Chapter 1

General introduction

Atherosclerosis

Atherosclerosis is a chronic lipid-driven inflammatory disease affecting arterial blood vessels. The onset of atherosclerosis already starts in the second decade of life and persists. Traditionally, atherosclerosis was seen as a lipid disorder in which the vascular wall became filled up with lipids, but nowadays it is widely accepted that inflammation plays an important role during atherosclerotic lesion formation. It is considered to be an enemy of human mankind and per year many people in the Western world die from cardiovascular events. In 2006, 33% (45,445) of all people that died that year in the Netherlands, died from a cardiovascular event. High fat intake, smoking, sedentary life-style and stress are only a few examples of factors that increase the risk for atherosclerosis. Besides these risk factors, other disorders of the body such as diabetes, dyslipidemia and obesity correlate with the incidence of atherosclerosis. The current therapies for atherosclerosis are mainly directed to these risk factors. The most prescribed medicines aimed at lowering plasma cholesterol levels are statins. Together with better life style advices, this has resulted in a strong decline in the incidence of atherosclerosis, but cardiovascular disease (CVD) is still the leading cause of death in Western societies. Therefore, additional therapies to treat atherosclerosis are highly relevant and will be beneficial in the further lowering of CVD.

Lesion development

Initial lesion formation

Atherosclerosis normally starts during adolescence in most major medium and large sized arteries and is in the beginning asymptomatic. Atherosclerosis starts with a dysfunctional endothelium at predisposed sites. A normal healthy endothelium plays an important role in maintaining vessel wall homeostasis and protects against the adhesion of platelets and inflammatory cells, thrombus formation and the proliferation of vascular cells.1 Dysfunction of the endothelium can be induced by turbulent or oscillatory shear stress, smoking, hypertension, obesity, atherogenic lipoproteins such as very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) and possibly by infectious microorganisms such as Chlamydia pneumoniae. These factors induce an increased permeability of the endothelial cell layer for lipoproteins and elevated expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1),
intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin. Endothelial cells also start producing chemokines (monocyte chemotactic protein-1 (MCP-1)), growth factors and vasoactive molecules which affect the blood pressure. Both the deposition of lipids and the expression of the adhesion molecules correspond with the location where a fatty streak, the first phase of the atherosclerotic plaque, is formed. The adhesion molecules are responsible for rolling of leukocytes on, and subsequent adhesion to, the endothelium. The leukocytes, monocytes or T cells, subsequently migrate through the endothelial layer into the intima (Figure 1.1). Under influence of different cytokines such as tumor necrosis factor-α (TNFα), macrophage colony stimulating factor (M-CSF), interferon-γ (IFN-γ), interleukin (IL)-1β, IL-2 and transforming growth factor-β (TGF-β), monocytes differentiate into macrophages. T cells can undergo antigen-dependent activation within the intima. Experiments using genetically altered mice show the importance of the adhesion molecules. E-selectin and P-selectin deficiency in apolipoprotein E deficient (apoE-/-) mice results in a reduced severity of atherosclerosis. LDL receptor deficient (LDLr-/-) mice depleted for functional VCAM-1 also show reduced plaque formation. Besides adhesion molecules, also chemokines contribute to the recruitment of the leukocytes into the vessel wall. Depletion of chemokine (CC) ligand 2 (CCL2) and its receptor CCR2 reduce atherosclerosis, while also CCL5 (RANTES), chemokine (CXCL) ligand 10 (CXCL10) and CXCL11 are produced within the atherosclerotic plaque. Blocking CXCR3 reduces the migration of effector T cells into the vessel wall and consequently results in an attenuation of atherosclerotic lesion formation (van Wanrooij et al., submitted for publication). All these data confirm the importance of the immune system in the early stages of atherosclerosis.

Figure 1.1: Effects of oxidized LDL on leukocyte recruitment into the vessel wall. LDL taken up in the vessel wall may get oxidized. The oxidized LDL stimulates the adhesion and infiltration of both T cells and monocytes into the vessel wall. The monocytes differentiate into macrophages and take up oxLDL which results in foam cell formation. T cells may be activated after presentation of the antigen by macrophages or DCs. This activation may result in inflammation and subsequently in plaque growth or it can result in a dampened inflammatory status (adapted from Robertson and Hansson)

The other important contributor to the development of atherosclerosis is the disturbed lipid metabolism. Due to the increased permeability of the vessel wall, lipids and especially LDL-cholesterol accumulate in the vessel wall. The cholesterol is incorporated in the monolayer surrounding the lipoproteins which
further consist of phospholipids and apolipoproteins. The core of the lipoprotein is filled with cholesteryl esters and triglycerides. The lipoprotein family can be divided in chylomicrons, VLDL, intermediate-density lipoprotein (IDL), LDL and high-density lipoprotein (HDL).\(^{14}\) The two most important lipoproteins are LDL, often seen as "bad cholesterol" and HDL also known as "good cholesterol". Both lipoproteins carry cholesterol in the blood stream. HDL transports cholesterol from the periphery to the liver where it is excreted from the body via the bile, while LDL transports the cholesterol from the liver and intestines to several tissues.

LDL is also entrapped within the vascular wall. Within the intima, the accumulated LDL may undergo modification by oxidation, glycation, aggregation, association with proteoglycans or incorporation into immune complexes.\(^{15-17}\) Oxidized LDL (oxLDL) can be internalized by the tissue macrophages via scavenger receptors.\(^{15,17-19}\) This internalization of oxLDL leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, because cholesterol derived from oxLDL cannot be mobilized from the cell sufficiently. This results in the formation of lipid-rich foam cells. The modified LDL is also chemotactic for other monocytes, attracting more and more of them into the intima. This may expand the inflammatory response and now also more T cells are attracted into the intima (Figure 1.1). In this stage, the lesion is called a "fatty streak" and remains asymptomatic.

**Progression of lesions**

Once smooth muscle cells start to migrate from the media into the lesion a progressive lesion is formed. The smooth muscle cells start to cover the fatty streak and extracellular matrix components are produced,\(^{20}\) resulting in a fibrous cap that covers a necrotic core which is composed of cell-free lipids and cholesterol crystals. The lesion is now called an advanced atherosclerotic lesion and grows into the lumen of the vessel.\(^{21,22}\) Consequently, the lesion is exposed to mechanical forces of the blood stream (shear stress) and the lesion is getting increasingly instable. Instability is also caused by other factors such as IFN-\(\gamma\) produced by T helper 1 (Th1) cells. IFN-\(\gamma\) inhibits both the proliferation of vascular smooth muscle cells and the production of collagen by these cells.\(^{23,24}\) In addition, macrophages are very important in reducing plaque stability via the production of matrix-degrading proteases such as matrix metalloproteinase (MMP)-1, MMP-8, MMP-9 and MMP-13.\(^{25}\) Also the presence of a number of immune cells and especially dendritic cells (DCs) in the shoulder regions of the plaque increase the vulnerability.\(^{26}\) The advanced lesions may become symptomatic when the fibrous cap ruptures and the necrotic core is exposed to the bloodstream.\(^{27}\) This induces thrombus formation which may occlude arteries in various organs and lead to lethal myocardial infarction or strokes and also in acute limb ischemia. Enhanced instability of the plaque increases the chance of a rupture (Figure 1.2). From all these data it can be concluded that the immune system is also accelerating the disease in advanced stages.
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Figure 1.2: The development of atherosclerosis: from a normal artery to a myocardial infarction. During the second decade of life normal arteries start to transform and early atheromas are formed. After this initial stage they can develop into stabilized or vulnerable plaques. The vulnerable plaque can rupture and cause thrombus formation. This can result in an acute myocardial infarction and subsequently in death (adapted from Libby).28

Immune cells involved in atherosclerosis

As mentioned above, a significant number of cells and molecules are involved in the immune response during the initiation and the progression of atherosclerosis. To discuss all cell types and molecules lies outside the scope of this thesis and therefore we focused on a few but very important cell types which will be described in separate sections below.

Monocytes/Macrophages

As described above, monocytes and macrophages play an important role in the initiation of atherosclerosis. Next to the vascular smooth muscle cells, macrophages are the most abundant cell types within the lesion. They are part of the innate immune response, normally responsible for the first line defense. The uptake of oxLDL by macrophages via scavenger receptors (CD36, CD68, CXCL16, lectin type oxLDL receptor 1, SR-A and SR-B1) not only leads to activation resulting in inflammatory macrophages but also in the formation of foam cells.15 Macrophages process the ingested oxLDL and oxLDL derived epitopes may be presented on MHC class II molecules present on the surface of macrophages. This may lead to the activation of antigen specific CD4+ T cells (Figure 1.1). Blocking MHC class II molecules with antibodies resulted in an inhibited oxLDL-induced IFN-γ secretion and T cell proliferation.29 Therefore, macrophages are considered to be one of the important antigen presenting cells (APCs) in atherosclerosis.
The role of T cells will be discussed in a separate section. Macrophages can also become activated via toll-like receptors (TLRs). These receptors recognize bacterial toxins such as LPS, stress proteins such as heat shock protein 60 (HSP60) and also oxLDL. The activated macrophages produce inflammatory cytokines such as TNF-$\alpha$ that is pro-atherogenic. In human atherosclerotic lesions many cells expressing a spectrum of TLRs are found. Intervention in the signaling pathway of TLRs and a lack of TLR4 reduces atherosclerosis in apoE$^{-/-}$ mice.

**Dendritic cells**

The most potent APCs are not macrophages but dendritic cells (DCs). DCs perform multiple important roles in both the innate and the adaptive immune responses. Immature DCs can capture antigens at the site of inflammation and subsequently migrate, triggered by pro-inflammatory signals, to lymphoid organs. During migration they mature and they subsequently present the captured antigen to naive T cells. Several chemokines such as CCR1, CCR2, CCR5, CCR6, CCR7, CXCR1 and CXCR2 are important regulators of DC-trafficking. An important step in the maturation of the DCs is the upregulation of CD40 and subsequently the interaction between CD40, expressed on the DCs and CD40L, mainly expressed on CD4$^+$ T cells. This ligation results in proliferation of T cells and plays a crucial role in immune responses. Upon maturation, DCs also upregulate the expression of several other maturation markers such as CD80 and CD86 and antigen presenting molecules such as MHC class I and II molecules and CD1 molecules. Depending on the local microenvironment of the DCs, they can induce Th1 or Th2 differentiation. In presence of Th1 cytokines such as IL-12, DCs induce a Th1 response. On the other hand, IL-6, IL-13 and OX40-ligand (OX40L) may induce Th2 responses. Chemokines are not only important in trafficking of DCs but also in regulating the maturation of DCs. Maturation of DCs is impaired when CCL3, CCL19 and CCL21 are not expressed. Importantly, IL-10 and TGF-$\beta$ are able to convert Th1-inducing DCs to DCs inducing Th2 or regulatory T cells. The DCs that can induce regulatory T cells are called tolerogenic DCs and produce high levels of IL-10 and TGF-$\beta$ and low levels of IL-12. These DCs, which are CD11c$^+$ and CD11b$^+$ are present in the Peyer’s patches in the intestines and are important in tolerance induction (see below).

In healthy arteries, low numbers of DCs are present within the intima, immediately beneath the endothelium and in the adventitia along the external elastic lamina. These subendothelial DCs may be part of the so-called vascular associated lymphoid tissue. The immature DCs scavenge the vessel wall for antigens and within the intima they may ingest atherosclerosis-related antigens. During atherosclerosis, the subendothelial DCs increase in number, especially in the shoulder regions of the plaque. Circulating monocytes attracted into the lesion due to chemokines can also differentiate into DCs and additionally, DCs can migrate from the adventitia into the intima. The presence of the DCs in the shoulder regions of the plaque may add to the induction of vulnerable lesions. Within the shoulder region of the lesions, DCs form clusters with activated T cells and NKT cells. During the maturation
process, the DCs normally migrate due to upregulation of CCR7 and return to secondary lymphoid organs (lymph nodes, spleen). In the lymphoid organs, the maturated DCs present their ingested antigens to T cells. However, due to inhibitory signals generated by PAF and oxLDL, hypercholesterolemia may lead to the activation within the atherosclerotic lesion and to reduced migration of monocyte-derived DC-like cells from the lesion to the secondary lymphoid organs. These DCs stay within the intima and interact with T cells which aggravate the inflammation within the lesion and promote thereby progression of atherosclerotic plaque formation.

Autoantigens in atherosclerosis

As described, both macrophages and DCs are important APCs in atherosclerosis. Both cell types can internalize various substances among which proteins and peptides, (glyco)lipids, etc. which can be processed by the APCs. Epitopes of these antigens can be presented on antigen presenting molecules. In atherosclerosis several antigens of both exogenous and endogenous sources have been identified. Studies indicate that possible antigens derived from exogenous sources play a contributory role in the inflammatory response in atherosclerosis. The most extensively studied pathogens in atherosclerosis are Helicobacter pylori, Cytomegalovirus (CMV) and Chlamydia pneumoniae. Patients with cardiovascular diseases have high antibody titers against these pathogens and Chlamydia pneumoniae, herpes simplex virus and CMV have been detected within the atherosclerotic plaque. The role of these bacteria and viruses remains however controversial because many contrasting studies exist. A T cell response to endogenous antigens can be elicited due to molecular mimicry, a cross reaction between the immune response to a pathogenic organism and homologous self-proteins. Heat shock proteins (HSPs) are expressed and secreted by pathogens such as Chlamydia pneumoniae and Helicobacter pylori. An immune response against microbial HSP65 can cross react with self-HSP60 and thereby induce an autoimmune response. This is due to the fact that HSPs form a family of evolutionary highly conserved proteins. In mammals, HSPs are expressed on endothelial cells and macrophages and can be induced by several stress factors such as fluid shear stress, oxidized lipoproteins and cytokines. Under these circumstances, HSPs repair or prevent degradation of denaturated proteins and increase the survival of a cell in response to stress stimuli. However, HSP, such as HSP60, are also involved in inflammatory diseases, probably resulting from their increased expression in cells exposed to pro-inflammatory mediators. Expression of HSP60 on endothelial, vascular smooth muscle and mononuclear cells is enhanced in human atherosclerotic lesions and patients with cardiovascular diseases have increased titers of HSP60-specific antibodies. These antibodies may contribute to endothelial damage and the inflammatory response in the vessel wall accelerating atherosclerosis. Additionally, HSP60 is expressed by macrophages exposed to oxLDL. Most importantly however, T cell clones with anti-self-HSP60 reactivity have been detected within atherosclerotic lesions. Further on, the induction of tolerance to HSP65 showed to be protective for
atherosclerosis in LDLr−/− mice while immunizing mice and rabbits with HSP60/65 accelerated fatty streak formation.77,78 Another possibility to develop an autoimmune response is by alteration of self-proteins. The modification of LDL in the vessel wall and in the circulation is thought to contribute to the immune response in atherosclerosis via this mechanism.81 For example, reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) produced by lipid peroxidation may bind to apolipoprotein B (apoB) resulting in immunogenic oxidative-specific neo-epitopes.82 The aldehydes can also cross-link amino acids creating many structural variations that may be identified as neo-epitopes. Oxidation of LDL can also result in oxidized phospholipids. The best-known altered self-proteins contributing to the immune response in atherosclerosis are therefore oxLDL and malondialdehyde modified LDL (MDA-LDL). In addition to activation of macrophages and endothelial cells, oxLDL is known to induce T cell activation.83 OxLDL-specific T cells are detected in the vessel wall and plaques of atherosclerosis patients.84,85 The presence of antibodies to oxLDL in plasma of animal models for atherosclerosis (LDLr−/− and apoE−/− mice)86 and in serum of patients with cardiovascular diseases87 again prove that oxLDL is one of the autoantigens in atherosclerosis. In animal models, the oxLDL-specific antibodies are mainly IgG2a antibodies, indicating Th1 assistance.88 Anti-oxLDL and anti-MDA-LDL IgG antibodies induced after immunization with oxLDL or MDA-LDL are shown to protect against atherosclerosis due to an enhanced Fc-dependent removal of the antigens by macrophages. This may prevent oxLDL and MDA-LDL from exerting pro-inflammatory and toxic effects in the vascular wall.17,88–90 In addition, IgM antibodies against oxidized phospholipids in oxLDL also protect against atherosclerosis.91 In most of these studies and in this thesis, oxLDL is made by copper oxidation or by modification with MDA. The important difference between these forms of oxidation is that copper oxidation results in many different specific epitopes, including MDA and HNE-epitopes and oxidized phospholipids, while MDA-modified LDL contains mainly MDA-epitopes. Immunization of atherosclerosis-prone mice with LDL and modified forms of LDL such as oxLDL or MDA-LDL and with peptide sequences of oxLDL resulted in a reduction of atherosclerosis.17,88–90,92 The third protein recognized as a possible autoantigen in atherosclerosis, is β2-glycoprotein I (β2GPI). Normally, this phospholipid-binding protein, which is present on platelets and endothelial cells, acts as an anti-coagulant molecule. However, β2GPI is abundantly expressed within subendothelial regions of human atherosclerotic plaques. Furthermore, CD4+ T cells colocalize with β2GPI and this supports the hypothesis that β2GPI initiates a chemoattractant process that results in the attraction of antigen-specific lymphocytes into the atherosclerotic plaque.93 Additionally, β2GPI colocalizes with oxLDL within the atherosclerotic plaque and these oxLDL/β2GPI complexes may be associated with systemic and chronic inflammation of the vasculature.94 Antibodies to β2GPI are correlated with the incidence of atherosclerosis in patients and autoantibodies to oxLDL/β2GPI complexes have been associated with arterial thrombosis.95 A role for β2GPI in atherosclerosis was further indicated by the fact that oral tolerance induction to β2GPI reduced atherosclerosis in LDLr−/−
mice, while immunization of LDLr\(^{-/-}\) mice with human \(\beta2\)GPI accelerated lesion formation. Most of the above mentioned antigens are based on a peptide structure. These epitopes are presented via MHC class I or II molecules and result in the activation of specific CD8\(^+\) or CD4\(^+\) T cells, respectively. The activation of these peptide-antigen-specific T cells most likely occurs in the secondary lymphoid organs such as lymph nodes and the spleen. The consequence of T cell activation on atherosclerosis is discussed in detail in the next section.

In addition to the expression of MHC class molecules, the APCs also express another antigen presenting class of molecules, the CD1 molecules. The family of CD1 molecules is related to MHC class I molecules but instead of presenting protein/peptide antigens they present lipid antigens to T cells. The CD1 family can be subdivided in two main groups. Group 1 molecules, CD1a, CD1b and CD1c, present foreign lipid-antigens to CD1 specific T cells. These molecules are absent in mice. Mice only express molecules from group 2, CD1d, which activates a specific subset of T cells; the natural killer T (NKT) cells, and also CD1d-restricted T cells. Because atherosclerosis is a lipidic disorder, one may imagine that lipid antigens exist in atherosclerosis. It was shown that within the atherosclerotic plaque, CD1d is expressed on DCs that colocalize with T and NKT cells in the shoulder regions of the plaque. In human atherosclerotic plaques, CD1d has been detected incidentally. The role of NKT cell activation by the presentation of (glyco)lipids and the role of CD1d in atherosclerosis will be discussed later.

**CD4\(^+\) and CD8\(^+\) T cells**

It was already described in 1985 that T cells are present within human atherosclerotic plaques and that they play a role in the disease process of atherosclerosis. Within human atherosclerotic plaques most of the T cells are CD45RO-expressing effector and/or memory T cells. Almost no naive T cells are found within lesions and the amount of activated T cells increases with the severity of coronary syndrome. Most of the T cells are CD4\(^+\) T cells and the majority of those are TCR\(\alpha/\beta\) positive, although TCR\(\gamma/\delta\) T cells are also present. In atherosclerotic lesions of apoE\(^{-/-}\) and LDLr\(^{-/-}\) mice, the CD4\(^+\) T cell is also the predominant T cell subset. A deficiency in CD4\(^+\) T cells or TCR\(\alpha/\beta\) T cells and thus a deficiency in adaptive immunity leads to reduced atherosclerosis in the atherosclerosis-prone mice. A transfer of CD4\(^+\) T cells to immune-deficient scid/scid mice accelerates atherosclerosis, indicating the important role of CD4\(^+\) T cells in the disease. Depletion of CD4\(^+\) T cells via antibody administration reduced fatty streak formation in C57Bl/6 mice, just like CD4\(^{-/-}\) C57Bl/6 mice were protected against fatty streak formation. The activation of the T cells is mainly important in the early progression of atherosclerosis, not in the initiation, CD8\(^+\) T cells have also been detected within the human atherosclerotic lesions, but contrasting data on their role in atherosclerosis exist. The absence of CD8\(^+\) T cells (apoE\(^{-/-}\)/CD8\(^{-/-}\) mice) has no effect on lesion formation, while in another study an acceleration of atherogenesis due to CD8\(^+\) T cell activation is observed. Altogether, their
The role of CD4+ T cells in atherosclerosis remains poorly understood. The TCRα/β positive CD4+ T cells can be subdivided into several subclasses depending on the cytokine secretion (Figure 1.3). Cytokines are small proteins and peptides that are used as signaling compounds between cells. Many cytokines, both pro-inflammatory and anti-inflammatory, are important regulators of the autoimmune process in atherosclerosis. T helper 1 (Th1) cells, partly responsible for cell-mediated immunity, secrete Th1-cytokines such as IL-1, IL-2, IFN-γ, TNF-α, IL-12 and IL-18, while Th2 cells produce IL-4, IL-5 and IL-13 and may be responsible for antibody production by B cells. Due to high IL-12 and IFN-γ levels during atherosclerosis, most of the CD4+ T cells within the plaque are shown to be of a Th1 cell type and produce TNF-α, IL-2 and IFN-γ. These Th1-cytokines stimulate macrophages and other cells within the lesion to secrete more pro-inflammatory cytokines. IFN-γ enhances the recruitment of T cells and macrophages to the plaque, increases lipid uptake by the macrophages and activates APCs. Deficiency in IFN-γ or the IFN-γ receptor resulted in attenuated atherosclerosis, while injections with IFN-γ accelerate the disease. Within the atherosclerotic plaque, IFN-γ is not only produced by Th1 cells, but also by the later on described NKT cells. Also deficiency in IL-12 and IL-18 in apoE-/- mice leads to an attenuation of atherosclerosis, whereas treatment with IL-12 and IL-18 accelerated atherosclerosis. Additionally, vaccination against IL-12 was successfully used to abrogate the Th1 mediated immune response in atherosclerosis. This effect was due to lowering of especially IFN-γ levels. TNF-α, also produced by macrophages and other cell types, show pro-inflammatory properties and promotes many autoimmune diseases. TNF-α deficiency also results in a reduction of atherosclerosis.

On the opposite side, Th2 cytokines are known for their atheroprotective characteristics, although there are some conflicting data. IL-5 is a promoter of B-1 cell development, the producer of natural antibodies. For example, some of the natural occurring (innate) IgM antibodies cross-react with oxLDL and inhibit cholesterol uptake and foam cell formation. Deficiency in IL-5 leads to an enhanced plaque formation in LDLr-/- mice. IL-4, a so-called Th2 cytokine, is able to inhibit Th1 induced immune responses and therefore protective against a number of Th1-mediated autoimmune diseases. In atherosclerosis, the role of IL-4 is however more complex. IL-4 has athero-promoting characteristics. Recent studies showed a reduced plaque formation in IL-4-/-LDLr-/- and IL-4-/-apoE-/- mice. Van Wanrooj et al. showed that blocking of the OX40 pathway results in a reduced plaque size in LDLr-/- mice due to lower levels of IL-4 and an increased formation of IgM antibodies. IL-4 can cause increased lipid oxidation, enhanced leukocyte adhesion and recruitment and increased lipid uptake and foam cell formation. IL-4 can also cause plaque destabilization by inducing apoptosis of SMCs and MMP-12 production. On the other hand, IL-4 also has some anti-atherogenic properties. Injecting C57Bl/6 mice with IL-4 decreased fatty streak formation. It seems that the effects of IL-4 are stage-dependent, being anti-atherogenic in early atherosclerosis and pro-atherogenic in advanced stages.
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Figure 1.3: An overview of the different CD4+ T cells in atherosclerosis. Upon recognition of a specific antigen presented on MHC molecules by APCs and in presence of co-stimulatory signals, Th1 and Th2 cells secrete specific cytokines. Upon activation Tregs secrete both IL-10 and TGF-β. Both cytokines can inhibit the activation of both Th1 cells producing IFN-γ, IL-2 and TNF-α and Th2 cells producing IL-4, IL-5 and IL-13. (adapted from Tedgui and Mallat) 120

From the discovery of the role of T cells in atherosclerosis it is thought that the main problem in the immune response is the disbalance between Th1 and Th2 cells. In atherosclerosis, this balance is directed towards Th1. This is supported by the fact that C57Bl/6 mice which are prone to a Th1 type of immune responses develop fatty streaks when they are fed a high cholesterol diet. In contrast, BALB/c mice which are prone to Th2 immune responses are protected against atherosclerosis.109,126 Many studies described in this section are performed to recover this balance and are done in either normal atherosclerosis-prone C57Bl/6 mice or in transgenic more atherosclerosis-susceptible apoE/- and LDLr/- mice.

Regulatory T cells

Recently there was however a change of view on the disturbed Th1/Th2 cytokine balance. IL-10 and TGF-β were found to be very effective in reducing atherosclerosis. Both are anti-inflammatory cytokines that are produced by a distinct subset of T cells; the regulatory T cells (Figure 1.3). IL-10, also produced by macrophages and DCs is produced within the plaque and inhibits oxLDL-induced production of IL-12 by human monocytes127 and it promotes plaque stability. In addition, endogenous IL-10128, systemically and locally administered IL-10129 and T cells overexpressing IL-10130 are protective against atherosclerosis. Deficiency in IL-10 in C57Bl/6 mice that are fed a atherogenic diet develop increased quantities of fatty streaks128,131, while IL-10 transgenic C57Bl/6 mice do not develop fatty streaks. Deficiency in IL-10 in apoE/- mice also results in bigger lesions.132 TGF-β is the rising star in anti-atherosclerotic
research. TGF-β neutralizing antibodies\textsuperscript{133}, soluble TGF-β receptors\textsuperscript{134} and adenovirus-mediated delivery of TGF-β\textsuperscript{135} demonstrates that TGF-β is a potent inhibitor of atherosclerosis. Crossbreeding of apoE\textsuperscript{-/-} mice with mice deficient in TGF-β receptors (T\textsubscript{β}RII\textsuperscript{-/-}) shows a five-fold increase in plaque size.\textsuperscript{136} The main functions of TGF-β are lesion stabilization through the induction of the synthesis of collagen and tissue inhibitors of MMPs, dampening atherogenic T cell responses and inhibition of leukocyte recruitment and foam cell formation. Many cells present in the atherosclerotic lesion are capable of producing TGF-β, but the most likely cell type producing this anti-atherogenic molecule is the regulatory T cell.\textsuperscript{137,138}

Mallat et al. now hypothesized that in atherosclerosis an imbalance exists between Th1/Th2 effector T cells and the regulatory T cells (Tregs)(Figure 1.4). In the past few years, these so-called Treg cells were identified to be responsible for a unique self-tolerance mechanism and control of autoimmunity. These cells can actively suppress immune activation and maintain immune homeostasis. They are specialized in suppression of both Th1 and Th2 pathogenic immune responses against self or foreign antigens.\textsuperscript{139} The majority of Tregs are CD4\textsuperscript{+} cells and constitutively express CD25 (IL-2R\textsubscript{α}). This CD25 is necessary for the development in the thymus, for survival and the function of Tregs. Activation of Tregs via IL-2, produced by non-Treg cells, results in the suppression of pathogenic T cells. The suppression of these T cells leads to the inhibition of IL-2 production and a reduction in the number of Tregs. Thus IL-2 is responsible for the feedback mechanism between pathogenic T cells and Tregs.

![Figure 1.4: The proposed anti-atherogenic properties of Tregs.](image)

Tregs may be activated in lymphoid organs by tolerogenic APCs and subsequently migrate to the atherosclerotic lesion just like Th1 and Th2 cells. Upon restimulation in the lesion the Tregs secrete IL-10 and TGF-β. These cytokines inhibit both Th1 and Th2 cells and also macrophages are affected. Altogether this results in a dampened inflammation in the lesion (adapted from Tedgui and Mallat)\textsuperscript{120}

Tregs can be subdivided in several types. The common ancestor is located in the
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thymus and there they can develop into Foxp3+ or Foxp3− Tregs. Subsequently, Foxp3+ Tregs, called natural Tregs, migrate out of the thymus into the periphery. The forkhead transcription factor Foxp3 is crucial in the development and function of these natural Tregs. FoXP3 controls several important genes such as CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-inducible tumor necrosis factor (GITR). The immunosuppressive function of these Tregs is partly mediated by TGF-β and IL-10 which can have direct effects on other cell types (bystander activation). TGF-β is also expressed on the surface of the natural Tregs instead of being secreted. The most important way of immuno-suppression by natural Tregs is through cell-cell contact via CTLA-4 on the Tregs and B7 on activated APCs and surface bound TGF-β on the Tregs and TβRII on other T cells, or it can be a combination of cell-cell contact and cytokine secretion. In vivo, activation of Tregs can result in the induction of IL-10 in CD4+CD25− T cells and TGF-β produced by the Tregs may inhibit pathogenic CD8+ T cells. The Foxp3− Tregs also migrate out of the thymus. These cells may be induced in the periphery and are called adaptive Tregs. These can be subdivided again, based on the cytokine they produce most excessively and on the surface markers they express. Tr1 cells (CD4+CD25−), which develop under influence of IL-10, produce mainly IL-10. Th3 cells (CD4+CD25+) are TGF-β producers. The Tr1 cells are CD4+CD25−Foxp3−. Th3 cells express both CD4 and CD25 and may express Foxp3 upon activation. There are contrasting studies about this! Foxp3-expressing T cells are found in human atherosclerotic plaques and Foxp3 mRNA is expressed in the aorta of apoE−/− mice. The protective action of CD4+CD25+ Tregs is proven by injections with anti-CD25 antibodies in apoE−/− mice. Anti-CD3 Fab antibodies stimulate the activation and proliferation of Tregs and treatment of LDLr−/− mice with these antibodies resulted in a reduction of the development and progression of atherosclerosis. Depletion of several co-stimulatory molecules such as CD28, CD80 and CD86 resulted in a reduction in numbers and function of Tregs and subsequently in higher atherosclerosis susceptibility. Furthermore, a transfer of Tr1 cells specific for ovalbumin elevated IL-10 levels in apoE−/− mice and reduced lesion formation. Recently it was shown that HSP60-specific regulatory T cells can be generated in vitro and injection of these cells in apoE−/− mice prevented atherosclerotic lesion development.

Th17 cells

As described, it was assumed for a long time that T cell-mediated tissue damage was caused by a disbalance in Th1 and Th2 cells (Th1/Th2 hypothesis). Later on, Mallat et al. introduced the new view on this balance in atherosclerosis. They postulated that a disbalance between pathogenic T cells and Tregs exist in atherosclerosis (Treg hypothesis). This view on the immunological process in atherosclerosis changed again very recently. Cua et al. revisited the immunopathological basis for diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA). Traditional blocking of IL-12 did not result in protection against these diseases, but blocking of a specific subunit of IL-23, which shares the subunit p35 with IL-12, resulted in
an attenuation of both diseases. Harrington et al. and Park et al. showed that development of Th17 cells from naive cells is potently inhibited by IFN-γ and IL-4, typical Th1 and Th2 cytokines, respectively, whereas already committed Th17 cells are resistant to suppression by Th1 or Th2 cytokines. Further studies defined more functional and phenotypic differences between the so-called Th17 cells and Th1 cells. This new lineage of T cells, Th17 cells, are now credited for causing tissue damage. Th17 cells are driven by IL-23 and TGF-β in presence of IL-6 and they mainly produce the pro-inflammatory cytokine IL-17. IL-17 deficient mice show attenuated disease progression of EAE and suppressed collagen-induced arthritis. Van Es et al. showed that vaccination against IL-17 protects against atherosclerosis. The new view on the balance of T cells is now called the Th17 hypothesis with Th17 cells on one side and Tregs on the other. IL-6 and TGF-β seems to be key-players in this balance since TGF-β plus IL-6 initiates Th17 cells, while TGF-β without IL-6 initiates Tregs (Figure 1.5).

**Figure 1.5: The new view on the balance between T cells in atherosclerosis.** Th17 cells and Tregs may develop from a common Foxp3−CD4+ ancestor. In presence of TGF-β and IL-6 the ancestor differentiates into Th17 cells, while without IL-6 they differentiate into Tregs. Th17 cells produce pro-atherogenic IL-17 and IL-6, while Tregs may produce NT-atherogenic TGF-β and IL-10. (adapted and modified from Weaver et al.)

**NKT cells**

Besides recognition of peptide antigens by T cells and Tregs, lipid antigens can be recognized by another specialized subset of T cells, the NKT cells. NKT cells are characterized by the expression of NK cell receptors such as CD1d (NK1.1 in mice) and an invariant TCR composed of Vα14 and Jα18 (previously known as Jα281) subunits paired with a restricted set of Vβ chains. Most of the NKT cells are CD4+ or CD4−CD8+. These classical NKT cells can recognize (glyco)lipid antigens presented by CD1d expressed on APCs. NKT cells are present in both humans and mice and in mice they are found most frequently within the liver (30-50% of the lymphocyte population) and bone-marrow (20-30%). They represent a smaller proportion of lymphocytes in the spleen (3%), lymph nodes (0.3%), blood (0.4%) and lung (7%). Within the liver they reside in
the sinusoids where they screen for possible ligands. Several subsets of NKT cells are known at the moment. The CD1d dependent NKT cells can be subdivided in NK1.1⁺ and NK1.1⁻ cells and there are also CD1d dependent NKT cells without expression of Vα14-Jα18 and CD1d independent but NK1.1⁺ NKT cells. The most common NKT cell is the invariant NKT cell, which is CD1d dependent and expresses NK1.1. Upon recognition of a glycolipid antigen, invariant NKT cells rapidly produce large amounts of both Th1 (IFN-γ, IL-12 and TNF-α) and Th2 (IL-4, IL-5, IL-10 and IL-13) cytokines and this makes them unique among lymphocytes (Figure 1.6). The question remains how this mix of cytokines can lead to a regulated immune response. One possible explanation could be the difference in the affinity of the ligand for CD1d, which can result in different signaling inside the NKT cell (see below). An important feature of the released cytokines from NKT cells is the bystander activation of adjacent NK cells, B cells and dendritic cells as well as activation of normal CD4⁺ and CD8⁺ T lymphocytes. Recent studies have shown that NKT cells can be activated by in vitro administration of marine sponge-derived α-galactosylceramide (α-GalCer) also known as KRN7000. Upon recognition of α-GalCer, mature NKT cells induce an early burst of IL-4 followed by a more prolonged burst of IFN-γ. However, repeated injections of α-GalCer have been shown to induce an adaptive immune response which is polarized towards production of Th2 cytokines (IL-4, IL-10). This results in the protection against autoimmune diabetes, experimental autoimmune encephalomyelitis and colitis in mice (Table 1.1). Interestingly some variants of α-GalCer exist that have decreased Th1 compared to Th2 cytokine induction. OCH, which has a truncated sphingosine chain, is one of these α-GalCer analogs. In vitro administration of OCH results in a higher ratio of IL-4 to IFN-γ secretion by NKT cells and in vivo it results in the prevention of experimental autoimmune encephalomyelitis and collagen-induced arthritis. Also the production of IL-10 by OCH-stimulated NKT cells is beneficial in Th1-mediated autoimmune diseases (Table 1.1). It has been suggested that the differences in cytokine profiles may be due to the length of the sphingosine chain. Due to the shorter lipid chain of OCH, it has a lower affinity for CD1d. This results in a shorter TCR ligation which results in a poor transcription of the NF-κB family member transcription factor c-Rel. c-Rel is identified as essential for IFN-γ production by NKT cells. It was observed that c-Rel is transcribed in α-GalCer-stimulated, but not in OCH-stimulated NKT cells so this may be responsible for the reduced IFN-γ gene transcription. An alternative hypothesis is that the different ligands reach different cell types upon injection. Th1 responses may result from the uptake of the ligand by for example IL-12 secreting cells such as certain DC-subsets, whereas Th2 responses may result from the uptake by non-IL-12 producing cells such as B cells or other DC-subsets.

The aforementioned antigens are all synthetically made. Recently some microbial ligands were found. Glycosphingolipids in the cell wall of Sphingomonas strongly activate NKT cells and NKT cells clear the infection of these bacteria. In addition, more recently also a self-ligand for NKT cells was found. The lysosomal glycosphingolipid iGb3 may activate mouse Vα14 and human Vα24 NKT cells. This iGb3 seems to be important during the natural development of NKT cells.
It is present within the thymus and is necessary during selection and is CD1d dependent.\textsuperscript{179,180} In addition to the microbial and lysosomal ligands, recently some plant- and bacteria-derived glycolipids were found to be possible ligands. Especially the phosphatidyl choline (PC) and phosphatidyl ethanolamin (PE) parts of these glycolipids are shown to activate NKT cells.\textsuperscript{181,182} Whether all these "natural" ligands affect the Th1/Th2 cytokine secretion of NKT cells and whether they can be used as treatment for diseases needs further investigation.\textsuperscript{183}

Because of the activation of NKT cells by (glyco)lipids and the fact that (glyco)lipids are a major part of the problems in atherosclerosis, NKT cells may play a role in atherosclerosis. Several groups are active in this field and both α-GalCer and OCH were used to study the effects of NKT cell activation on atherosclerosis. The first publication on a possible role for NKT cells in atherosclerosis showed that apoE\textsuperscript{−/−} mice crossed with CD1d\textsuperscript{−/−} mice exhibit a 25% reduction in lesion size. α-GalCer treatment resulted in a burst of cytokines and induced a 50% increase in lesion size.\textsuperscript{184} These data are confirmed by the study of Major et al.\textsuperscript{185} In both studies IL-4 and IFN-γ production was increased after treatment. LDLr\textsuperscript{−/−} mice crossed with CD1d\textsuperscript{−/−} mice also have smaller lesions compared with LDLr\textsuperscript{−/−} mice.\textsuperscript{186,187} It is however mentioned that this effect was only present during the initial stages of atherosclerosis. There was an effect after 4 weeks of diet, while the effect of CD1d deficiency was lost after 8 and 12 weeks of diet.\textsuperscript{187} Nakai et al. repeatedly injected apoE\textsuperscript{−/−} mice with both α-GalCer

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**Figure 1.6: Production of both Th1 and Th2 cytokines by activated NKT cells.** Upon recognition of ligands such as α-GalCer and OCH by CD1d on APCs, NKT cells may produce a large diversity on both Th1 (IFN-γ) and Th2 (IL-4, IL-10 and IL-13) cytokines. These cytokines may induce bystander activation of Th1 and Th2 cells and this results in the initiation or prevention of different inflammatory diseases.
### Table 1.1: The effect of α-GalCer treatment on different inflammatory diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mouse strain</th>
<th>Model</th>
<th>Treatment protocol</th>
<th>Treatment Outcome</th>
<th>Proposed mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1D</td>
<td>NOD spontaneous</td>
<td>α-GalCer, multiple i.p.</td>
<td>protection</td>
<td>Th2 deviation, MDC recruitment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NOD spontaneous</td>
<td>α-GalCer + IL-7, multiple i.p.</td>
<td>superior protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NOD in situ transplant</td>
<td>α-GalCer, multiple i.p.</td>
<td>protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td>EAE</td>
<td>B6 MOG(35-55)</td>
<td>α-GalCer, multiple s.c./p.</td>
<td>protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 MOG(35-55)</td>
<td>α-GalCer, multiple s.c./p.</td>
<td>slight exacerbation</td>
<td>competition for CD1d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 MOG(35-55)</td>
<td>α-GalCer, single i.p.</td>
<td>no effect or protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 MOG(35-55)</td>
<td>APC + α-GalCer + B7.2 blockade, single i.p.</td>
<td>protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 MOG(35-55)</td>
<td>APC + α-GalCer + CD40 activation, single i.p.</td>
<td>exacerbation</td>
<td>Th1 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 MOG(35-55)</td>
<td>OCH, single i.p. or p.o.</td>
<td>protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PL/PL MBP</td>
<td>α-GalCer, multiple s.c./p.</td>
<td>protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B10.PL MBP(AC1-9)</td>
<td>α-GalCer, co-immun., single i.p.</td>
<td>exacerbation</td>
<td>Th1 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B10.PL MBP(AC1-9)</td>
<td>α-GalCer, pre-immun., i.p.</td>
<td>protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B10.PL MBP(AC1-9)</td>
<td>α-GalCer, multiple s.c./p.</td>
<td>slightly delay, increased mortality</td>
<td>Th1 bias of NKT cells</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>B6, SJL/J collagen</td>
<td>α-GalCer, multiple i.p.</td>
<td>weak or no protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6, SJL/J collagen</td>
<td>OCH, multiple i.p.</td>
<td>protection</td>
<td>Th2 deviation</td>
<td></td>
</tr>
<tr>
<td>SLE</td>
<td>NZB/W F1 spontaneous</td>
<td>α-GalCer, multiple i.p. at adult age</td>
<td>exacerbation</td>
<td>Th1 deviation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRL-lpr/lpr spontaneous</td>
<td>α-GalCer, multiple i.p.</td>
<td>decreased dermatitis, no effect on lupus nephritis</td>
<td>regulatory cytokines</td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>B6 DSS</td>
<td>α-GalCer, multiple i.p.</td>
<td>weak protection</td>
<td>regulatory cytokines</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 ApoE^{-/-} spontaneous</td>
<td>α-GalCer, multiple i.p.</td>
<td>exacerbation</td>
<td>NKT cell recruitment and activation in lesion</td>
<td></td>
</tr>
</tbody>
</table>

In most inflammatory diseases, multiple injections of α-GalCer or OCH resulted in a protection against the disease. In studies that observed an exacerbation of the disease the ligand was injected only once or was injected at adult age. The only exception in this table is atherosclerosis in which multiple injections of α-GalCer accelerated the disease (adapted from van Kaer).175

and OCH during the early phase of atherosclerosis. Both ligands caused an increase in plaque size which was dedicated to an increase in the production of pro-atherogenic cytokines (IL-4 and IFN-γ).186 The effect of α-GalCer on the development of atherosclerosis and the produced pro-atherogenic cytokines by NKT cells suggests that CD1d-dependent NKT cells may play a specific role in atherogenesis. Furthermore, the results with CD1d^{-/-} mice suggest that the absence of CD1d-reactive NKT cells attenuates atherosclerotic lesion formation in mice during early fatty streak formation.

In addition, NKT cells are found in the aortic arch of LDLr^{-/-} and apoE^{-/-} mice fed a Western-type diet.184-186,188 In advanced human lesions NKT cells have been identified in the regions bordering the shoulder of the lipid core and the fibrous cap,189 especially there where DCs are present.50 In these regions, NKT cells represent 2% of the total lymphocyte population.50,189
Immunotherapies as treatment for atherosclerosis

Currently, many research groups around the whole world are struggling to find the best treatment for atherosclerosis. From multiple sides the disease process is “attacked”. Lipid lowering therapies, promotion of physical activity, blood-pressure lowering methods and many other techniques are still not sufficient enough to win this battle. With statins and anticoagulants the development of atherosclerosis and its consequences can be inhibited, but still the disease is manifest. Since the knowledge that atherosclerosis has an important inflammatory component, major research effort has been done to interrupt the inflammatory response and thereby reduce the severity of the inflammation and consequently the severity of atherosclerosis. Many approaches are focused on the role of certain cytokines or cell types. Depletion or overexpression of athero-promoting cytokines and athero-protective cytokines, respectively, is shown to be successful in atherosclerosis-prone LDLr-/- and apoE-/- mice (described above). Another upcoming technique to block key players of atherosclerosis is via active vaccination. Using a novel vaccination technique, antibodies to IL-12 can be induced which specifically block the function of IL-12 and protect against atherosclerosis in LDLr-/- mice. This vaccination strategy was based on a protein vaccine. Another protein vaccination against cholesteryl ester transfer protein (CETP) altered the lipoprotein profile in cholesterol-fed rabbits and reduced the lesion formation. Hauer et al. also showed that DNA vaccination against the vascular endothelial growth factor receptor 2 (VEGFR2) blocked the initiation and progression of atherosclerosis, whereas van Es et al. showed that DNA-vaccination against IL-17 blocked the initiation of atherosclerosis. Another important way to interrupt the disease process is by reducing the antigen-specific immune responses in atherosclerosis. This can be done by removing the antigens which is hard to achieve in humans since many antigens are self-proteins. Two other ways are immunization and tolerance induction to specific atherosclerotic antigens. Immunization with oxLDL protects against atherosclerosis in hypercholesterolemic rabbits, while immunization with MDA-LDL and apoB100 peptide sequences reduces atherosclerosis in LDLr-/- and apoE-/- mice, respectively. The different approaches used in this thesis are described below. First tolerance induction to auto-antigens of atherosclerosis is mentioned. This was already successful in several other publications in which oral and nasal tolerance to HSP65 and β2GPI protected against lesion formation in LDLr-/- mice, while induction of neonatal tolerance to oxLDL reduced atherosclerosis in apoE-/- mice. Secondly, a new vaccination approach is introduced to reduce atherosclerosis. This vaccination technique uses DCs as transfer units of specific antigens and ligands. The principles of DC vaccination will be clarified in the second part. At last different strategies to activate NKT cells are mentioned.

Tolerance induction

It is known that Tregs can be induced via mucosal tolerance induction. The classical definition of mucosal tolerance is a specific suppression of cellular
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and/or humoral immune responses to an antigen by administration of the antigen via a mucosal surface. It is a form of peripheral tolerance that evolved to treat external agents that gain access to the body via a natural route. It is of unique immunologic importance, because it is a continuous natural immunologic event driven by exogenous antigens. Many processes, both Th1 and Th2-driven, can be suppressed or induced by mucosal tolerance induction. To induce mucosal tolerance, the antigen has to be administered to a mucosal surface. This can be achieved in two ways; nasal or oral administration. The main pathways in both tolerance mechanisms are the same. It is shown that oral tolerance induction mainly induced Th3 cells, while nasal tolerance induction mainly resulted in the activation of Tr1 cells. In recent studies it is shown that oral tolerance induction also results in an increase in CD4+CD25+ cells expressing either CTLA-4 and/or FoxP3. In this thesis the method of oral tolerance induction is used in two chapters and therefore this section of the introduction is focused on this method of tolerance induction.

The effects of oral tolerance induction are mainly determined by the dose of the antigen. Low dose feeding results in Treg activation in the gut, while high doses result in deletion/anergy of the antigen specific T cells. The antigen-specific Tregs are activated in the gut due to presentation of the fed antigens by tolerogenic DCs. As mentioned earlier, the Tregs mainly producing IL-10 and TGF-β, may migrate to lymphoid organs and target organs to suppress the disease both in an antigen-specific and an antigen-non-specific way. The induced Tregs are specific for the fed antigen and can recognize the antigen in the lymphoid organs or target organs and secrete their cytokines locally. Additionally they can suppress the disease by releasing antigen-non-specific cytokines, which is called bystander suppression.

The method of tolerance induction to treat atherosclerosis is used in several studies. Harats et al. used HSP65 and feeding of certain doses of this antigen induced a specific immune suppression and a reduction in lesion size in LDLr−/−mice which were immunized with Mycobacterium tuberculosis or fed an atherogenic diet to induce atherosclerosis. The mechanism underlying this effect was however not clearly defined. Low doses of HSP65 reduced plaque size but without inducing a specific immune suppression. Maron et al. showed a reduction in atherosclerosis after nasal and oral administration of HSP65, but only nasal administration led to a change in T cell phenotype. In a study by George et al, β2GPI was used as atherosclerosis-specific antigen. Oral feeding of this antigen also resulted in a reduction of plaque size. They also show a lowered lymph node cell reactivity to β2GPI and an upregulation in anti-atherogenic cytokines but again the underlying method remains to be clarified.

DC vaccination

DCs are more and more used in vaccination strategies to treat not only cancer but also autoimmune responses in animal models. DCs can be isolated and cultured from different sources such as bone marrow, blood and lymphoid organs. The immature DCs can be loaded with different compounds ex vivo. Proteins, peptides and lipids can be taken up and presented via antigen presenting
molecules. DCs can also be loaded with DNA or mRNA, viral vectors, tumor cells and tumor cell lysates. Transferring peptide- or lipid-loaded DCs can result in the induction of a number of responses. In one study, DCs were pulsed with bovine collagen type II and injection of the DCs in mice protected against collagen-induced arthritis.\textsuperscript{205} The observed effect was dedicated to a decrease in the collagen-specific Th1-associated IgG2a response. Loading immature DCs with a peptide of glutamic acid decarboxylase and injection of these DCs in non-obese diabetic mice protected against type I diabetes.\textsuperscript{206} Furthermore and of importance for this thesis, lipids can be loaded on DCs. This may result in an activation of lipid-specific NKT cells and a prolonged production of IFN-$\gamma$ by these cells. These DCs were protective against several forms of cancer.\textsuperscript{207,208}

\textbf{α-GalCer and OCH administration}

As described above, α-GalCer and OCH are injected to activate NKT cells. In most studies on autoimmune diseases, both ligands are administered intravenously (i.v.), intraperitoneally (i.p.) or a combination of both (Table 1.1). One single injection of α-GalCer mostly results in the activation of NKT cells producing more Th1 than Th2 cytokines. This results in a exacerbation of different diseases in mouse models. Multiple injections however switch the NKT cell activation towards a Th2 phenotype resulting in protection against many autoimmune diseases except atherosclerosis (Table 1.1). The ligands for NKT cells can also be administered via the above described vaccination technique. Two studies showed that NKT cells can be activated by injecting α-GalCer loaded on mature dendritic cells. This resulted in a sustained expansion of NKT cells and a prolonged production of IFN-$\gamma$.\textsuperscript{207,208} Both ways of administering NKT cell ligands may be useful to modify the immune response in atherosclerosis.

\textbf{Outline of this thesis}

In this thesis several therapeutic strategies are used to treat atherosclerosis. All these strategies are focused on interruption of the harmful Th1 immune response in atherosclerosis. In all strategies there is a central role for the DCs. First of all DCs were used as a vaccination unit to induce an atheroprotective antibody response and to induce a protective NKT cell activation. Additionally, α-GalCer was used to treat atherosclerosis and since DCs express CD1d they will be very important in this process. Two studies are focused on the induction of oral tolerance to atherosclerosis-specific antigens. Tolerogenic DCs present in the Peyer’s patches in the gut are important regulators of the activation of Tregs after oral feeding of the antigens. In the last study, the faith of NKT cells after Western type diet feeding is described. DCs which engulf lipids can be responsible for the activation of NKT cells in atherosclerosis. Overall, the focus of this thesis was to develop novel experimental therapeutic strategies to treat atherosclerosis and to increase insight in the role of Tregs and NKT cells in atherosclerosis.

\textbf{Chapter 2} describes a study in which oral tolerance induction to oxLDL leads to an increase in oxLDL-specific Tregs in several organs and leads to an amelioration
of early atherosclerotic (30-71%) and advanced atherosclerotic lesions (45%). Furthermore, an increase in Tregs within the atherosclerotic lesion and an increased oxLDL-specific TGF-β production was observed.

Chapter 3 also describes a study in which oral tolerance induction was used to inhibit atherosclerosis. In this study it is shown that tolerance induction to HSP60 or an HSP60 peptide (253-268) reduces atherosclerosis with 81%. After treatment with HSP60, an increase in Foxp3+ Tregs was observed in several organs and the atherosclerotic lesion. In addition, treatment with HSP60 increased TGF-β and IL-10 production by HSP60 specific cells, while splenocytes from HSP60-treated mice show lower proliferation in response to HSP60.

Chapter 4 describes a study in which mice were treated with oxLDL-pulsed DCs. Atherosclerosis was dramatically reduced which may be due to an increased plaque stability, lowered plasma cholesterol levels and increased titers of anti-oxLDL IgG levels in serum of mice treated with oxLDL-pulsed DCs. This anti-oxLDL IgG participates in immune-complex formation and reduces foam cell development.

Chapter 5 describes a study in which the athero-protective effects of α-GalCer were investigated. In contrast with other studies, a reduction in atherosclerosis was found after multiple injections with α-GalCer in high fat diet fed LDLr-/- mice. This effect was not observed in apoE-/- mice. Splenocytes of LDLr-/- mice were more responsive to α-GalCer and Western-type diet feeding induced a more abundant increase in NKT cells in LDLr-/- mice when compared to apoE-/- mice.

Chapter 6 describes another DC-vaccination study in which mice were treated with OCH-pulsed DCs. OCH-pulsed DCs induce an activation of NKT cells, which are increased in the liver and blood. Additionally we observed a Th2 profile of cytokines in the spleen and a lowering of total plasma cholesterol after treatment with OCH-pulsed DCs.

Chapter 7 describes the search for natural ligands of NKT cells. Western-type diet feeding in LDLr-/- mice resulted in an increased number of NKT cells in the liver and spleen. Proliferation studies with LDLr-/- and LDLr-/-Jα281-/- mice showed that oxLDL or at least a part of it can activate NKT cells. However, a similar lesion size in LDLr-/-Jα281-/- and LDLr-/- mice fed a Western-type diet for 12 weeks was observed.

Chapter 8 discusses the findings of this thesis and future perspectives of these studies. Also the therapeutic implications will be evaluated.

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