral blood monocytes.[23]

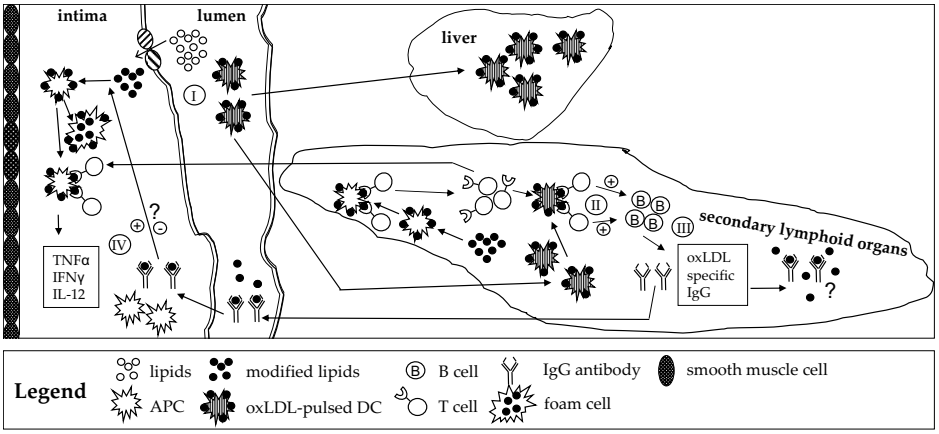

Figure 8.3: Dendritic cells pulsed with oxLDL are used to modulate the immune response in atherosclerosis. Several critical steps are indicated by numbers: I=Intravenous injection of oxLDL-pulsed DCs II=activation of oxLDL-specific T cells III=production of oxLDL-specific IgG antibodies by B cells IV=protective effect of oxLDL-specific IgG on the immune response in the atherosclerotic plaque.

NKT cell activation in atherosclerosis

In addition to MHC molecules, DCs also express CD1, another class of antigen presenting molecules. In mice only CD1d is expressed and this molecule binds glycolipid antigens instead of peptide antigens like MHC molecules do. Antigens presented by CD1d are recognized by a specialized subset of T cells, the NKT cells, which express a T cell receptor (TCR) composed of Vα14 and Jα18 (previously known as Jα281) subunits paired with a restricted set of Vβ chains. CD1d expressing DCs were found in the atherosclerotic lesions together with NKT cells.[24–27] NKT cells are known for their unique capacity to produce a diverse spectrum of cytokines, both Th1 and Th2.[28] Since many studies on other Th1 mediated diseases showed that repetitive injections with α-GalCer, which is a strong synthetic activator of NKT cells, was protective, it was expected that α-GalCer activation of NKT cells would also be protective in atherosclerosis. Accumulating data however proves that NKT cells accelerate atherosclerosis.

Depletion of CD1d by crossbreeding CD1d$^{-/-}$ mice with LDLr$^{-/-}$ and apoE$^{-/-}$ mice decreased lesion formation while activation of NKT cells by injecting α-GalCer in apoE$^{-/-}$ mice resulted in an acceleration of lesion formation.[24–26,29] NKT cells were found in atherosclerotic lesions, especially co-localized with DCs, and the general idea is that the NKT cells within the lesion are activated by an endogenous ligand. The endogenous ligand for NKT cells in atherosclerosis is however not known but since NKT cells are activated by lipidic antigens it is assumed that one of the lipids excessively present in atherosclerotic lesions and the liver may be able to activate NKT cells. It is suggested that this activation may result in Th1-biased NKT cells which may promote lesion formation (Figure 8.4, process I). In **chapters 5 and 6** we describe for the first time that NKT cells may however protect against atherosclerosis. In **chapter 5**, atherosclerosis was induced in LDLr$^{-/-}$ and apoE$^{-/-}$ mice by a combination of collar placement around the carotid arteries and feeding a Western-type diet. Subsequently, the mice were treated with α-GalCer twice a week for 7 weeks by i.v. and i.p. injections (Figure 8.4, process II). This resulted in a reduced lesion formation in LDLr$^{-/-}$ mice whereas there was no effect in apoE$^{-/-}$ mice. The difference between both mouse models is further elucidated by the observation of a lower proliferative response and a lower cytokine production in response to α-GalCer by apoE$^{-/-}$ splenocytes than LDLr$^{-/-}$ splenocytes. Additionally, the *in vivo* cytokine response in apoE$^{-/-}$ mice treated with α-GalCer was dampened when compared with LDLr$^{-/-}$ mice which showed an increased production of IL-10 and IL-4 in secondary lymphoid organs when compared with β-GalCer-treated mice. The difference in responses to α-GalCer in the two mouse models may be explained by two statements. First of all, apoE is an important mediator of lipid antigen presentation on CD1 molecules. ApoE$^{-/-}$ mice lack this important protein and consequently have an impaired ability to present lipid antigens to NKT cells (Figure 8.4, process III). This however does not explain why an increased plaque size was observed in other studies on α-GalCer treatment in apoE$^{-/-}$ mice. Another difference between our study and the other studies could however form an explanation for this. The most important difference between the studies is that our mice were fed a Western-type diet in stead of a normal chow diet. We now hypothesize that this high fat diet may influence the NKT cells before they were treated with α-GalCer. This is confirmed by the observation that a Western-type diet results in an increased NKT cell number in liver after 1.5 weeks followed by an increase in the spleen after 4.5 weeks (Figure 8.4, process IV). Due to this pre-activation of NKT cells, which may result in NKT cells that accelerate the disease (Figure 8.4, process I), α-GalCer may switch these pre-activated Th1 cytokine producing NKT cells into Th2 cytokine producing NKT cells (Figure 8.4, process V). These "double"-activated NKT cells may migrate to the lesion and secondary lymphoid organs and dampen the Th1-response via re-stimulation by endogenous ligands or α-GalCer (Figure 8.4, process VI). This may be explained by the fact that repetitive activation of NKT cells results in a more Th2-phenotype. We suggest that the repetitive injection of α-GalCer after continuous Western-type diet feeding induced a switch in cytokine-profile. In apoE$^{-/-}$ mice, a delayed response to the Western-type diet was observed. After two weeks of diet feeding and at the beginning of α-GalCer administration the hepatic and splenic NKT

cells are not endogenously triggered by the diet. NKT cells in this mouse model are activated by the injected α-GalCer but this was not protective. However, we did not observe an increase in lesion size in apoE$^{-/-}$ mice like it was observed in previous studies and therefore, it is concluded that the endogenous activation of NKT cells, which is delayed in apoE$^{-/-}$ mice, still affects the previously described negative effect of α-GalCer activation of NKT cells in a beneficial way, but not sufficiently enough to reduce atherosclerosis. From this study we can conclude that the vision on NKT cells accelerating atherosclerosis should be reconsidered. ApoE$^{-/-}$ mice are not the best model to investigate the role of NKT cells.

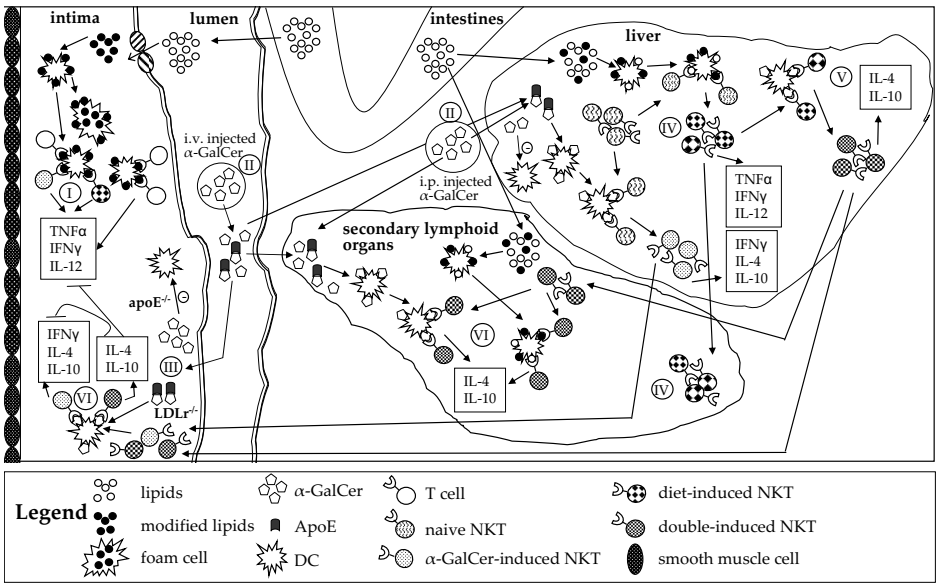

Figure 8.4: NKT cell activation via α-GalCer and its effect on atherosclerosis; an important role for high fat diet and apoE. Several critical steps are indicated by numbers: I=suggested atherosclerosis-promoting effect of NKT cell activation by lipids II=intraperitoneal and intravenous injection of α-GalCer III=lack of apoE results in an impaired ability to present lipids via CD1d to NKT cells IV=activation and proliferation of NKT cells due to a high fat diet V=proposed effect of α-GalCer; a switch from NKT cells producing Th1-cytokines to NKT cells producing Th2-cytokines VI=proposed effect of "double activated" NKT cells on the immune response in the atherosclerotic plaque.

Dendritic cells used to activate NKT cells

Ex vivo pulsed DCs can also be used as activators for NKT cells. Loading of α-GalCer on DCs and a subsequent injection of these DCs in mice and patients resulted in a prolonged activation of NKT cells producing a large amount of IFN-γ.[30,31] In **chapter 6** we used an analogue of α-GalCer, OCH, which triggers the NKT cells to a more Th2-phenotype.[32,33] OCH was administered intraperitoneally to LDLr$^{-/-}$ mice but this had no effect on atherosclerotic lesion formation. However, intravenous injection of DCs pulsed *ex vivo* with OCH (Figure 8.5, process I) reduced the lesion size dramatically. This reduction may be due to the migration of a high number of OCH-loaded DCs to the liver. There the

DCs may present OCH to the largest NKT cell pool of the body. This induced a proliferation and activation of hepatic NKT cells (Figure 8.5, process II) and these NKT cells produced more IL-10 while the same amount of IFN-γ was produced when compared with NKT cells from control-treated mice (Figure 8.5, process III). This whole process may also occur within the spleen, but this needs further investigation. After activation, the NKT cells may migrate out of the liver (and spleen) to other locations. This may explain the increased NKT cell number in the blood of mice treated with OCH-pulsed DCs. Since NKT cells are also found in atherosclerotic lesions we hypothesize that NKT cells induced in the liver migrate towards the site of inflammation. There they may be re-stimulated by OCH on injected DCs or by DCs already present in the lesion which present endogenous ligands (Figure 8.5, process IV). Upon re-stimulation the NKT cells secrete Th2 cytokines, especially IL-10, and inhibit the Th1 immune response within the lesion directly or via a bystander activation (Figure 8.4, process V). In addition, lower cholesterol levels were observed in the mice treated with OCH-pulsed DCs. This difference was only present in later stages of atherosclerosis and therefore can not explain the difference in plaque size, but it may contribute to the reduction. It is known that IL-10 influences the cholesterol levels in LDLr$^{-/-}$ mice.[34] We now suggest that the increased production of IL-10 by NKT cells in the liver stimulates the uptake of cholesterol from the blood by parenchymal cells and the subsequent secretion of cholesterol in the bile.

Endogenous ligands for NKT cells

In **chapter 5** we observed that high fat diet feeding may influence the NKT cell population in LDLr$^{-/-}$ mice. A natural endogenous ligand for NKT cells is however still not known. Recent publications suggest that some plant- and bacteria-derived glycolipids may be able to activate the NKT cells.[35,36] These glycolipids contain phosphatidyl choline (PC) and phosphatidyl ethanolamin (PE) of which the first one is also present in some lipids important in atherosclerosis; POV-PC and oxPAPC. In **chapter 7**, LDLr$^{-/-}$ mice were fed a Western-type diet and again an increase in NKT cell frequency was observed in the liver followed by the spleen and the iliac lymph nodes (Figure 8.3, process IV). These data confirm the fact that NKT cells play an important role in the initiation of atherosclerosis[29] since the increase in NKT cells is within 4.5 weeks of diet feeding. During progressive stages no differences in the number of NKT cells was observed. This is also shown by a deficiency in NKT cells, which has no effect on atherosclerosis after 12 weeks of Western-type diet feeding. OxLDL is one of the major lipids in atherosclerosis and a deficiency in NKT cells reduced the proliferative response of splenocytes to oxLDL. This indicates that oxLDL or at least one of its lipid components may be an endogenous ligand for NKT cells. We now suggest that PC units in oxLDL may be responsible for this; especially oxPAPC and POVPC are interesting candidates. These data are encouraging to continue the search for endogenous ligands in atherosclerosis. A plethora of lipids will be tested in the future which may possibly lead to a specific endogenous ligand for NKT cells. This may consequently lead to a better insight in the role of NKT cells and to new targets for therapeutic intervention in atherosclerosis. Overall, from the data in

chapters 5-7 it is suggested that NKT cells are activated by an endogenous ligand which may be one of the (PC-containing) lipids important in atherosclerosis. This activation may result in Th1-biased NKT cells accelerating atherosclerotic lesion development. The activation of the NKT cells may start in the liver, where also dendritic cells which ingest and process lipid antigens are present. The activation of NKT cells may subsequently spread through the whole body including the atherosclerotic lesion. Within the lesion they may be re-stimulated, resulting in the production of Th1 cytokines, especially IFN-γ, and this may contribute to the ongoing inflammation. However, re-stimulating the pre-activated NKT cells with α-GalCer switched the cytokine profile of NKT cells to a Th2-like profile, and they produce especially IL-10. These Th2 cytokines may dampen the Th1-mediated immune-response in atherosclerosis. This effect is only observed in $LDLr^{-/-}$ mice and is absent in $apoE^{-/-}$ mice because apoE is an important mediator of lipid antigen presentation on CD1 molecules. Activation of NKT cells by OCH, loaded on DCs, also reduced atherosclerotic lesion formation. This study was also performed in $LDLr^{-/-}$ mice that were fed a Western-type diet, further suggesting that a combination of a Western-type diet and a synthetic ligand are important to skew NKT cells to a Th2 phenotype.

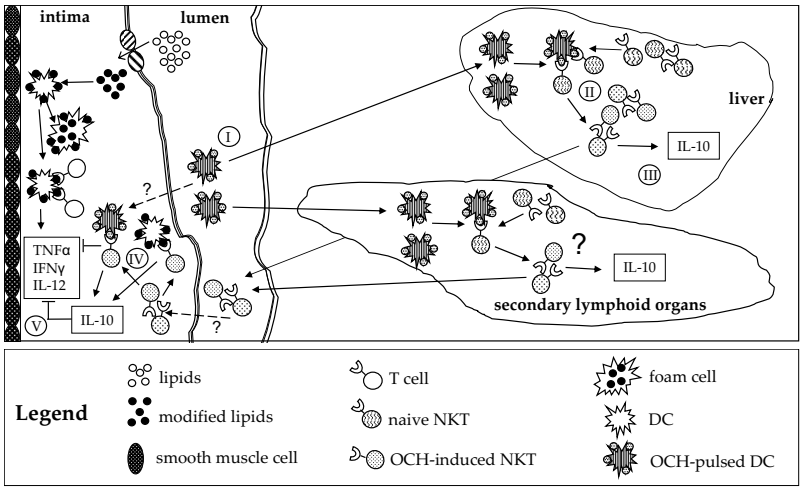

Figure 8.5: The effect of OCH-pulsed DCs on atherosclerosis; altered cytokine profile of NKT cells. Several critical steps are indicated by numbers: I=intravenous injection of OCH-pulsed DCs II=activation and proliferation of NKT cells via presentation of OCH by the injected DCs III=increased IL-10 production by NKT cells IV=restimulation of activated NKT cells within the atherosclerotic plaque by OCH or lipids presented by APCs V=proposed inhibition of the Th1-mediated immune response by IL-10 produced by the restimulated NKT cells within the atherosclerotic plaque.

Perspectives

In this thesis, several strategies to modulate the immune system during atherosclerosis have been studied in experimental therapies. These strategies provide different forms of immunotherapy and the goal of these strategies is to achieve a very efficient treatment for atherosclerosis that may be applied in

addition to the use of statins, aspirin and blood pressure lowering medication. The necessity for such as therapy is high because the current therapies in atherosclerosis are still insufficient. Statins are prescribed to patients to lower the cholesterol levels and aspirin is prescribed because of the anti-coagulative effects. Both medications resulted in a decline in the incidence of cardiovascular disease (CVD) during the last decade, but the fact that CVD is still the leading cause of death in the Western world implies that new therapies that regulate the immune response in atherosclerosis may be able to substantially reduce the incidence of CVD. This forms the reason why a substantial effort is put into the development of immunotherapies for atherosclerosis.
One of the strategies described in this thesis is vaccination using oxLDL-loaded DCs. This technique has some benefits when compared with existing vaccination/immunization protocols against oxLDL. DC-vaccination as used in **chapter 4** was very efficient, reducing atherosclerosis with more than 85%, and no adjuvant is needed to activate the immune system, which will reduce the time needed to optimize the vaccination protocol. When immunizing mice with modified LDL, an adjuvant is needed and the reduction in atherosclerosis was less prominent than in the DC vaccination study. An adjuvant gives an extra boost of the immune system and more specific strategies without an adjuvant are supposed to lead to less side effects. Therefore, the use of DCs as a vaccination unit is considered to be promising. At the moment, DC vaccination therapies are tested in clinical trials to treat several types of tumors. A complication of this technique is however that the DC production is labor-intensive and expensive, requiring specialized and costly manufacturing facilities.

A second strategy used in this thesis is the induction of oral tolerance and activation of Tregs. Tregs are increasingly used as therapeutic intervention method. In several diseases the number or function of Tregs is decreased and restoring the normal numbers may be protective. Adoptive transfer of Tregs could be successful but no clinical trials in humans have been reported yet. A major problem in this strategy is to obtain large numbers of these cells as per patient a high number of cells will be needed. Another method to induce Tregs is oral tolerance induction. Oral tolerance has been used in mouse models to treat several autoimmune diseases and it is currently tested in several clinical trials to treat type 1 diabetes mellitus, allergy, arthritis, multiple sclerosis etc. In **chapter 2 and 3** oral tolerance was induced to two important auto-antigens in atherosclerosis; HSP60 and oxLDL. The results of the clinical trials on other autoimmune disorders are very diverse. Some are successful, while others have no effect. Most of the clinical trials to treat several autoimmune disorders that are performed nowadays have however one common problem, which is the fact that the administered antigens are non-self molecules. Examples are clinical trials with myelin in patients with multiple sclerosis and collagen in patients with arthritis. In this thesis, oxLDL is used, which can be generated via oxidation of the patient's own LDL. This will be an advantage in clinical trials, because less side effects will be induced. We now intend to test oral tolerance to oxLDL in a clinical trial.

The third strategy used as an immunotherapy in this thesis was the activation of NKT cells. α-GalCer and OCH are both strong activators of NKT cells with protective effects in mouse models for several autoimmune diseases. Because α-GalCer had anti-tumor effects in mice, α-GalCer as used in **chapter 6** is tested in clinical trials to treat several types of cancer. In **chapter 5** DC-vaccination was combined with NKT cell activation via loading of OCH on the DCs. NKT cell activation via α-GalCer loaded DCs is also tested in clinical trials to treat lung cancer and it is shown that this activates NKT cells very efficiently. The only problem however is that the natural activator of NKT cells in atherosclerosis is not known. The discovery of this ligand will be a breakthrough in atherosclerosis research and will result in more opportunities for therapies in atherosclerosis. In **chapter 7** it is observed that oxLDL may be one of these ligands but it needs further investigation to what extent this oxLDL is responsible for activation of NKT cells in atherosclerosis.

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