

The potential use of dendritic cells in mouse models of atherosclerosis

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Therapeutic potential of dendritic cells in mouse models of atherosclerosis

Kim L.L. Habets

Therapeutic potential of dendritic cells in mouse models of atherosclerosis

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Chapter 1

Introduction

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1. Cardiovascular disease

Cardiovascular disease (CVD) refers to a group of diseases related to the cardiovascular system. An unhealthy lifestyle and physical inactivity result in elevated blood pressure, raised cholesterol levels, increased blood glucose, overweight, and obesity. All these features are recognized as the most important behavioral risk factors of CVD. CVD can be subdivided in three groups: heart failure, aneurysm formation and atherosclerosis.

1.1 Heart Failure

Heart failure is a chronic, progressive condition in which the heart muscle is unable to pump enough blood through the heart to meet the body's needs for blood and oxygen. At first, the heart tries to make up for this by enlarging the heart chambers, developing more muscle mass and increasing the heart rate. Also blood vessels are narrowed to keep blood pressure up and blood is diverted away from organs to maintain flow to the heart and brain. However, these temporary measurements are not sufficient and finally persons experience fatigue and breathing problems. Heart failure can involve the heart's left side, right side or both sides. However, it usually affects the left side first. Two types of left-sided heart failure are discriminated. The first is systolic failure in which the left ventricle loses its ability to contract normally with a reduced ejection fraction as result. The second is diastolic failure in which the ventricle loses its relaxation capacity but the ejection fraction is not affected. Right ventricular heart failure usually occurs as a result of left-sided failure. As blood flow out of the heart reduces, venous blood returning to the heart backs up, causing congestion in the body's tissue which often results in edema. The prevalence of heart failure increases with age. The condition is not curable but with the appropriate medical treatment and a drastic change in lifestyle, the condition is manageable.[1, 2]

1.2 Aneurysm formation

An aneurysm of the abdominal aortic (AAA) is a focal balloon-like dilation of the aorta (Figure 1). The expansion of the aorta is caused by a chronic inflammation which leads to degradation of the elastic lamina and collagen and subsequent further dilatation and even rupture.[3, 4] AAA is a common disease which mainly affects men aged 60 or older. Mostly, aneurysms remain undiscovered and they grow approximately 0.3 cm each year. Rupture of aneurysm has a high mortality and now ranks number 12 of the most common causes of death in the Western world.[5] Risk factors for the development of AAA are age, sex, smoking, and atherosclerosis. Treatment of AAA mainly focuses on the control of risk factors or, when the AAA is growing larger then 5.5 cm, the surgical removal of the aneurysm such as stent graft repair or complete replacement of the diseased part with an artificial vascular graft. Suitable pharmacological treatment is currently unavailable, although inhibitors of Angiotensin converting enzyme (ACE) and statins have been shown to prevent progression or rupture of an aneurysm in patients.[6]

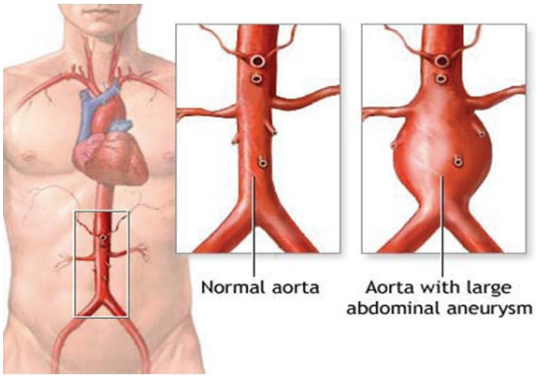


Figure 1: Abdominal Aortic Aneurysm (AAA)
AAA is a localized dilatation of the abdominal aorta that exceeds the normal diameter of 2 cm by more than 50%

1.3 Atherosclerosis

Atherosclerosis, the main underlying pathology of cardiovascular disease, is a multifactorial, chronic, auto-immune-like disease initiated by lipid accumulation and inflammatory processes.[7-9] Atherosclerosis affects the medium and large-sized arteries and is already initiated during early adolescence. The flow-limiting stenosis can cause discomfort to the patient but most clinical manifestations are stroke and acute myocardial infarction. In the last decades, a wide variety of risk factors have been identified. Behavioral factors such as smoking, high fat intake, stress, and a sedentary life style are identified, while clinical symptoms such as hypertension, diabetes, obesity, and dyslipidemia generate an increased risk for cardiovascular disease.[10-12]

Current therapies mainly focus on decreasing risk factors. Lowering cholesterol levels with statins or aspirin, improving the life style and lowering blood pressure but also surgical interventions such as bypasses or dottering in combination with stent placement have resulted in a strong decline in the number of deaths caused by cardiovascular disease. However, atherosclerosis continues to be the leading cause of morbidity and mortality in the Western world and this will probably not change in the near future because of the alarming rising numbers of diabetic and obese people.[13] Therefore, the search for disease-specific therapies is still a clinically highly relevant challenge.

2. Atherosclerotic lesion development

2.1 Lesion initiation

Atherosclerotic plaque formation occurs mainly at high risk areas such as branching points and bifurcations of the arterial tree.[14-16]Low shear stress, oscillatory or turbulent flow in combination with atherogenic risk factors, cause a dysfunction of the endothelial layer which results in an increased endothelial permeability. In hypercholesteremic patients, Low Density Lipoprotein (LDL) infiltrates into the intima. The interaction with oxidatively and enzymatically modified LDL leads to the expression of adhesion molecules by endothelial cells. Increased expression of vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin result in the rolling and firm adhesion of leukocytes to the endothelial layer.[17, 18] Adhesion is followed by transmigration through the endothelial layer into the subendothelial space. This process of diapedesis is induced by the presence of chemotactic proteins, also known as chemokines. Several chemokines are produced by endothelial cells, smooth muscle cells, and intimal macrophages. One of the best characterized chemokines is Monocyte chemotactic protein-1 (MCP-1) which promotes the recruitment of both monocytes and T cells.[19] In addition, modified LDL is also chemotactic for monocytes and T cells, attracting more cells into the intima.

Under the influence of cytokines such as Tumor Necrosis Factor-α (TNF-α), Macrophage Colony Stimulating Factor (M-CSF), Interferon-γ (IFN-γ), Interleukin-1 (IL-1) which are produced in the inflamed intima, monocytes will differentiate into macrophages (MΦ).[20, 21] During differentiation, macrophages upregulate the expression of scavenger receptors and Toll-Like Receptors (TLRs). Oxidized LDL (OxLDL) is internalized by tissue macrophages through scavenger receptors and if cholesterol cannot be secreted from the cell, it accumulates as cytosolic droplets. Ultimately, this results in the formation of lipid-rich foam cells. At this stage, the lesion is called a "fatty streak".

2.2 Lesion progression

Fatty streaks, which are clinically asymptomatic, can progress into intermediate lesions. Vascular smooth muscle cells (VSMCs) migrate from the media into the lesion under the influence of growth factors and start to cover the foam cells. These pre-atheroma plaques further progress to advanced and more complex lesions. The intimal lipid pool will expand into a large necrotic core containing cell-free lipids and cholesterol crystals. VSMCs and fibroblasts start to produce collagen and proteoglycans forming a thick fibrous cap which covers the necrotic core. The lesions will further develop by outwards remodeling, resulting in a reduced vessel lumen. At this stage the lesion is called an advanced plaque (Figure 2).

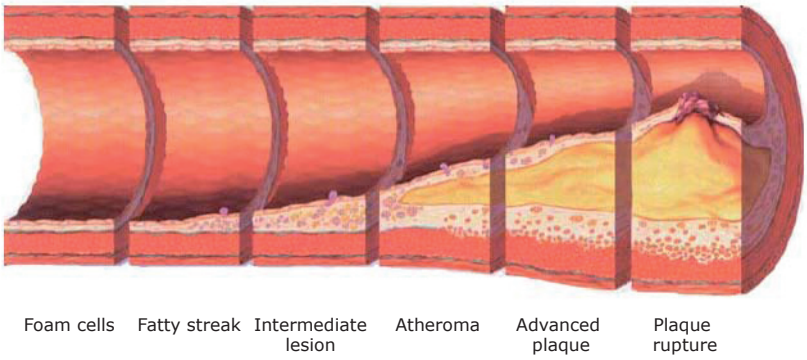


Figure 2: The different stages of atherosclerotic lesion development.
Early atheroma's are formed during the second decade of life. After this initial phase they can develop into stabilized or vulnerable plaques. When a vulnerable plaque ruptures, a thrombus can be formed which can block off the blood supply to for example the heart leading to myocardial infarction and possibly death.

These advanced plaques are exposed to mechanical forces of the blood (shear stress) leading to fibrous cap erosion. In addition, IFN-γ produced by Th1 T cells will inhibit the proliferation of VSMCs and their production of collagen[22, 23] while, matrix metalloproteinases produced by macrophages attribute to the thinning of the fibrous cap.[24, 25] These processes lead to the formation of vulnerable plaques which contain many leukocyte subsets (e.g. T cells, macrophages, dendritic cells, mast cells) that can produce chemokines and pro-inflammatory cytokines.[26, 27] Upon rupture of the thinned fibrous cap, the content of the necrotic core will be released into the bloodstream leading to coagulation and the formation of a thrombus. The thrombus can occlude arteries leading to lethal myocardial infarction, stroke or acute limb ischemia.

3. The different leukocyte subsets involved in atherosclerosis

3.1 Monocytes/Macrophages

As described, monocytes and macrophages play an important role in the initiation of atherosclerosis. Next to the VSMCs, macrophages are the most abundant cell type within the lesion. Macrophages are key players in inflammation and innate immune responses as they form the first line of defense against pathogens. Macrophages express pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) which recognize pathogen-associated molecular patterns (PAMPs). These PAMPs can be ligands derived from pathogens but also bacterial toxins such as lipopolysaccharide (LPS), stress proteins such as heat shock protein 60 (HSP60) and oxLDL. Macrophages also express scavenger receptors such as CD36, SR-BI, SR-A and CD68 which allow them to take up oxLDL. This leads to the activation of the macrophages and to their transformation into foam cells. Upon activation,

macrophages produce free oxygen radicals, proteases, complement factors and cytokines. More importantly, macrophages may also initiate adaptive immune response by presenting antigens to T cells. All these characteristics/qualities make them important key players in the process of atherosclerosis.

3.2 Dendritic cells

Dendritic cells (DCs) are the most potent and versatile antigen-presenting cells (APCs). Like the macrophages, immature DCs (ImDCs) are positioned as sentinels in the periphery where they frequently come into contact with antigens. DCs have a superior capacity to engulf antigens and after the uptake of antigens, DCs rapidly migrate towards secondary lymph nodes.[28] During migration DCs process the antigens within the lysosomal compartment and undergo a process of maturation. Therefore, upon arrival in lymphoid organs, DCs are capable to present epitopes on MHC class II, class I molecules or CD1d. These molecules are expressed on the cell surface and are required for antigen presentation. In combination with high levels of costimulatory molecules, DCs are then fully competent to drive T-cell activation.[29, 30] Consequently, DCs have the capacity to bring naïve or central memory T cells into contact with peripheral antigens. It has been established that there are many different subpopulations with distinct functions which are related to their location and their maturation state.[31]

Table 1: Organ distribution of mouse dendritic cells

Organ	$CD8^-$ DCs	$CD8^+$ DCs	$CD8^{interm}$ DCs	Langerhans cells	Dermal DCs	$B220^+$ DCs
Thymus	low	+	-	-	-	+
Spleen	+	+	-	-	-	+
Lymph Node	+	+	+	-	-	+
Peyer's Patch	+	+	-	-	-	+
Skin	-	-	-	+	+	-
Liver	+	+	-	-	-	ND

+: present; -: not present; ND: not dermined

Effective priming of naive T cells results in a clonal expansion and differentiation into effector and memory T cells. DCs are capable of activating cells from both the innate and adaptive immune system (Figure 3). The activation of $CD4^+$ or $CD8^+$ T cells and the activation of Natural Killer T (NKT) cells through the presentation of lipid antigens on CD1d[32] can have major implications in the treatment of atherosclerosis. Another important feature of DCs is their capacity to sense the environment and to translate these cues.[33] Consequently, depending on the local cytokine surrounding, DCs can determine the fate of the T cells in either Th1, Th2 or cytotoxic T lymphocytes. IL-12, IL-18 production by DCs polarizes T cells into pro-inflammatory Th1 T cells. IL-4, -5 -9 and IL-13 lead to Th2 cells. In addition to the classical Th1 and Th2 cells, DCs can also induce regulatory T cells (Tregs). Tolerogenic DCs produce large amounts of IL-10, TGF-β and low levels of IL-12 and induce regulatory T cells. Furthermore, DCs have also been implicated in the induction of the newly discovered IL-17 producing CD4 T cells (Th17 cells).[34-36] The roles of these T cell subsets in atherosclerosis will be discussed later.

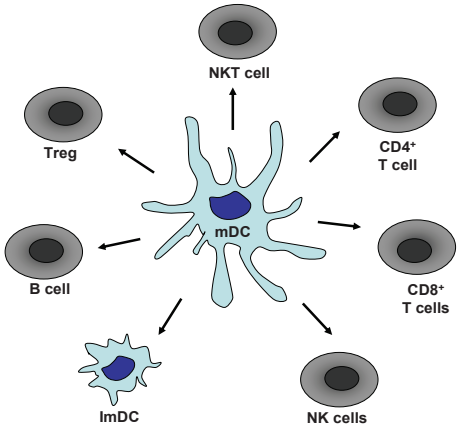


Figure 3: A central role for mature DCs in stimulation of cells of the innate and adaptive immune system

DCs in the steady-state (in the absence of maturation stimuli) can also induce tolerance. When DCs are not fully activated after the uptake of antigens, they express low levels of costimulatory molecules and will induce anergy of the antigen-specific T cells. This is an important process that leads to the deletion or suppression of e.g. autoreactive T cells that have escaped thymic deletion.[37]

Taken together, DCs play an important role in all different routes of the immune system and they form a promising alternative tool for vaccination, immunotherapy in cancer, autoimmunity and allergy. The potential to use DCs in immunotherapy will be discussed in more detail in chapter 4: Treatment strategies.

3.3 Dendritic cells and atherosclerosis

The induction of oxLDL-specific T cells has raised the question whether DCs play a role in the pathogenesis of atherosclerosis. Dendritic cells in the arteries were first described in 1998 by Bobryshev.[38] In non-diseased arteries small numbers of DCs are present in the intima immediately beneath the endothelial layer in the so-called vascular associated lymphoid tissue (VALT). The VALT consists of DCs, macrophages and T cells and may play an important role in the screening of the vascular tissue for potential harmful antigens such as oxLDL. DCs are also present in the adventitia where they are located in proximity to neovascularization suggesting that neovascularization acts as a tract for the entry of DCs as well as other inflammatory cells.[38, 39] During lesion development, plaques become enriched by DCs invading from the adventitia into the intima. In addition to these resident DCs, lesions may also be invaded by blood $CD11c^+$ cells attracted by chemokines such as fraktalkine, produced by the inflammatory component of the plaque. Recently it has been shown that deficiency in the fraktalkine receptor resulted in decreased atherosclerosis development and decreased numbers of DCs in the atheroma.[40] In the arterial wall DCs engulf antigens and migrate towards regional lymph nodes where they activate T cells. Maintaining the ability of DCs to migrate outwards of the lesion may diminish plaque size through the clearance of harmful

antigens. However, it has been shown that dyslipidemia impairs DC migration and may thereby contributes to the activated immune status in the plaque.[41] Indeed, it has been shown that not all DCs migrate towards the lymph nodes and that some DCs stay in the intima where they may activate resident T cells.[38] Studies have shown that PAF and 18:1 lysophosphatidic acid are responsible for retaining DCs within the plaque.[41, 42] In addition, it has been shown that entrapment of activated DCs in the adventitia leads to a loss of local tolerance against vascular self-antigens thereby attributing to a more activated immune status.[43]

A number of antigens that have been identified as risk factors for atherosclerosis can modulate the immature DCs present in the healthy vessel or developing lesions. Nicotine activates DCs and augments their capacity to stimulate T cell proliferation and cytokine secretion.[44] Modified lipoproteins like oxLDL have been shown to trigger DC maturation *in vitro* and oxLDL increases the adherence of DCs to the vascular endothelial cells.[45, 46] In addition, DCs are also activated by HSP60[47] and by glycated proteins that are formed during diabetes.[48, 49] The highest intensity of DCs within the human lesions has been found in areas enriched in T cells such as the shoulder region of rupture prone plaques. The DCs not only form clusters with convential T cells but also with NKT cells.[38, 50] DC-induced T cell activation may further stimulates vascular inflammation and it has been suggested that clustering of DCs with T cells in rupture prone regions is associated with plaque destabilization.[26, 50] Indeed, recently Ranjit *et al.* reported that patients with unstable angina pectoris have functionally altered DCs.[51] The DCs have an enhanced expression of CD86 and are more able to stimulate T cells compared to DCs from healthy individuals.[51] In contrast, statin-treated patients have lower numbers of DCs in the lesions compared to patients without statins.[52] *In vitro* studies have shown that statins reduce DC adhesion and transmigration through the endothelial layer, inhibit DC maturation and diminishes their antigen-presenting capacities thus possibly contributing to the beneficial effects of statins in atherosclerosis.[50, 53] Interestingly, statins also diminish the production of pro-inflammatory cytokines by DCs but this effect is dependent on the maturation state.[52]

3.4 T cells

The presence of T cells in human atherosclerotic lesions has already been described in 1985.[54] A T cell infiltrate is observed in all stages of lesion formation. Within human plaques, most of the T cells are $CD45RO^+$ effector/memory T cells and the number of T cells increases with the severity of the coronary syndrome.[55] The majority of these T cells are $CD4^+$ T cells which recognize epitopes presented by MHC-II molecules. $CD4^+$ T cells reactive for disease-related antigens such as oxLDL, HSP60 and Chlamydia have been found in human plaques.[56-58] $CD8^+$ cytotoxic T cells are also identified in atherosclerotic lesions but there is contrasting data about their role in atherosclerosis. The absence of $CD8^+$ T cells in $ApoE^{-/-}$ $CD8^{-/-}$ mice has no effect on lesion development, while in another study increased lesion size is observed after $CD8^+$ activation.[59, 60] The role of $CD4^+$ T cells in atherosclerosis is more apparent. Ablation of $CD4^+$ T cells attenuates lesion formation in $ApoE^{-/-}$ mice while adoptive transfer of $CD4^+$ T cells to $RAG^{-/-}$ mice which lack T and B cells,

accelerates atherogenesis.[61-63]

Classically, $CD4^+$ T cells are categorized into 2 subtypes based on their cytokine profile. T-helper 1 (Th1) T cells produce mainly pro-inflammatory cytokines (IFN-γ IL-1, IL-2 and TNF-α) and induce a cellular immunity while T-helper 2 (Th2) T cells produce mainly anti-inflammatory cytokines (IL-10, IL-4, IL-5, IL-9, IL-13) and induce a humoral response. Analysis of the cytokine expression in atherosclerotic plaques suggests a dominance of Th1 cells.[64] This is supported by the fact that C57Bl/6 mice (more prone to develop a Th1-response) develop fatty streaks on high cholesterol diet while BALBc mice (prone to develop a Th2 response) are protected for atherosclerotic development.[65, 66] In addition, most of the $CD4^+$ T cells within the plaque are shown to produce TNF-α, IFN-γ and IL-2.[64] These pro-inflammatory cytokines activate other cell types present in the lesion which will lead to the production of even more pro-atherogenic cytokines. Deficiency of IFN-γ, IL-12 or IL-18 or the IFN-γ receptor in $ApoE^{-/-}$ mice leads to attenuated atherosclerosis. On the other hand, injections of recombinant IFN-γ, IL-12 and IL-18 in $ApoE^{-/-}$ mice accelerated the disease. [67-70] These treatments have an indirect effect on T cells as IL-12 and IL-18 are mainly produced by DCs and are key activators of IFN-γ producing T cells. Also vaccination against IL-12 is successful in preventing atherosclerotic development due to the lowering of IFN-γ production.[71]

Th2 cytokines are considered mostly to be atheroprotective. IL-5 has been shown to induce B-1 cells which produce protective oxLDL-specific IgM antibodies and IL-5 deficiency in $LDLr^{-/-}$ mice led to increased lesion size. Because of its ability to inhibit Th1 differentiation, IL-4 is protective in many Th1-driven immune diseases but the role of IL-4 in atherosclerosis is more complex. $IL\text{-}4^{-/-}$ $ApoE^{-/-}$ and $LDLr^{-/-}$ dKO mice have reduced lesion size. [72, 73] Wanrooij *et al.* showed that $LDLr^{-/-}$ mice deficient in OX40 signaling have reduced lesion size due to lower IL-4 levels, higher IL-5 levels and higher levels of protective anti-oxLDL IgM antibodies.[74] IL-4 is able to activate mast cells, which leads to apoptosis of VSMCs, reduced collagen production and increased production of proteases. In addition, IL-4 induces MMP-12. Together these events lead to destabilization and rupture of the plaque. [75, 76] It appears that the function of IL-4 is dependent on the stage of atherosclerosis being atheroprotective in the early stages and pro-atherogenic in later stages. The role of IL-13 in atherosclerosis has not been investigated but several properties of IL-4 are shared with IL-13. Taken into account the dual role of IL-4 and IL-13, switching the immune system towards a Th2 profile does not necessarily lead to a reduction in atherosclerosis.

3.5 Regulatory T cells

Regulatory T cells (Tregs) form of a diverse group of lymphocytes with regulatory properties. Since their discovery, Tregs have been implicated in immunosuppression associated with cancer and chronic infections. At the same time, deficiency in Tregs has been considered as a cause for immune hyperactivity, allergic reactions and auto-immune diseases. Tregs can suppress activation, proliferation, differentiation and even effector functions of multiple immune cells such as $CD4^+CD25^-$ (both Th1 and Th2), $CD8^+$, B cells, natural killer cells and dendritic cells.[77-79] The best defined Tregs are the naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs. The forkhead transcription factor Foxp3 controls several genes such as CD25 (IL-2 receptor-α), cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-inducible tumor necrosis factor (GITR) which are important in the development of Tregs. Naturally occurring Tregs leave the thymus and are thought to be self-antigen specific. Several potential mechanisms of Treg suppression have been suggested. The production of immunosuppressive cytokines such as IL-10 and cell surface bound transforming growth factor-β (TGF-β) or cell-cell contact dependent suppression mediated through CTLA-4 are the main means of action of natural Tregs.[80-84] An additional suppression mechanism is granzyme or perforin-dependent killing of effector cells.[85, 86]

Other $CD4^+$ populations also have regulatory properties. These regulatory T cells are called adaptive Tregs and do not always express Foxp3. Adaptive Tregs can be divided into Tr1 and Th3 cells based on their cytokine production and their surface markers. Tr1 cells are $CD4^+CD25^-Foxp3^-$ which develop under the influence of IL-10 and mainly produce IL-10. Th3 cells on the other hand, produce TGF-β, are $CD4^+CD25^+$ and may express Foxp3 upon activation. Tregs play an important role in atherosclerosis. Foxp3 has been found both in the aorta of $ApoE^{-/-}$ mice as in human atherosclerotic plaques.[87, 88] The injection of depleting anti-CD25 antibodies increases lesion size, showing that Tregs have a protective role in atherosclerosis.[89] In another study anti-CD3 Fab antibodies were used to induce the activation and proliferation of Tregs and showed to be protective for atherosclerosis in $LDLr^{-/-}$ mice.[90] Also the induction of oral tolerance against HSP60 and oxLDL attenuates atherosclerosis by an induction of Tregs. [91, 92]

Ample data describe the protective role of the cytokines produced by Tregs (IL-10 en TGF-β) on atherosclerosis. TGF-β inhibits the maturation of DCs and the upregulation of MHC-II molecules thereby reducing T cell stimulation and differentiation.[93, 94] In addition, TGF-β may target endothelial cells, DCs, macrophages and VSMCs.[95, 96] Recruitment of leukocytes into the lesion, foam cell formation, and T cell activation are therefore possibly all targeted by TGF-β. Moreover TGF-β is a potent inducer of collagen production and the production of inhibitors of MMPs therefore contributing to a more stable plaque phenotype. Also, $ApoE^{-/-}$ TGF-β receptor (TβRII) dKO mice show a five-fold increase in plaque size.[97] TGF-β neutralizing antibodies, soluble TGF-β receptors and adenovirus-mediated delivery of TGF-β demonstrated that TGF-β is a potent inhibitor of atherosclerosis.[98-100]

IL-10 is, in addition to Tregs, also produced by DCs and macrophages and has an inhibitory effect on lesion size and promotes plaque stabilization. Overexpression of IL-10 leads to reduced IFN-γ production. In addition, it also inhibits apoptosis contributing to a stable plaque phenotype. IL-10 has been found to be produced in the plaque where it inhibits oxLDL-induced production of IL-12 by human macrophages.[101] Many studies demonstrated the protective effect of IL-10. Deficiency of IL-10 in both ApoE$^{-/-}$ [102] and C57BL/6 mice resulted in larger plaques.[103, 104] In addition, endogenous IL-10, locally or systemically administrated IL-10 and T cells overexpressing IL-10 are protective against atherosclerosis.[103-105] Furthermore, IL-10 enhances TGF-β production and vice versa. Taken together, these data suggest an atheroprotective role of Tregs and their regulatory cytokines in atherosclerosis.

3.6 Natural Killer T cells

The term NKT cells was first used to define a subset of T cells in mice that express the natural killer (NK) cell-associated marker NK1.1 (CD161) and an invariant T cell receptor (TCR) composed of Vα14 and Jα18 subunits paired with a restricted set of Vβ chains (Vβ8, Vβ7 and Vβ2).[106-108] This property distinguishes NKT cells from normal T cells that use a diverse TCR repertoire. In mice, NKT cells are found most frequently in the liver (30-50% of T lymphocytes) and bone-marrow (20-30%). They represent a smaller fraction of T lymphocytes in spleen (3%), blood (4%), lymph nodes (0.3%) and lung (7%).[106] Within the liver they reside in the sinusoids where they screen for possible ligands presented to them via CD1d. NKT cells are activated by glycolipid antigens presented in the MHC class I like molecule CD1d. The most common NKT cell is the invariant NKT cell which is CD1d dependent and expresses NK1.1.

NKT cells can be subdivided in CD4^{+}CD8^{-} and CD4^{-}CD8^{-} (double negative) cells which differ in their functional properties. The CD4^{+} subset potently induces both Th1 and Th2 whereas the DN NKT cells selectively produce IFN-γ and TNF-α, preferentially upregulate perforin in response to IL-2 and IL-12 and are not CD1d-dependent. The most striking property of NKT cells is their capacity to secrete large amounts of cytokines (IFN-γ, TNF-α, IL-4, IL-2, IL-5, IL-10, IL-13 and GM-CSF) within minutes after TCR stimulation. This distinguishes them from naïve T cells that require more time to secrete cytokines after stimulation.[109] An important consequence of the large amount of cytokines produced by NKT cells is the bystander activation of neighboring NK cells, B cells and DCs as well as the activation of CD4^{+} and CD8^{+} T cells.[110] Upon activation, NKT cells also have cytotoxic properties through the release of perforin and granzymes and by the expression of FasL.[111, 112]

There is still little known about the natural ligands of NKT cells. Recently some microbial ligands were found: Glycosphingolipids in the cell wall of *Sphingomonas* strongly activates NKT cells.[113] Also some plant and bacteria-derived glycolipids such as phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) parts of these glycolipids are shown to activate NKT cells.[114, 115] Nevertheless, the most studied ligand for NKT cells is the marine sponge derived α-galactosylceramide

(α-GalCer). NKT cell activation through a-GalCer, leads to a burst of IL-4 and IFN-γ production. However, it is still not clear how repeated injections of α-GalCer can suppress Th1-mediated autoimmune diseases such as type 1 diabetes, experimental autoimmune encephalomyelitis and colitis in mice.[116-118] It seems that timing and dosage of α-GalCer have a significant impact on the outcome of the disease. This illustrates the possible risk of augmenting an unwanted Th1 response that can worsen the disease. Interestingly, there are some variants of α-GalCer that have decreased Th1 cytokine production. OCH is one of these ligands. OCH has a truncated sphingosine chain and therefore has a lower affinity for CD1d. This results in a shorter TCR ligation and activation of the transcription factor c-Rel. c-Rel is identified as being essential in IFN-γ production by NKT cells.[119] *In vitro* stimulation of NKT cells with OCH leads to a higher ratio of IL-4 to IFN-γ production. Also *in vivo* OCH-stimulation of NKT cells results in the prevention of experimental autoimmune encephalomyelitis, diabetes and collagen-induced arthritis. [120-122]

Taken into account that NKT cells are activated by (glyco) lipids and the fact that lipids are the main culprits in atherosclerosis, NKT cells are an interesting target in atherosclerosis. Several groups are active in this field and both α-GalCer and OCH are used to study the effect of NKT cell activation in atherosclerosis. ApoE$^{-/-}$ CD1d$^{-/-}$ dKO mice have a 25% reduction in plaque size.[123] LDLr$^{-/-}$ CD1d$^{-/-}$ KO mice have 50% smaller lesions compared to LDLr$^{-/-}$ mice when fed a western type diet for 4 weeks.[124, 125] Nakai *et al.* repeatedly injected ApoE$^{-/-}$ mice with both α-GalCer and OCH during the early stages of atherosclerosis.

Both ligands induced an increase in lesion size due to the production of IL-4 and IFN-γ.[125] These data suggest that the absence of NKT cells attenuates lesion formation during early fatty streak formation. In addition, NKT cells have been found in the aortic arch of both LDLr$^{-/-}$ and ApoE$^{-/-}$ mice after feeding a western type diet.[123, 125, 126] Also in advanced human lesions, NKT cells have been identified in the shoulder region of the plaque. In this region, NKT cells represent 2% of the total T lymphocyte population.[26] Furthermore, adaptive transfer of bone-marrow from NKT cell-enriched (Vα14Jα18 TCR transgenic) mice into LDLr$^{-/-}$Rag$^{-/-}$ KO mice resulted in a 70% increase in plaque size compared to mice which have been reconstituted with bone-marrow from NKT cell deficient mice (CD1d$^{-/-}$). These studies demonstrated that NKT cells are proatherogenic even in the absence of exogenous stimulation and that the NKT cell activity is likely associated with endogenous lipids presented to them in atherosclerotic plaques.[127]

4. Treatment strategies

4.1 Research models

Over the last decade, the mouse has become the predominant model for research in atherosclerosis. Mice are small, have a short life-time, are relatively cheap and many transgenic and knock-out mice are currently available. All of the current mouse models of atherosclerosis are based on perturbations of lipoprotein metabolism through dietary and/or genetic manipulations. Although hyperlipidemia is necessary for the development of atherosclerosis, mouse models have demonstrated that many non-lipid factors can also influence the severity and characteristics of lesions.[128]

Because C57BL/6 mice only develop small fatty streaks after being fed a high cholesterol cholate containing diet, hyperlipidemic mice such as LDLr$^{-/-}$, ApoE$^{-/-}$ and ApoE*3-Leiden transgenic mice have been created to study atherosclerosis. In humans there are 3 major isoforms for ApoE (apoE2, -3, and -4). ApoE3 is the most common allelic form. ApoE*3-Leiden mice develop a type III hyperlipidemia like phenotype and this occurs despite the continued presence of endogenous ApoE.[129] The LDLr$^{-/-}$ and ApoE*3-Leiden mouse only develop lesions after feeding a Western-type diet. ApoE$^{-/-}$ mice however, are already hypercholesteremic while being fed a chow diet and spontaneously develop large plaques with age. The LDLr$^{-/-}$ mice have higher cholesterol levels after western-type diet and develop lesions at a slower pace then ApoE$^{-/-}$ mice. Nevertheless, lesion development does still take months to develop in these mice models. Therefore strategies have been developed to speed up the progress. The model of collar induced atherosclerosis has been described and validated.[130] Upon placement of the mildly constrictive silicone collars around carotid arteries, shear stress is being induced by the narrowing of the carotid arteries. Proximal from the collar a significant 2.5-fold decrease in shear stress has been observed, while the shear stress remained high within the collar (unpublished data). This is in line with the observed expression of KLF2, which is a transcription factor that is related to the function of endothelial cells. KLF2 expression was high within the collar and almost not detectable proximal to the collar. The low KLF2 expression proximal to the collar coincides with the enhanced expression of VCAM-1 and is exactly the site were the atherosclerotic lesions are formed.[131]

To study aneurysm formation Daugherty *et al.* developed a mouse model using ApoE$^{-/-}$ mice that are infused with Angiotensin II (AngII).[132] AngII-infusion did not influence blood pressure, serum cholesterol or the distribution of lipoproteins. However, AngII infusion increased plaque size and induced abdominal aortic aneurysms.[132] So to induce aneurysms, we put ApoE$^{-/-}$ mice on a western-type diet for 4 weeks and than mice receive an osmotic pump releasing 1.44 mg/kg AngII per day. Pumps are placed subcutaneously in anesthetized mice through a small incision in the back. After 4 weeks of AngII infusion, mice are sacrificed and aneurysm formation can be studied.

4.2 Dendritic cell vaccination

As we discovere more about the importance of immunity in atherosclerosis, it becomes increasingly interesting to apply this knowledge in new treatments for atherosclerosis. Several studies have revealed the relevance of oxLDL-immunization in different animal models yielding between 40 and 70 % reduction of lesion size.[133-140] This suggests that oxLDL is a promising target for immunotherapy and that it could be possible to develop a vaccine based on antigens present in oxLDL. However, oxLDL is a very complex particle containing many different epitopes that potentially be pro-atherogenic or atheroprotective. Furthermore, the therapeutical potential of anti-oxLDL antibody treatment is currently being investigated in a phase II clinical trails (Genentech, Roche). The remarkable capacity of DCs to induce an immune response and the rapidly increasing knowledge of DC biology offers a new perspective for the generation of immunotherapeutic interventions. DCs can be isolated from different sources such as the blood, bone-marrow and lymphoid organs. DC-based vaccines used in early patient studies are safe and have minimal side-effects. The usefulness of DC-based immunotherapy has been established in many different diseases ranging from cancer, collagen-induced arthritis, type I diabetes and experimental autoimmune encephalomyelitis.[141-149] An increasing number of preclinical studies are also focusing on the use of immature DCs to induce antigen-specific tolerance. DCs in steady state or immature DCs can silence immunity by either inducing T cell anergy or by expanding regulatory T cells. This feature of silencing the immune response suggests a potential role for tolerogenic DCs in the treatment of auto-immune and chronic inflammatory diseases.[149-152]

5. Outline of this thesis

In this thesis we have explored the effectiveness of DC-immunotherapy in atherosclerosis. We have used different strategies to target the immune component in different stages of atherosclerosis. First we used DCs as a vaccination strategy to induce a protective antibody response through the injection of oxLDL-pulsed DCs or to target NKT cells by the injection of OCH-pulsed DCs. Next we assessed the potential of DC-immunotherapy in a model of established atherosclerosis. We also evaluated the effects of a disturbed TGF-β signaling in DCs and the subsequent effects on atherosclerosis by using $ApoE^{-/-}$ which have a dysfunctional TGF-β Receptor II under the CD11c promoter. Next, we were interested in the effect of foam-cell formation on the antigen-presenting capacity of DCs and macrophages. Therefore we studied the effect of oxLDL-loading on antigen uptake and antigen presentation by DCs and macrophages. Finally, by depleting or inducing Tregs we investigated the potential role of regulatory T cells in a mouse model for aneurysm formation.

6. References

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Chapter 2

Vaccination using oxLDL-pulsed DCs reduces atherosclerosis in LDLr$^{-/-}$mice

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Abstract

Modification of lipoproteins plays an important role in the development of atherosclerosis. Oxidatively modified low-density lipoprotein (oxLDL) has a number of pro-inflammatory effects, whereas immunization with various forms of oxLDL is able to reduce atherosclerosis. The uptake of modified LDL by dendritic cells (DCs) and the presentation of epitopes thereof may form an important step in the immunomodulatory effects of LDL. In this study we transferred oxLDL-pulsed mature DCs (mDCs) to $LDLr^{-/-}$ mice and examined the effects on atherosclerosis. Bone-marrow derived DCs were cultured for 10 days in the presence of granulocyte-macrophage colony-stimulating factor. Immature DCs were matured by lipopolysaccharide and pulsed with copper oxidized LDL. These DCs were transferred three times to $LDLr^{-/-}$ mice before the induction of atherosclerosis by Western-type diet feeding. Transfer of oxLDL-pulsed mDCs resulted in an 87 % reduction in carotid artery lesion size ($P<0.01$) with a concurrent increase in plaque stability while treatment with mDCs pulsed with the atherosclerosis irrelevant antigen, ovalbumin, did not influence lesion size or stability. Furthermore, the vaccination procedure resulted in the induction of oxLDL-specific T cells with a reduced Th1 profile and an increase in oxLDL-specific IgG levels, which contributed to a reduction in foam cell formation. In conclusion, these data indicate that vaccination with oxLDL-pulsed mDCs provides a novel and powerful strategy for the immunomodulation of atherosclerosis.

Introduction

Atherosclerosis is a slowly progressing disease that develops at sites of lipid accumulation in large and medium sized arteries, which can lead to infarction of the heart or the brain. Over the past several years, accumulating data has identified a key role for inflammation in atherosclerosis and both innate and adaptive immune responses are involved.[1-5] Several antigens have been implicated in the initiation of immune responses during atherosclerosis including exogenous infectious pathogens such as *Chlamydia pneumoniae*, and cytomegalovirus but also endogenous proteins such as heat-shock proteins and β2-glycoprotein-Ib.[6-8] The most intensively studied endogenous antigen is oxidized low-density lipoprotein (oxLDL). Oxidation of lipoproteins in the arterial intima, followed by their uptake by macrophages and subsequent foam cell formation, plays an important role in the development of atherosclerosis. In addition, oxidation of LDL results in many structural modifications of apoB-100 and thus the formation of many neo-epitopes, which renders the modified LDL immunogenic and leads to both a cellular and humoral response. Since the different epitopes of oxLDL induce atherogenic immune responses, it is attractive to modulate the immune response towards oxLDL. Also, a number of studies show that immunization against oxLDL reduces atherosclerosis in several animal models.[9-11]

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system.[12-14] Immature DCs differentiate from bone-marrow progenitors or circulating blood monocytes. They reside in the blood stream or peripheral tissues where they survey incoming pathogens. An interaction with pathogens induces maturation during which DCs generate MHC-peptide complexes and upregulate the expression of costimulatory molecules such as CD40, CD80 and CD86. These changes render the DCs fully competent to activate T cells. Several studies showed that oxLDL induces several changes, characteristic for DC maturation, including a higher expression of costimulatory molecules and the increased ability to stimulate T cells. [15, 16]

Due to their potent capacity to stimulate T cells, DCs are being investigated in vaccine and therapy approaches.[17, 18] We aimed to assess the use of oxLDL-pulsed mature DCs (mDCs) as an immunotherapy for atherosclerosis. DCs obtained from the bone-marrow can be pulsed *ex vivo* by inducing maturation in the presence of oxLDL. In this study we show that oxLDL-pulsed mDCs induce oxLDL-specific T cells, a lowered Th1-response and an increased production of oxLDL-specific antibodies and this accumulates in a reduction in lesion size. In conclusion, these data indicate that vaccination with oxLDL-pulsed mDCs triggers the immune system against oxLDL, which proves to be beneficial for the treatment of initial phases of atherosclerosis.

Materials and methods

Media and reagents

Cell culture medium for the dendritic cells was IMDM (Cambrex, Belgium) supplemented with 8 % FCS (PAA, Germany), 100 U/ml streptomycin/penicillin (PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μM β-mercaptoethanol. LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave *et al*.[19] The isolated LDL was dialyzed against phosphate buffered saline (PBS) with 10 μM EDTA (pH 7.4) for 24 hours at 4°C and oxidized by exposure to 10 μM $CuSO_4$ at 37°C for 20 hours as previously described.[20] For the proliferation studies and the induction of foam cells we used RPMI 1640 medium supplemented with 2 mM L-Glutamine, 10% FCS and 100 U/ml streptomycin/penicillin (PAA, Germany) (cRPMI). For the culturing of Bone-marrow derived macrophages cRPMI with 20% FCS was used.

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male LDLr-/- mice and UBC-GFP mice were from Jackson Laboratories on a C57Bl6 background and bred in-house. Male C57BL/6J mice were from Charles River Laboratories. Mice were kept under standard laboratory conditions and were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Mice were 10-12 weeks old at the start of the experiment. Diet and water were administered ad libitum.

Generation and injection of BM-DCs

For each injection time-point, bone marrow cells were isolated from the tibia and femora of 3 C57BL/6 mice. Cells were immediately pooled and cultured during 10 days in complete IMDM in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). After 10 days of culture, immature DCs were activated with 1 μg/ml of lipopolysaccharide (LPS from Salmonella typhosa, Sigma Aldrich, Zwijndrecht, The Netherlands) during 24 hours. Simultaneously, DCs were pulsed with or without 7.5 μg/ml of copper-oxidized LDL or 1 mg/ml of ovalbumin. Purity and functionality of the DCs were assessed using flow cytometry. CD11c was used as a specific DC marker (purity > 90%). Functionality was determined by the expression of several costimulatory molecules (CD40, CD80, CD86) and the expression of MHC-II and CD1d. Mice were injected intravenously (8, 6 and 3 days prior to atherosclerosis induction by Western-type diet feeding) with 200 μl of PBS (n = 8) or 1.5 x 10^6 DCs (oxLDL-pulsed or unpulsed mDCs) in 200 μl PBS (n = 11). DCs from the UBC-GFP mice were identically isolated and cultured.

Assessment of atherosclerosis

Male $LDLr^{-/-}$ mice were injected 3 times intravenously (1 injection every other day) with 1.5 x10^6 DCs or saline prior to Western-type diet feeding. After 3 weeks of diet, atherosclerosis was induced by bilateral perivascular collar placement (2 mm

long, diameter 0.3 mm) around both carotid arteries and continuous Western-type diet feeding.[21] Seven weeks after collar placement, mice were sacrificed and tissues were harvested after *in situ* perfusion using PBS and subsequent perfusion using formalin. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use.

Histological analysis and morphometry

Transverse cryosections were prepared in a proximal direction from the carotid bifurcation. Cryosections were stained with hematoxylin (Sigma Aldrich, Zwijndrecht, The Netherlands) and eosin (Merck Diagnostica, Germany) or with hematoxylin and Oil-Red-O. Hematoxylin-eosin stained sections of right carotid artery (5 µm) and Oil-Red-O stained sections of heart valves (10 µm) were used for morphometric analysis of atherosclerotic lesions as described previously.[21] Corresponding sections were stained immunohistochemically with antibodies against a macrophage specific antigen, (MOMA-2, polyclonal rat IgG2b, Research Diagnostics Inc, NJ) or were stained for collagen fibers using the Masson's Trichrome method (Sigma Aldrich, Zwijndrecht, The Netherlands). The images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, Cambridge, UK).

Proliferation assay

Spleens from mice treated with oxLDL-pulsed mDCs (n=5) or mDCs (n=5) were isolated and smashed through a 70 µm filter. Red blood cells were lysed using 0.83% NH_4Cl in 0.01 M tris/HCL, pH 7.2. Splenocytes were cultured in triplicates at $2x10^5$ cells/well in the presence or absence of oxLDL (5 µg/ml) in complete RPMI 1640 (cRPMI). Concanavalin A (ConA, Sigma-Diagnostics, MO) (2 µg/ml) was used as a positive control. After 24 hours cytokine levels were determined in the supernatant according to the manufacturer's protocol (Mouse Th1/Th2 ELISA, Ebioscience, Belgium). Parallel cultures were pulsed for an additional 16 hours with 1 µCi/well of ^{3}H-thymidine (Amersham Biosciences, The Netherlands) and the amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R) as the number of disintegrations per minute (DPM).

Foam cell formation

To obtain macrophages, bone-marrow cells from C57BL/6 mice were resuspended in complete RPMI supplemented with 20% FCS and 30% of L929-conditioned medium (source for monocyte-colony-stimulating factor, M-CSF). After 7 days of culture, macrophages were seeded in Lab-Tek chamber slides (BD Falcon, The Netherlands) ($0.8 x10^6$ cells/well) in cRPMI. Cells were starved for 4 hours after which a mixture of oxLDL (10 µg/ml) + an equal volume of mouse serum (2 fold diluted, sera from 2 mice were pooled) was added. This mixture of oxLDL and mouse serum was made 30 minutes before addition to the cells. After overnight culture, cells were fixed using zinc formal Fixx, stained for lipids using Oil-Red-O and counter stained with hematoxylin. The amount of Oil-Red-O staining was corrected for the number of cells as indicated by the hematoxylin staining. 8 random fields per condition were analyzed using the Leica DM-RE microscope and

LeicaQwin software.

Cholesterol assay
Blood was collected at several time points during the experiment by tail vein bleeding. Plasma was obtained after centrifugation and total plasma cholesterol levels were measured using enzymatic procedures and a spectrophotometer (Roche Diagnostics, The Netherlands). Precipath was used as an internal standard (Boehringer, Mannheim, Germany). The cholesterol distribution over the different lipoproteins was analyzed by fractionation using a Superose 6 column (3.2 x 30 mm, Smart-System, Pharmacia).

Cytospin
After 24 hours of incubation in the presence of oxLDL, DCs were centrifuged for 5 min at 500 rpm using the Thermo Shandon Cytospin 4. After centrifugation, slides were fixed for 30 minutes using zinc formal Fixx (Shandon, Pittsburgh, USA). Lipid loading in the DCs was visualised using Oil-Red-O staining.

Flow Cytometry
After the culture period, dendritic cells or macrophages were stained with the appropriate Ab (CD11c, CD11b, F4/80, MHC-II, CD40, CD80, CD86 and CD1d, all obtained form Ebioscience, Belgium) to check purity or the maturation status.
After sacrificing the mice, blood, spleen, liver and lymph nodes were isolated (n=5 per group). Single cell suspensions were obtained by smashing the cells through a 70 µm cell strainer (Falcon, The Netherlands). Blood and spleen cells were lysed using 0.83% NH_4CL in 0.01 M Tris/HCL pH 7.2. Mononuclear cells from the liver were isolated from non-parenchymal cells using Lympholyte (Cederlane, Canada). Subsequently 300.000 cells were stained with antibodies (Ebioscience, Belgium) in the presence of 1 % mouse serum. For the detection of subclasses specific Ab, spleen cells were treated as stated above and stained with IgG1 and IgG2c (BD Bioscience, Belgium). FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software.

ELISA for IgM and IgG antibodies against LDL, MDA-LDL and Cuox-LDL
Antibodies against LDL, MDA-LDL and Cuox-LDL were determined by ELISA. MaxiSorp 96 well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µg native LDL or oxLDL in 100 µl PBS at 4°C. Plates were washed 5 times with 0.01M Tris, 0.15 M NaCl and 0.05% Tween20 (pH 8.0). Mouse serum was added in duplicate at a 1:50 dilution in incubation buffer (0.1 M Tris, 0.3 M NaCl and 0.05% Tween20 (pH 8.0) overnight at 4°C. After washing, plates were incubated with either alkaline phosphatase-labelled anti-mouse IgM or IgG (Jackson Immuno-Research, Pennsylvania) both at a 1:4000 dilution in incubation buffer for 1 hour at 37°C. After washing, substrate (1 mg/ml disodium p-nitrophenyl phosphate, Sigma, The Netherlands) was added. After 2 hours at room temperature, absorbance was read at 405 nm.

ELISA for detecting subclasses specific antibodies

IgG1 and IgG2c levels were detected using the Mouse MonoAb ID kit (Zymed Laboratories Inc., South San Francisco, USA) according to manufacturer's instructions. Briefly, high binding plates were coated overnight with either Cuox-LDL (5 µg/ml) or ovalbumin (10 µg/ml). Serum samples were 1:1 diluted in PBS. Absorbance was read at 405 nm.

ELISA for detecting cytokines

Levels of IFN-γ, IL-10 and IL-4 were measured in the supernatants of the ConA stimulated spleen using the Th1/Th2 Ready-Set-Go Kit (Ebioscience, Belgium). Detection limits are 15 pg/ml; 30 pg/ml and 4 pg/ml, respectively. In short, triplo cultures from the spleen cells treated with ConA were pooled and cytokine levels were determined in the undiluted supernatant following the manufacturer's instructions.

Statistical analysis

Values are expressed as mean ± SEM. Data were analyzed with either a parametric or non-parametric ANOVA when comparing three groups or a two-tailed Student's t-test or the Mann-Withey U test when comparing two groups. Statistical analysis was performed using the Instat3 software. Probability values of $P<0.05$ were considered significant.

Results

Effect of oxLDL on dendritic cells

We investigated whether DCs engulfed oxLDL and if the uptake of lipoproteins affected the maturation of DCs. Increasing concentrations of oxLDL (up to 20 µg/ml) were added to immature DCs. After 24 hours of incubation we stained the intracellular lipid accumulation in the DCs with Oil-Red-O and analyzed for surface markers by flow cytometry. We found that increasing oxLDL concentrations induced an increasing lipid accumulation, but oxLDL levels exceeding 7.5 µg/ml induced a concentration dependent increase in cell death and a lowering of the expression of the DC-specific markers (data not shown). Therefore we chose a concentration of 7.5 µg/ml which induced optimal lipid loading and minimal cell death. The addition of oxLDL to both imDCs and mDCs resulted in lipid accumulation in more than 95% of the DCs (Figure 1B and 1C) compared to the immature control incubated DCs (Figure 1A). During maturation, DCs upregulate the expression of costimulatory molecules and the maturation level of DCs can thus be followed by flow cytometry for several specific markers (CD40, CD80, CD86, MHC-II, CD1d and CCR7). We treated immature DCs with the TLR4 agonist, LPS (1 µg/ml) in the absence or presence of oxLDL (7.5 µg/ml). Treatment with LPS resulted in a typical mature DC cell surface phenotype showing high expression of CD11c (a specific marker for mouse DCs), CCR7 (necessary for migration towards lymphoid organs) MHC-II, CD1d and the costimulatory molecules CD40, CD80, CD86. The addition of oxLDL during maturation had no effect on the expression level of these molecules and thus did not affect the maturation state of the DCs (Figure 1D).

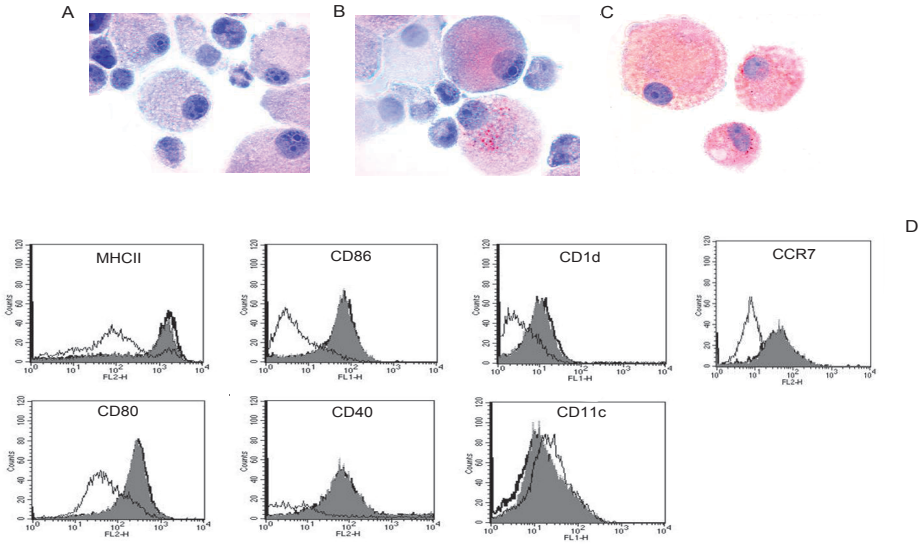


Figure 1: The effect of oxLDL on dendritic cells
Immature DCs were treated with LPS (1 µg/ml) in the absence or presence of oxLDL (7.5 µg/ml). After 24 hours, DCs were collected. Oil-Red-O staining of DCs: immature DCs in the absence of oxLDL (A), in the presence of oxLDL (B) and mature DCs in the presence of oxLDL (C). Figure D: Expression levels of surface markers analyzed by flow cytometry on immature DCs (dotted line), mature DCs (thick line) and mature oxLDL-pulsed DCs (grey).

Dendritic cells home towards lymphoid organs after *i.v.* injection

To determine the fate of DCs *in vivo*, we cultured DCs from UBC-GFP mice, which have a high and constitutive expression of GFP, and injected 1.5 x 10^6 DCs into $LDLr^{-/-}$ mice. We injected both mature unpulsed and mature oxLDL-pulsed GFP-DCs. 72 hours after injection, mice were sacrificed and GFP expression was determined in different tissues using flow cytometry. While there were almost no GFP^+ DCs present in the circulation, DCs did migrate towards the lung and liver, and towards lymphoid organs such as spleen, mediastinal and inguinal lymph nodes (Figure 2). Mature unpulsed and mature oxLDL-pulsed DCs displayed an identical distribution pattern.

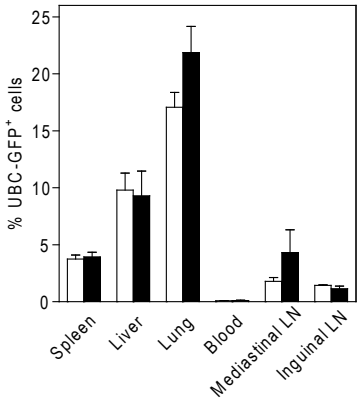


Figure 2: Homing of GFP^+-DCs
1.5 x 10^6 mature GFP^+-DCs (white bars, n=4) and mature oxLDL-pulsed GFP^+-DCs (black bars, n=4) were injected *i.v.* into $LDLr^{-/-}$ mice. Mice were sacrificed after 72 hours. The percentage of GFP^+ cells in different organs are shown.

Vaccination using oxLDL-pulsed mDCs attenuates atherosclerotic lesion formation

To evaluate the effect of vaccination using oxLDL-pulsed mDCs on de novo atherosclerosis, LDLr$^{-/-}$ mice were injected intravenously 3 times (day –8, –6 and –3) with either PBS, mDCs, or oxLDL-pulsed mDCs. On day 0, mice were put on a Western-type diet. Three weeks thereafter, atherosclerosis was induced by perivascular collar placement around the carotid arteries as described[21] and Western-type diet was continued for seven weeks where after mice were sacrificed. Plaque formation in the carotid artery and the aortic root was analyzed using the hematoxylin/eosin staining and hematoxylin/Oil-Red-O staining, respectively. Plaque sizes in mice treated with PBS did not significantly differ from the plaque sizes upon treatment with mDCs. In addition cholesterol levels and morphology of the plaque (see below) were also identical in these groups. Figure 3A-C shows representative examples of hematoxylin/eosin staining in the carotid arteries. Injection of oxLDL-pulsed DCs resulted in a 87 % reduction in plaque size in the carotid arteries (PBS: 48,578±9,231 μm^2; mDCs: 31,919±7,914 μm^2; mDCs+oxLDL: 4,023±504,1 μm^2; P_{anova}=0.0009) (Figure 3D). Also a concomitant 85 % reduction in intima/lumen ratio (PBS: 0.560±0.097; mDCs: 0.461±0.089; mDCs+oxLDL: 0.071±0.009; P_{anova}=0.0003) was observed (Figure 3E). In the aortic root the treatment with oxLDL-pulsed DCs induced a non-significant 24% decrease in plaque size (PBS: 228,667±32,747 μm^2; mDCs: 293,181±36,193 μm^2; mDCs+oxLDL 224,983±48,546 μm^2) (Figure 3F).

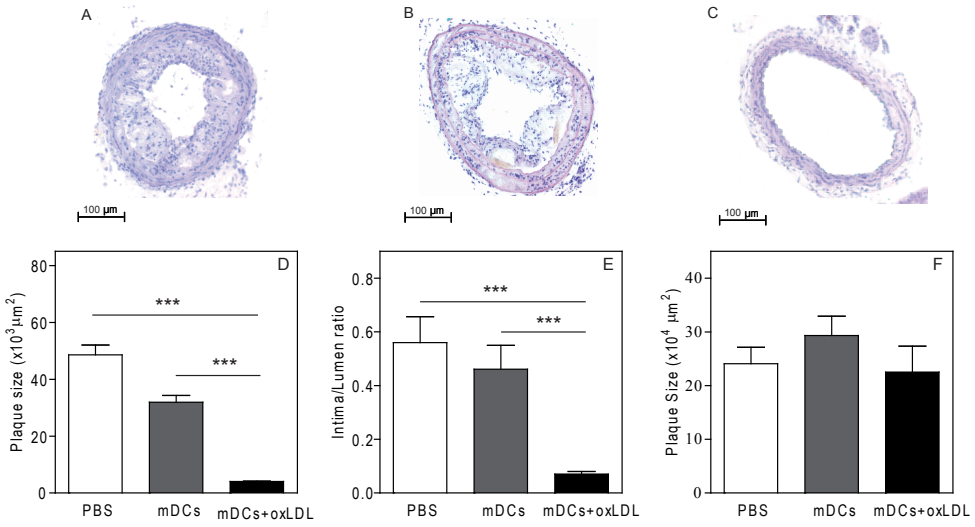


Figure 3: Effect of vaccination on atherosclerosis
Representative sections of lesions in the carotid arteries of PBS, (A; n=9), mDCs (B; n=11) and oxLDL-pulsed mDCs (C; n=11) treated mice. Lesion size (D) and intima/lumen ratio (E) in the carotid arteries were determined. Plaque size in the aortic root was quantified (F)(***P<0.001).

Plaque morphology was assessed by the number of macrophages and the presence of collagen fibers. When comparing mice in the oxLDL-pulsed mDCs group to mice in the mDCs group, we observed a 50% reduction in the MOMA-2/Intima ratio in both the carotid arteries (Figure 4A) and the aortic root (Figure 4D), while the collagen content of the intima was increased in both the carotid arteries (P_{anova}=0.0145) (Figure 4B) and the aortic root (Figure 4E). In addition, we calculated the MOMA-2/collagen ratio as an indicator for plaque stability. A significant 3.9- and 2.8-fold reduction was observed in both the carotid arteries (P_{anova}=0.05) and aortic root, respectively (P_{anova}=0.0097) (Figure 4C and 4F). This reduced MOMA-2/collagen ratio indicates a more stable plaque phenotype in the mice treated with oxLDL-pulsed DCs in both the carotid arteries and the aortic root.

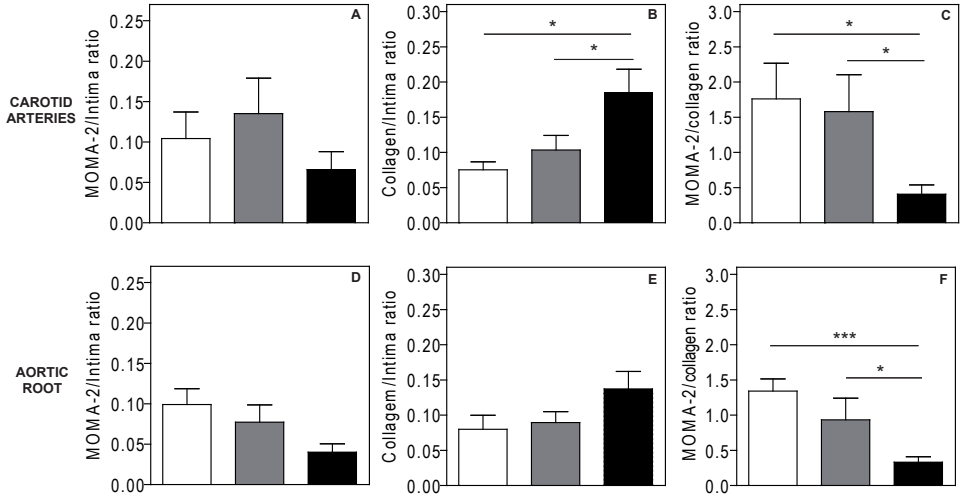


Figure 4: Plaque morphology
Plaque composition was assessed in the carotid artery (A-C) and in the aortic root (D-F). The amount of macrophages was determined as the MOMA-2 positive area per intima area, the amount of collagen was determined by quantifying the blue collagen staining of the Masson's Trichrome stain within the plaque (*P<0.05; ***P<0.001).

Since treatment with mDCs induced a slight, non-significant reduction in atherosclerosis, we additionally tested whether treatment with DCs pulsed with an irrelevant antigen affected atherosclerosis. To that end we treated mice with ovalbumin-pulsed DCs next to oxLDL-pulsed DCs and PBS treatment. Treatment with ovalbumin-pulsed DCs had no effect on plaque size in the carotid artery nor in the aortic root (Figure 5), while it also did not affect plaque composition at these sites (Figure 6). In contrast, oxLDL-pulsed DCs did lower the plaque size in the carotid artery. In addition, the macrophage/collagen ratio in the lesions in both the carotid artery and the aortic root were beneficially lowered in oxLDL-pulsed mDCs treated animals.

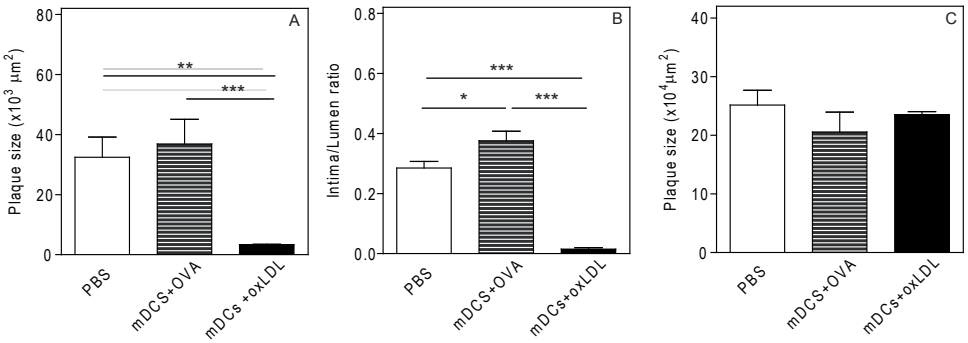


Figure 5: Effect of antigen-pulsed dendritic cells on lesion formation
Lesion size (A) and intima/lumen ratio (B) in the carotid arteries were determined. Plaque size in the aortic root was quantified (C) (**P*<0.05; ***P*<0.01; ****P*<0.001).

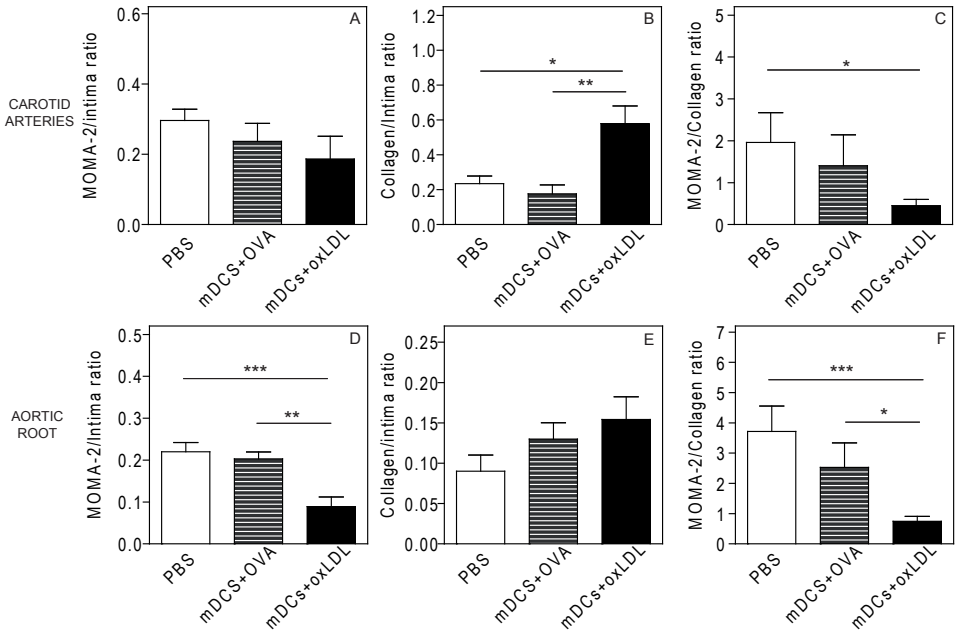


Figure 6: Effect of antigen pulsed dendritic cells on lesion stability
Plaque composition was assessed in the carotid artery (A-C) and in the aortic root (D-F) (**P*<0.05; ***P*<0.01; ****P*<0.001).

Effect on body weight and cholesterol levels

Both body weight and total cholesterol levels were measured at different time points during the experiment. Throughout the experiment, differently treated mice did not vary in body weight (data not shown). Also cholesterol levels were not affected by the DC-treatment. Only at sacrifice, mice treated with oxLDL-pulsed mDCs showed a 27% (P_{anova}=0.0066) lower plasma cholesterol level compared to mice treated with mDCs (PBS: 1538±158 mg/dl; mDCs: 1483±66 mg/dl; mDCs+oxLDL: 1076±101 mg/dl). This reduction did not result from cholesterol lowering in a particular class of lipoproteins (VLDL, LDL or HDL). Nevertheless, we calculated the area under the curve for the cholesterol burden during the entire experiment and again there was no difference between mice treated with mDCs or oxLDL-pulsed mDCs (data not shown). Also, cholesterol levels were not affected by treatment with ovalbumin-pulsed DCs (1492±115 mg/dl).

Induction of an oxLDL-specific humoral response after vaccination

Plasma samples from each mouse were obtained after sacrifice and IgG, IgM and subclass specific antibodies were determined. The T cell-dependent IgG antibodies against oxLDL were increased in mice vaccinated with oxLDL-pulsed DCs (Figure 7A) (P_{anova}<0.0001). Interestingly no significant effect was found on the IgG levels against MDA-LDL which indicates a highly specific response against oxLDL (Figure 7B). No differences in titers of IgM against oxLDL or MDA-LDL were detected (Figure 7C, D).

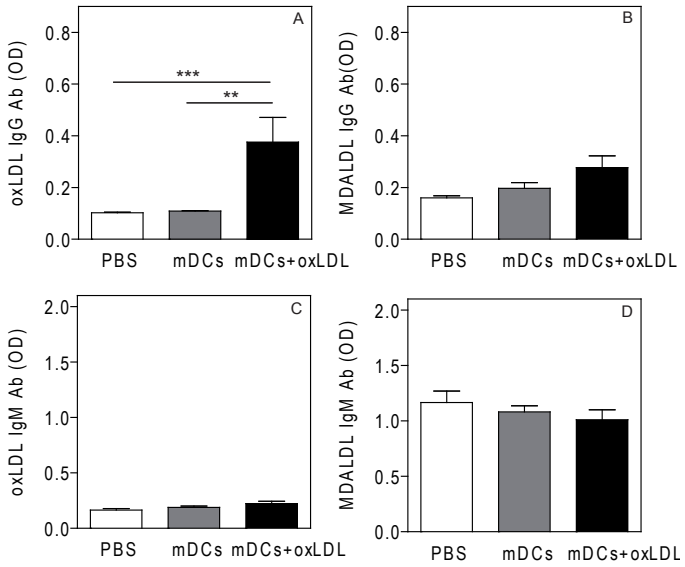


Figure 7: Humoral response against oxLDL and MDA-LDL

IgG and IgM titers were determined in serum in mice treated with PBS (n=9), mDCs (n=11) and oxLDL-pulsed mDCs (n=11) using an ELISA. IgG titers against oxLDL and MDA-LDL are presented in A and B, respectively. Figures C and D show IgM levels against oxLDL and MDA-LDL, respectively (**P<0.01; ***P<0.001).

We also analyzed whether IgG1 or IgG2c mainly contributed to the rise in anti-

oxLDL IgG levels. We observed significantly higher levels of both IgG1 and IgG2c in serum of mice vaccinated with oxLDL pulsed mDCs ($P<0.001$) as determined by ELISA (Figure 8A). Also, IgG1 levels in spleen were elevated ($P<0.01$) as determined by flow cytometry (Figure 8B). There were no differences in the number of IgM producing cells, the number of B cells nor the number of T cells in spleen was affected (data not shown). When we calculated the IgG2c/IgG1 ratio we observed a tendency, especially in the spleen, towards a reduced ratio indicative of a Th2 response ($P=0.08$) (Figure 8C and 8D). We observed that treatment with ovalbumin-pulsed mDCs induced a significant increase in the IgG titers for ovalbumin showing that the ovalbumin-pulsed mDCs effectively induced an immune response against ovalbumin (Figure 9). In addition, the IgG1/IgG2c ratio was not changed, suggesting that the Th1/Th2 ratio was not disturbed in mice treated with ovalbumin-pulsed mDCs.

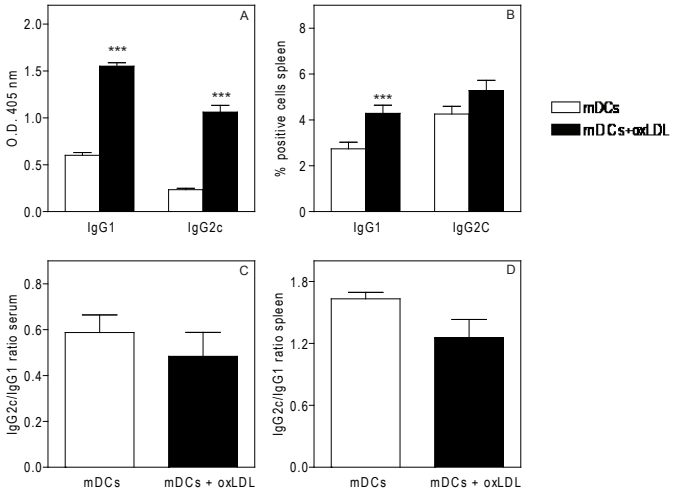


Figure 8: IgG1 and IgG2c response against oxLDL
IgG1 and IgG2c levels in serum (n=10) and spleen (n=5) were determined by ELISA or flow cytometry (A and B, respectively). The ratio of IgG2c to IgG1 in serum and spleen are depicted in C and D, respectively (***$P<0.001$).

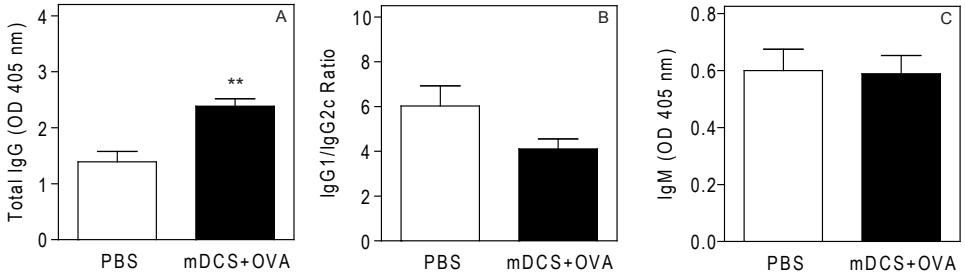


Figure 9: Induction of ovalbumin specific antibodies after injection of ovalbumin-pulsed DCs
Ovalbumin specific Total IgG; IgG1/IgG2c ratio and IgM titers were determined in serum in mice treated with PBS (n=12) mDCs and OVA-pulsed mDCs (n=13) using an ELISA (**$P<0.001$).

Induction of oxLDL-specific T cells in mice treated with oxLDL-pulsed mDCs

We have previously shown that oxLDL induced proliferation of $CD4^+$ T cells and not $CD8^+$ T cells or $CD19^+$ B cells in whole spleen cell cultures after oxLDL treatment (Figure 10).[22] To investigate whether we have induced an oxLDL-specific T cell response, we therefore re-stimulated splenic leukocytes *in vitro* with oxLDL (5 µg/ml) and determined the proliferation of splenic T cells by incorporation with ^{3}H-thymidine. The incubation of oxLDL to splenocytes resulted in a more pronounced T cell proliferation in mice treated with oxLDL-pulsed mDCs (1.8-fold increase, $P<0.01$) and the proliferation was significantly higher than the oxLDL-induced proliferation in the mice treated with mDCs ($P<0.05$) (Figure 11).

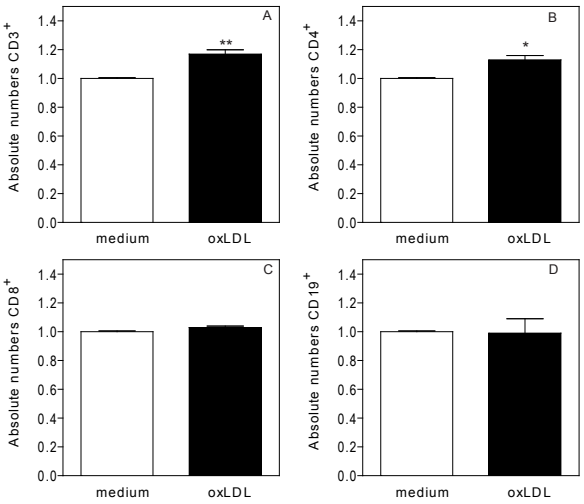


Figure 10: FACS data of spleen cells after oxLDL treatment
Spleen cells were cultured in the presence of oxLDL (5 µg/ml) during 48 hours (n=3) and absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells were determined. Then the stimulation index per spleen was calculated (*$P<0.05$; **$P<0.01$).

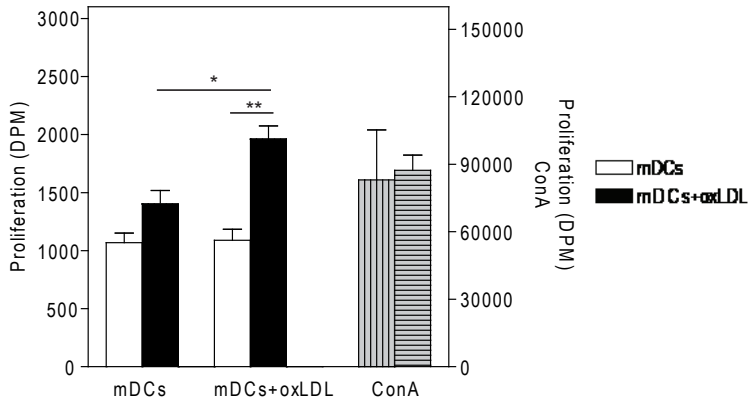


Figure 11: oxLDL-specific proliferation response
Total splenocytes were *in vitro* cultured in the absence (white bars) or presence of oxLDL (black bars). Proliferation was measured by ^{3}H-thymidine incorporation. ConA was used as a positive control (n=5;*P<0.05;**P<0.01).

Cytokine production after *in vitro* stimulation of splenic T cells

We also determined cytokine production by T cells from mice treated with oxLDL-pulsed DCs or unpulsed mDCs 24 hours after stimulation of splenocytes with ConA. We observed a 75% reduction in IFN-γ production by splenic T cells from mice vaccinated with oxLDL-pulsed mDCs compared to T cells from mice treated with mDCs (P=0.001) (Figure 12A). The level of IL-10 and IL-4 production by T cells was equal in both groups (Figure 12B and 12C, respectively).

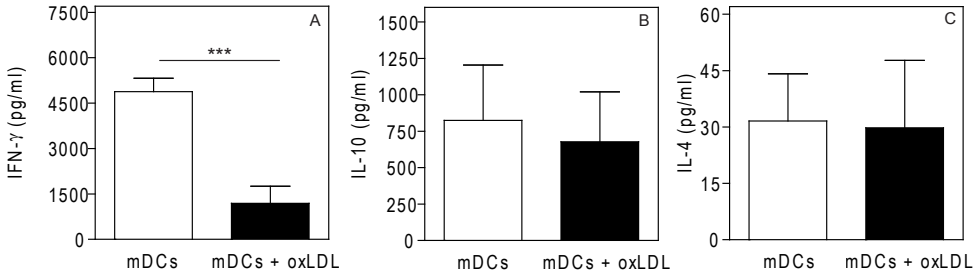


Figure 12: Cytokine production of splenocytes after *in vitro* stimulation
Total splenocytes were *in vitro* cultured with ConA during 24 hours. Cytokine levels were determined in the supernatants (n=5; ***P<0.001).

Cellular response to vaccination strategy

To evaluate the effect of DC treatment on the numbers of different subsets of leukocytes, blood was collected at key time points during the experiment (before injection of DCs, after each injection, before collar placement and at sacrifice) and FACS analysis was performed on leukocytes. We analyzed the numbers of T cells (CD4 and CD8), regulatory T cells ($CD4^+CD25^{high}$), NKT cells ($CD3^+NK1.1^+$). We observed no significant differences in the numbers of blood leukocytes at any time point during the experiment (Table1). We also performed FACS analysis on leukocytes in spleen, liver and mediastinal lymph nodes after sacrifice and again observed no differences between the various groups (data not shown).

Table 1: % of leukocytes in the blood after sacrifice

Markers	PBS	mDCs	mDCs+oxLDL
$CD4^+$	13,32 ± 2,24	12,01 ± 0,37	14,33 ± 1,96
$CD4^+CD25^{high}$	0,87 ± 0,11	1,28 ± 0,08	1,2 ± 0,17
$CD4^+CD62L^{low}$	8,02 ± 2,22	8,52 ± 2,23	8,29 ± 1,36
$CD8^+$	6,73 ± 0,54	6,88 ± 0,61	7,96 ± 0,78
$CD8^+CD62L^{low}$	3,21 ± 0,43	3,55 ± 0,6	3,77 ± 0,13
$CD3^+NK1.1^+$	1,98 ± 0,23	2,78 ± 0,51	2,36 ± 0,12

Inhibition of foam cell formation

Bone-marrow derived macrophages were cultured using L929-conditioned medium (source of M-CSF). The phenotype of the macrophages was tested using flow cytometry and the cultured cells were for more than 90% positive for the macrophage specific markers F4/80 and CD11b, whereas they were less than 10% positive for the DC marker CD11c (Figure 13A). Foam cell formation by the addition of oxLDL was 2.5-fold (P<0.001) lower when the macrophages were incubated with serum from oxLDL-pulsed DC compared to the addition of serum from mice treated with mDCs (Figure 13B), indicating that treatment with oxLDL-pulsed mDCs results in the formation of oxLDL-specific Ab that reduce foam cell formation.

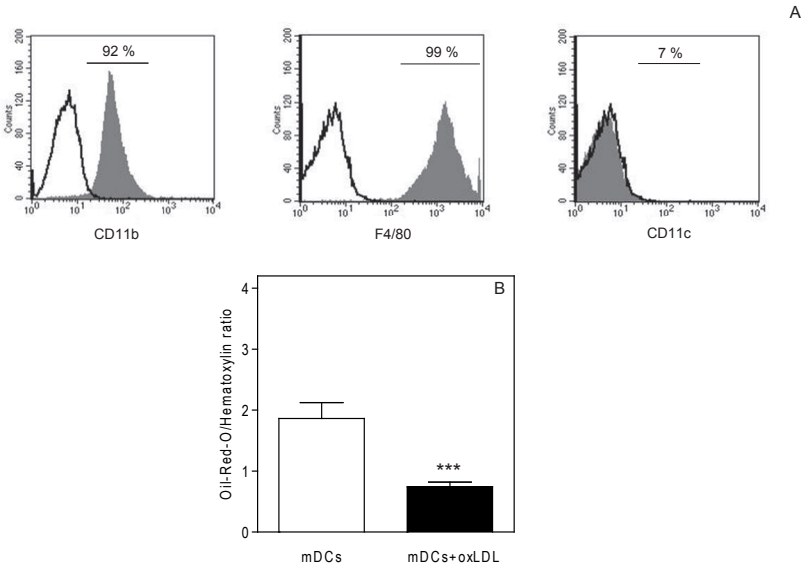


Figure 13: Inhibition of foam cell formation

Bone-marrow derived macrophages were analyzed by flow cytometry. The fluorescence intensity of respectively CD11b, F4/80 and CD11c (grey curves) are depicted in A. The white curves represent the isotype control. B. The amount of Oil-Red-O staining was corrected for the number of cells as indicated by the hematoxylin staining. (pooled sera; n=3), 8 fields per condition (***P<0.001).

Discussion

Oxidation of lipoproteins and oxidative processes play an important role in the initiation and progression of atherosclerosis. In this study we show that vaccination using oxLDL-pulsed DCs effectively reduces atherosclerotic lesion formation in $LDLr^{-/-}$ mice. In healthy vascular tissue, low numbers of DCs reside within the intima, immediately beneath the endothelium and in the adventitia along the external elastic lamina.[23, 24] In atherosclerotic-prone regions of healthy carotid arteries, the DCs accumulate and form clusters at sites subjected to major haemodynamic stress. It has been suggested that the migratory routes of vascular DCs are similar to those in other peripheral tissues. After uptake of antigen, vascular DCs migrate towards regional lymph nodes where they activate T cells. Importantly, plaque progression is linked to a reduced emigration of monocyte-derived DC-like cells from developing lesions.[25] In accordance, histopathological studies on human arterial tissues demonstrate that occasional DCs migrate towards the lymph nodes whereas other DCs stay within the plaques and locally interact with T cells.[26] Also, hyperlipidemia suppresses the migration of skin DCs.[27] Taken together, these studies emphasize the important immunoregulating role of vascular DCs and that the impaired migration of DCs may play an important role in the development of atherosclerosis.

We therefore treated atherosclerosis prone mice before the induction of atherosclerosis with DCs that were loaded *ex vivo* with cupper oxidized LDL. The advantage of using mature pulsed-DCs is that these cells do not need to migrate towards the plaque to engulf oxLDL. The oxLDL-pulsed mDCs used in this study are ready to migrate directly towards the lymphoid tissues where they can interact with lymphocytes and induce oxLDL-specific immune responses.[28] Indeed we showed that CCR7, necessary for migration towards lymphoid organs, is upregulated in the mature DCs used in this study and that injected GFP^{+} DCs homed towards the spleen and lymph nodes. In contrast to pulsing of DCs with oxidized lipids (Bluml *et al.)*[29] we did not observe an effect of oxLDL-priming on LPS-induced DC maturation, which may result from the fact that the amount of added oxLDL (and thus of oxidized lipids) by us was too low to affect the DC maturation. A recent paper by Packard *et al.* shows that foam cell formation as such does not affect the antigen presenting capacity of dendritic cells.[30]

We now show that vaccination using oxLDL-pulsed mDCs reduced atherosclerotic lesion formation in the carotid arteries for 87% after collar placement. Since the initial study of Palinski a number of studies have shown the effectiveness of immunization against oxLDL or apoB-100 peptides in experimental animal models.[31] Immunization leads to a reduction in atherosclerosis ranging from 40 to 70% in mice and rabbits.[9-11, 32] We chose to use oxLDL-loaded DCs for vaccination since it provides us with a very effective method to initiate immune responses to oxLDL. The oxLDL-pulsed DCs present a broad spectrum of epitopes after internalizing and processing the Ag. Therefore DCs are capable of activating a wide range of oxLDL-specific T cells and immune escape is minimized. The use of oxLDL-pulsed mDCs provided us therefore with a very efficient method of manipulating the immune system against multiple epitopes of oxLDL without the requirement of an adjuvant.

The observed reduction in lesion size in the carotid arteries was accompanied by a decrease in macrophage numbers and an increase in collagen, leading to a significantly more stable plaque phenotype**.** Although we only observed a 24% reduction of lesion size in the aortic valve leaflets, the injection of oxLDL-pulsed mDCs did result in more stable atherosclerotic lesions in the aortic root thereby confirming the effect of the treatment on plaque composition in the carotid arteries. The discrepancy in the effect of immunization against oxLDL on plaque size between aortic root and arteries is similar to the observations of Fredrikson *et al.* who showed that immunization against apoB-100 induced a 60% reduction in plaque size in the descending aorta but did not affect the size of the plaques in the aortic root.[10] The differential effect of the treatment using oxLDL-pulsed DCs on atherosclerosis in the carotid arteries and the aortic valves is an interesting, but not entirely new finding. A review paper of Getz *et al.* nicely describes that initiation of atherosclerosis at different sites in the vasculature may involve different molecular pathways.[33] We have however shown that the effect of regulatory T cells on atherosclerosis is similar in the collar-induced atherosclerosis in the carotid arteries and in the aortic root.[22, 34] Therefore it should be noted that the observed difference between the carotid artery and the heart valves is not necessarily the consequence of the collar model. The collar model used in this study is based on the induction of shear stress by the narrowing of the carotid arteries upon placement of the mildly constrictive silicone collars around carotid arteries. Proximal to the collar a decrease in shear stress is observed and the expression of KLF-2 is almost undetectable at this site. Endothelial KLF-2 links local arterial shear stress levels to the expression of vascular tone-regulating genes.[35] The low KLF-2 expression proximal to the collar coincides with the enhanced expression of VCAM-1 and is exactly the site were the atherosclerotic lesions are formed. The collar model is therefore injury-driven but comparable to the human situation where endothelial KLF-2 expression is lowered at the site of atherosclerosis.

During the experiment, cholesterol levels were evaluated. There was no difference in cholesterol burden during the entire experiment but at sacrifice, mice treated with oxLDL-pulsed DCs showed lowered cholesterol levels. This may form an additional interesting long-term effect of the treatment with oxLDL-pulsed DC. However, it cannot explain the enormous decrease in atherosclerotic lesion formation because up to 3 weeks prior to sacrifice, there were no differences in cholesterol levels. *Ex-vivo* generated and antigen-loaded DCs have been used in vaccination protocols in many animal models[36, 37] and have been used to improve immunity in cancer[38] and HIV-infected patients by inducing specific T cells.[39, 40] The attenuation of atherogenesis in our study was accompanied by an increase of T cell-dependent IgG Ab towards oxLDL and not towards MDA-LDL. This indicates that the treatment with oxLDL-pulsed mDCs induced an activation of ox-LDL-specific T cells. Indeed we show that spleen cells from mice treated with oxLDL-pulsed mDCs displayed an increased *in vitro* proliferation towards oxLDL as compared to spleen cells from mDCs-treated mice. In addition, both the enhanced IgG1/IgG2c ratio and the cytokine production of T cells point towards an anti-inflammatory response in the oxLDL-pulsed DCs vaccinated mice as shown by the clear inhibition of the IFN-γ production by ConA stimulated cells in these mice. We and others have previously shown that a reduction in IFN-γ contributes

to a reduction in atherosclerosis.[41] Interestingly, we now observe that the T cell response induced by oxLDL-pulsed DCs shows a reduced Th1 response. By using mDCs loaded with an atherosclerosis-irrelevant antigen, ovalbumin, we showed that the effects observed using oxLDL-pulsed mDCs were due to oxLDL-specific responses and not to side-effects inflicted by the use of antigen-pulsed mDCs. Indeed, although ovalbumin-specific IgG antibodies were induced, lesion size and composition were not changed in mice treated with ovalbumin-pulsed mDCs, nor were cholesterol levels affected.

In a previous study we showed that oral tolerance against oxLDL in $LDLr^{-/-}$ mice results in the induction of TGF-β producing regulatory $CD4^{+}CD25^{+}FoxP3^{+}$ T cells that inhibit lesion formation.[22] Oral tolerance to HSP60 also reduces atherosclerosis by inducing HSP60 specific regulatory T cells that produce TGF-beta and IL-10. The induction of oral tolerance to oxLDL and HSP60 was not accompanied by the induction of IgG or IgM specific for the oraly administered antigen.[34] These findings are in contrast to the present study where the treatment with oxLDL-pulsed mDCs induced anti-oxLDL antibodies, a reduction in IFN-gamma production by oxLDL-specific T cells and we did not observe an effect on the number of Tregs. We can conclude from these studies that both oral tolerance towards oxLDL as inducing a oxLDL-specific immune response can be beneficial in the treatment of atherosclerosis.

We also showed that serum of mice treated with oxLDL-pulsed mDCs reduced the formation of foam cells. These mice had higher titers of oxLDL-specific IgG Ab and we argue that the oxLDL-specific IgG can inhibit foam cell formation via complex formation with oxLDL. This is in agreement with the findings of the group of Witztum who showed that monoclonal IgG Fab Ab directed to oxLDL blocked foam cell formation in macrophages.[42] Also, Caligiuri *et al.* showed that sera from $ApoE^{-/-}$ mice, immunized with phosphorylcholine reduced the uptake of oxLDL by macrophages.[43] In addition, both Zhou *et al.* and Freigang *et al.* demonstrated an inverse correlation between lesion size and anti-MDA-LDL IgG levels in mice immunized with MDA-LDL.[11, 44] These data suggest that the presence of oxLDL-immune complexes play an atheroprotective role and it is suggested that the protective effect may be partially mediated via the inhibitory Fc receptor IIB.[45]

Taken together, this strategy of DC vaccination is a new powerful vaccination method which provides us with a highly efficient route to modulate the immune responses to oxLDL by inducing oxLDL-specific T cells with a reduced Th1 response and enhanced levels of anti-oxLDL IgG. Therefore, the approach of DC mediated vaccination against oxLDL is possibly applicable in young adults to inhibit the initiation of atherosclerosis. Additional studies on the effect of DC therapy on progression of atherosclerosis to eventually implement the therapy in patients need to be performed, but studies using anti-apoB100 antibodies indicate that these antibodies may induce inhibition of plaque progression or may even induce plaque regression, indicating that an effect of DC therapy on pre-existing lesions may be relevant.[32, 46]

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Chapter 3

Immunomodulation with OCH-pulsed dendritic cells attenuates atherosclerosis

-Manuscript submitted-

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Abstract

Natural killer T (NKT) cells contribute significantly to the inflammatory response in atherosclerosis. In atheroprone apolipoprotein E (apoE) deficient mice, NKT cells have an atherogenic phenotype and their activation with the synthetic ligand α-Galactosylceramide (α-GalCer) causes an increase in atherosclerotic plaque formation. Stimulation with the α-GalCer analog OCH is shown to provoke a T helper 2 (Th2) cytokine phenotype in NKT cells. In this study, we observed an increased interleukin-10 (IL-10) production after intraperitoneal treatment with OCH, but no effect on atherosclerosis. Therefore we used mature dendritic cells (mDCs) to deliver the OCH to the NKT cells in the liver. Treatment with OCH-pulsed mDCs resulted in an increased percentage of IL-10 producing NKT cells in the liver and in a subsequent reduction in atherosclerotic plaque formation. Additionally a reduction in cholesterol levels was observed in mice treated with OCH-pulsed mDCs. Altogether, this strategy of immunomodulation with mDCs loaded with OCH may form a new therapeutical approach to prevent atherosclerosis.

Introduction

In the chronic inflammatory response that underlies atherosclerosis, both innate and adaptive mechanisms are very important.[1] Antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), are reported to initiate the autoimmune response by the uptake of autoantigens, such as oxidized low density lipoprotein (oxLDL) and heat shock proteins. Peptides of these antigens are presented to T cells via MHC class I and II molecules.[2, 3] By this, the peptides elicit the activation of T cells, especially T helper 1 (Th1) cytokine producing $CD4^+$ T cells.[4-7] T cell triggering naturally occurs within the lymphatic system, especially within the lymph nodes and spleen. After triggering, T cells migrate to the site of inflammation *i.e.* infiltrate the atherosclerotic plaque, where they may re-encounter their specific antigen, become activated and mediate their inflammatory damage. This is the onset of a process in which increasing numbers of immune cells are attracted to the atherosclerotic plaque and can result in occlusion of the vessels and severe cardiovascular disorders.

APCs such as macrophages and DCs are also attracted into the atherosclerotic plaque. APCs and especially the DCs express CD1 molecules, MHC class I like molecules, which specifically present lipid antigens to T cells.[8] In mice, only CD1d, a CD1 family member, is expressed on DCs[9] and Bobryshev et al. observed that CD1d is expressed within the atherosclerotic plaque.[10] The complex of CD1d with a lipid antigen can be recognized by invariant natural killer T (NKT) cells. NKT cells are a specialized subset of T cells expressing both an invariant T cell receptor (TCR) α chain composed of Vα14-Jα18 segments and the NK cell marker NK1.1. In atherosclerosis a colocalization of NKT cells and DCs within the shoulder regions of the plaque is observed, suggesting that NKT cells are activated by antigen presentation on the DCs.[11]

NKT cells can be activated using synthetic ligands such as α-galactosylceramide[9] (α-GalCer) and the α-GalCer analog ((2S,3S,4R)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-tetracosanoylamino)-1,3,4-nonanetriol) (OCH).[12, 13] Activation by α-GalCer induces a rapid aspecific mixed Th1/Th2 response, in which the NKT cells produce large amounts of IL-4, IL-10, IL-12, IL-13, and IFN-γ. Recent studies showed that in contrast with other Th1-mediated autoimmune-like diseases such as autoimmune diabetes,[14-16] experimental autoimmune encephalomyelitis[17, 18] and colitis,[19, 20] treatment with α-GalCer accelerated the disease process in atherosclerosis-prone apoE deficient mice.[21-23] OCH, which has a truncated sphingosine chain and therefore a lower affinity for CD1d, induces a more Th2-like cytokine profile. OCH-activated NKT cells produce predominantly IL-4, IL-10, and IL-13 and low levels of IFN-γ.[12, 13] This feature makes OCH an interesting glycolipid, which abrogates Th1-mediated immune responses.[12, 19, 24, 25]

Because of their capacity to stimulate T and NKT cells, DCs are widely used in vaccination therapies. DCs may be pulsed with an antigen *ex vivo* and subsequently these "pulsed" DCs are returned into the bloodstream. This approach is successfully used in cancer[26] and in several autoimmune diseases. DCs pulsed with bovine collagen type II protect mice from collagen-induced arthritis[27] and immature DCs (imDCs) pulsed with a peptide of glutamic acid decarboxylase protect nonobese diabetic (NOD) mice against type I diabetes.[28] In studies on cancer it was observed

that DCs pulsed with α-GalCer induced a prolonged IFN-γ-producing NKT cell response.[29, 30]

In this study we observed that intraperitoneal injections of OCH in low density lipoprotein receptor (LDLr) deficient mice had no effect on atherosclerosis despite an increased IL-10 production by splenic $CD4^+$ lymphocytes. In contrast, injection of OCH-pulsed mDCs in LDLr deficient mice reduced atherosclerotic plaque formation which may be explained by a significant increase in $IL\text{-}10^+$ producing NKT cells in the liver. Additionally a significant reduction in serum cholesterol levels was observed after treatment with OCH-pulsed mDCs.

Materials and methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male $LDLr^{-/-}$ and GFP^+ mice were obtained from Jackson's Laboratory and male C57BL/6j mice from Charles River Laboratories (Maastricht). $Jα281^{-/-}$ mice on a C57BL/6 background were obtained from Dr. M. Taniguchi. $Jα281^{-/-}LDLr^{-/-}$ double knockout mice were generated by crossing $Jα281^{-/-}$ mice with $LDLr^{-/-}$ mice. The offspring was crossed to produce mice with homozygous deletion in both LDLr and Jα281 genes. All mice were kept under standard laboratory conditions and bred in-house. The mice were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Water and food were administered *ad libitum*.

Media and reagents

R1 cells, producing Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), and dendritic cells were cultured in IMDM (Cambrex, Belgium) supplemented with 8% FBS, 100 U/ml penicillin/streptomycin (both from PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μm β-mercaptoethanol (Sigma Aldrich, The Netherlands). β-GalCer was obtained from the Kirin Brewery Co Ltd, (Gunma, Japan). OCH ((2S,3S,4R)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-tetracosanoylamino)-1,3,4-nonanetriol) was synthesized as previously described by Fan et al. and dissolved in dimethyl sulphoxide (DMSO).[31] The synthesis of OCH was analyzed via nuclear magnetic resonance (NMR).

Intraperitoneal treatment with OCH

To test the direct effect of OCH on atherosclerosis, $LDLr^{-/-}$ mice were injected intraperitoneally with either 100 μg/kg β-GalCer (n=11) or OCH (n=13), twice a week for 7 weeks. Both β-GalCer and OCH were dissolved in PBS with 1% DMSO. After 3 weeks of Western-type diet feeding and prior to the treatment with β-GalCer and OCH, atherosclerosis was induced in both carotid arteries by bilateral perivascular collar placement as described previously.[32] The mice were fed a Western-type diet during the whole experiment (10 weeks). At the end of the experiment, mice were sacrificed and tissues were harvested after *in situ* perfusion with PBS and FormalFixx. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and

stored at -20°C until further use.

Treatment with OCH-pulsed mDCs

To test the effect of OCH-pulsed mDCs, bone marrow cells were harvested from the femur and tibia of C57BL/6j mice and were cultured for 10 days in complete IMDM supplemented with GM-CSF. Maturation of the DCs was accomplished via the addition of 1 μg/ml of LPS (from Salmonella Typhosa, Sigma Aldrich, The Netherlands) to the medium for 24 hours. Together with LPS, 100 ng/ml of OCH was added to a fraction of the DCs. Control DCs were incubated with LPS only. After 24 hours, the DCs were harvested and diluted in PBS. Subsequently, $LDLr^{-/-}$ mice were injected intravenously 8, 6 and 3 days before the mice were put on a Western-type diet. The mice received PBS (n=13), $1.5x10^6$ mature DCs (n=11) or $1.5x10^6$ OCH-pulsed mDCs (n=11). Next, the mice were either sacrificed one day after the last injection with DCs or the mice were fed a Western-type diet for 3 weeks and atherosclerosis was induced in both carotid arteries by bilateral perivascular collar placement as described previously.[32] Seven weeks after collar placement, the mice were sacrificed and tissues were harvested after *in situ* perfusion with PBS and FormalFixx. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use. In case of the $Jα281^{-/-}LDLr^{-/-}$ mice, mDCs and OCH-pulsed mDCs were administered and atherosclerosis was induced by feeding these mice a Western-type diet for 10 weeks without placement of perivascular collars.

Homing of injected DCs

To investigate the homing of injected DCs, bone marrow DCs were isolated from GFP^+ mice and were cultured for 10 days in presence of GM-CSF. Subsequently, the cells were maturated with LPS (1 μg/ml) and injected intravenously in $LDLr^{-/-}$ mice fed a normal chow diet or a Western-type diet 48 hours after injection, the mice were sacrificed and the distribution of GFP^+-DCs was determined via FACS-analysis of several organs. These organs were isolated and mononuclear cells were isolated using Lympholyte according to the manufacturer's protocol (Cedarlane, Hornby, Ontario, Canada).

Histological analysis

After sacrificing the mice, the carotid arteries were sliced (5 μm) proximal of the collar and the cryosections were stained with hematoxylin (Sigma Aldrich, The Netherlands) and eosin (Merck Diagnostica, Germany). Cryosections of the aortic root (10 μm) were stained with Oil-red-O and hematoxylin to determine plaque size. Corresponding sections of carotid arteries and aortic root were stained with a macrophage specific marker (MOMA-2, Research Diagnostic Inc., New Jersey) and a collagen specific marker (Masson's Trichrome, Sigma Aldrich, The Netherlands). All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Cholesterol and triglyceride levels

During the experiment, plasma samples obtained by tail vein bleeding were used to determine the total plasma cholesterol and triglyceride levels. Cholesterol

levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Triglyceride levels were quantified by using a ready-made kit (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard. Via SMART-analysis (3.2 x 30 mm, Smart System, Pharmacia) using the Superose 6 column the cholesterol distribution over different lipoproteins was analyzed.

Flow Cytometry

To check the maturation status of the DCs, cells were stained with the antibodies CD80-PE, CD86-FITC, CD40-PE, MHC II-PE, CD1d-FITC and CD11c-FITC (eBioscience, Belgium). In order to detect effects on cytokine production, 3 days after the last injection with β-GalCer or OCH, leukocytes were isolated from spleens using Lympholyte (Cedarlane, Canada). Per well of a 96-wells plate, $5x10^5$ of these mononuclear cells were stimulated for 24 hours with αCD3/CD28 (5 μg/ml) and 100 ng/ml OCH. Next the splenocytes were incubated with a leukocyte activation cocktail containing Golgi-stop (Becton Dickinson, CA). After 4 hours we performed intracellular staining as suggested by the manufacturer's protocol. In this experiment CD4-PerCP, IFNγ-APC, IL-10-PE and IL-4-FITC antibodies were used. To determine NKT cells in blood during DC treatment, blood was collected at several time points in EDTA-coated tubes. Red blood cells were lyzed using a lysis buffer containing 0.83% NH_4Cl in 0.01M Tris/HCL (pH 7.2). Subsequently the cells were stained with CD3-PerCP, NK1.1-FITC or PE-labeled α-GalCer loaded CD1d tetramer antibodies to detect NKT cells. To detect intracellular cytokines after the DC treatment, the mice were sacrificed one day after the last injection with DCs. Leukocytes were isolated using Lympholyte (Cedarlane, Hornby, Ontario, Canada) and CD3-PerCP, NK1.1-FITC, IL-10-APC and IFNγ-APC antibodies were used to detect intracellular antibodies in NKT cells. All antibodies were purchased from eBioscience (Belgium). Staining of the cells was done in PBS with 1% normal mouse serum. FACS analysis was performed on a FACSCalibur (Becton Dickinson, CA). Data were analyzed with Cell Quest software.

Antibody detection

Cuox-LDL was synthesized as described previously[33, 34]. MDA-LDL was made by addition of 0.5 M MDA to 10 mg of LDL for 3 hours at 37ºC. Antibodies against MDA-LDL and ox-LDL were determined according to Damoiseaux et al.[35] Briefly, maxiSorp 96 well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μg MDA-LDL or oxLDL in 100 μl PBS at 4°C. Plates were washed 5 times with 0.01M Tris, 0.15 M NaCl and 0.05% Tween20 (pH 8.0). Mouse serum was added in duplicate at a 1:50 dilution in incubation buffer (0.1 M Tris, 0.3 M NaCl and 0.05% Tween20 (pH 8.0) overnight at 4°C. After washing, plates were incubated with either alkaline phosphatase-labelled anti-mouse IgM or IgG (Jackson Immuno-Research, Pennsylvania) both at a 1:4000 dilution in incubation buffer for 1 hour at 37°C. After washing, substrate (1 mg/ml disodium p-nitrophenyl phosphate, Sigma, The Netherlands) was added. After 2 hours at room temperature, absorbance was read at 405 nm.

Statistical analysis

Values are expressed as mean ± SEM. A two tailed Student's t-test was performed to compare data. When necessary a Mann-Whitney test was performed. Probability (*P)* values below 0.05 were considered significant.

Results

Effect of multiple intraperitoneal injections with OCH on atherosclerosis

To investigate the effect of OCH on atherosclerosis, $LDLr^{-/-}$ mice in which atherosclerosis was induced by Western-type diet feeding and perivascular collar placement around the carotid arteries, were used. Following collar placement, β-Galactosylceramide (β-GalCer) and OCH (100 μg/kg) were administered intraperitoneally twice a week for 7 weeks. The control, β-GalCer, has a β-linkage of galactose to the ceramide group and binds to CD1d but is not able to activate proliferation of and cytokine production by NKT cells.[9, 36] Although NKT cells are not completely inert to β-GalCer[36], β-GalCer is used as a control because it has no effect on atherosclerosis as shown by our group (unpublished results). After treatment, the mice were sacrificed and the degree of atherosclerotic plaque formation in β-GalCer-treated mice and OCH-treated mice was determined in the carotid arteries (Figure 1A and 1B, respectively) and in the aortic root (Figure 1D and 1E, respectively). Treatment with OCH had no significant effect on plaque size in both the carotid arteries (Figure 1C; 38,758±6,937 μm^2 versus 39,211±9,363 μm^2) or the aortic root (Figure 1F; 438,163±41,923 μm^2 versus 499,456±52,2998 μm^2) when compared with β-GalCer treated mice. During the experiment, total plasma cholesterol levels and body weight were not significantly different between both groups of mice (data not shown).

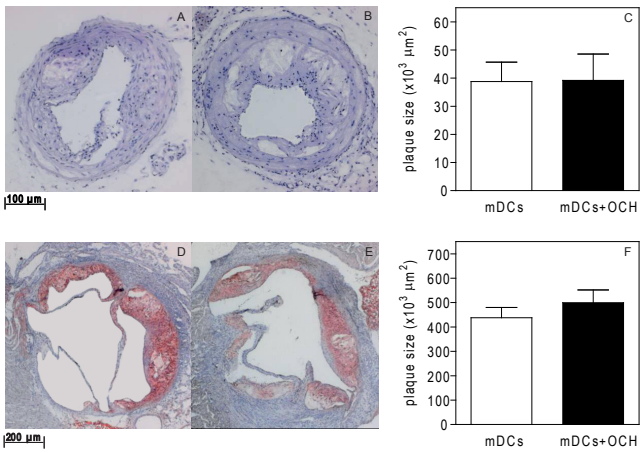


Figure 1. Effect of intraperitoneal injections with OCH on atherosclerotic plaque formation.
Atherosclerosis was induced in $LDLr^{-/-}$ mice by Western-type diet feeding and collar placement around both carotid arteries. Next, mice were treated with β-GalCer (n=11) or OCH (n=13) twice a week during seven weeks after which mice were sacrificed. Lesion size was determined in the carotid arteries of β-GalCer-treated (A) and OCH-treated mice (B) and in the aortic root of β-GalCer-treated (D) and OCH-treated (E) mice.

Multiple intraperitoneal injections with OCH influence the cytokine profile

OCH is reported to induce a Th2-like cytokine profile in NKT cells. Therefore we isolated spleens of β-GalCer-treated and OCH-treated mice 3 days after the last injection with β-GalCer and OCH and performed an intracellular FACS staining to determine the cytokine profile. Following restimulation with OCH *in vitro*, the percentage of IFN-γ producing cells within the $CD4^+$ population of the splenocytes decreased significantly with 34% from 1.20±0.14% in the β-GalCer-treated mice to 0.79±0.08% in the OCH-treated mice (Figure 2; $P<0.05$). Furthermore, a 76% increase in IL-10 producing cells within the $CD4^+$ population was observed (Online Figure 2; 0.76±0.14% versus 1.34±0.15%; $P<0.05$), while there was no effect on the percentage of IL-4 producing $CD4^+$ cells.

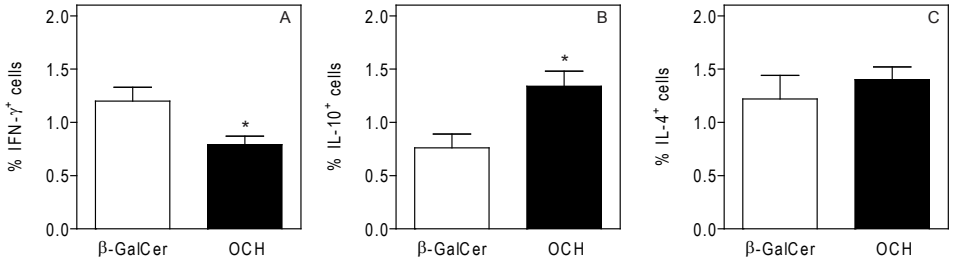


Figure 2. *In vivo* administration of OCH affects the cytokine profile within the spleen.

After multiple treatments with OCH, spleens of $LDLr^{-/-}$ mice fed a Western-type diet and equipped with collars around both carotid arteries were dissected and splenocytes were isolated. The splenocytes of β-GalCer-treated (n=5) and OCH-treated mice (n=6) were re-stimulated with αCD3/CD28 (5 μg/ml) and OCH (100 ng/ml) *in vitro*. After 24 hours the percentage of CD4+ T cells producing IFN-γ, IL-10 and IL-4 was determined via intracellular FACS analysis after gating for $CD4^+$ lymphocytes (*$P<0.05$).

OCH priming of DCs

In order to test the effect of OCH-pulsed mDCs on atherosclerosis, DCs had to be pulsed with OCH first. Therefore, bone marrow cells were isolated from the femur and tibia of C57Bl/6 mice and cultured for 10 days in presence of GM-CSF. To maturate the DCs, LPS (1 μg/ml) was added to the culture for 24 hours. The maturation status of the DCs was checked by FACS analysis. CD11c was constitutively expressed on all DCs *i.e.* imDCs, mDCs and OCH-pulsed mDCs. CD40, CD80 and CD86, well known maturation markers for DCs are upregulated after the addition of LPS. The peptide-antigen presenting molecule MHC class II was already present on imDCs and is only slightly upregulated due to maturation, while the lipid-antigen presenting molecule CD1d is upregulated due to the addition of LPS. OCH, when added together with LPS, had no additional influence on the maturation of the DCs (Figure 3).

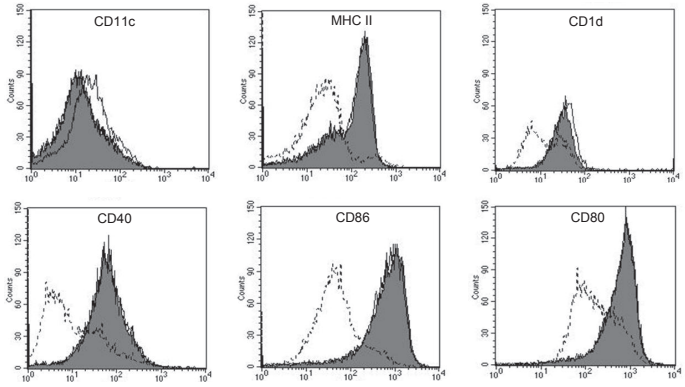


Figure 3. The effect of OCH on the maturation of DCs.
Bone marrow derived DCs were incubated with GM-CSF for 10 days. Subsequently, maturation was induced by LPS (1 µg/ml) in the presence or absence of OCH (100 ng/ml). After 24 hours expression of maturation markers was determined by FACS analysis. (dashed line: immature DCs, full black line:mature DCs, full black line with grey area: mature DCs pulsed with OCH).

Accumulation of i*ntravenously* injected mDCs in different organs

To investigate the homing of intravenously injected DCs, DCs were isolated from GFP$^+$ mice and maturated by the addition of LPS. Forty-eight hours after injection of mature GFP$^+$ DCs in chow diet fed and Western-type diet fed LDLr$^{-/-}$ mice, DCs were recovered in the lung, liver, spleen and lymph nodes around the heart (HLN). When fed a chow diet, 18.6% of the lymphocytes in the lung were GFP$^+$, while in the liver 3.8% was GFP$^+$. Relatively low percentages of the injected mDCs were found in spleen and lymph nodes (Figure 4A). When mice were fed a Western-type diet, 16.5% of the lymphocytes in the liver were GFP$^+$. The number of GFP$^+$ cells in other organs was not really different from that in the chow diet fed mice (Figure 4B). Taken into account the large number of leukocytes within the liver, we conclude that large numbers of the injected mDCs home to the liver.

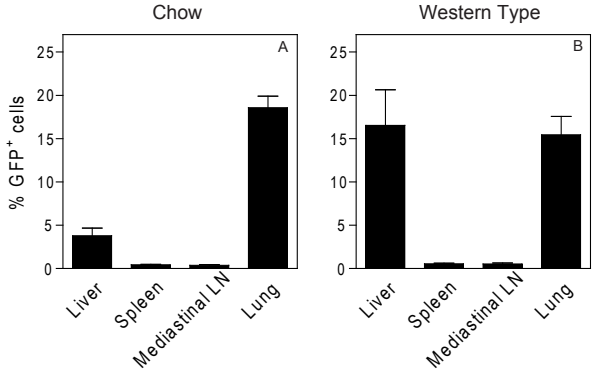


Figure 4. Homing of dendritic cells.
1.5x10^6 of mature GFP$^+$ DCs were administered intravenously to LDLr$^{-/-}$ mice fed a chow diet (A) or a Western-type diet (B) (n=4). After 48 hours the mice were sacrificed and the percentage of GFP$^+$ cells in organs were determined by FACS analysis

Repetitive injection of OCH-pulsed mDCs reduces atherosclerosis

To determine the effect of OCH-pulsed mDCs on atherosclerosis, PBS, mDCs and OCH-pulsed mDCs were administered 3 times in 8 days intravenously to LDLr$^{-/-}$ mice. After the treatment, atherosclerosis was induced by Western-type diet feeding and perivascular collar placement around both carotid arteries. Representative examples of hematoxylin-eosin stained atherosclerotic lesions in the carotid arteries of mDC-treated and OCH-pulsed mDC-treated mice are shown in Figure 5A and 5B, respectively. Injection of OCH-pulsed mDCs (9,400±2185 μm^2) resulted in a significant 70.6% reduction in plaque size in the carotid arteries when compared to mDC-treated mice (Figure 5C; 31,920±7,914 μm^2; P<0.05).

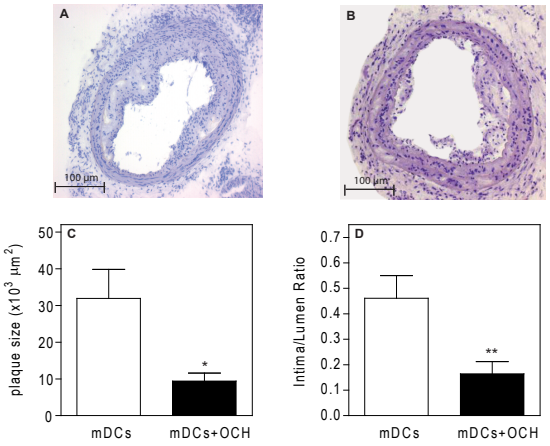


Figure 5. Immunomodulation with OCH-pulsed mDCs reduces lesion size in the carotid arteries.
LDLr$^{-/-}$ mice were three times injected with mDCs or OCH-pulsed mDCs prior to atherosclerosis induction. After a total of ten weeks on WT diet, mice were sacrificed and sections of the carotid arteries of mDC-treated mice (A, n=11) and OCH-pulsed mDC-treated mice (B, n=9) were stained with hematoxylin and eosin. Plaque size (C) and intima/lumen ratio (D) were determined (*P<0.05 **P<0.01).

Injection of OCH-pulsed mDCs (0.164±0.048) also reduced the intima/lumen ratio with 64.4% (Figure 5D; 0.461±0.089; P<0.01). Additionally, the treatment with mDCs did not differ from the treatment with PBS in plaque size (48,578±9,231 μm^2; P=0.20) and intima/lumen ratio (0.560±0.097; P=0.48) (not shown). A significant 58.1% reduction in plaque formation at the aortic root was observed when comparing OCH-pulsed mDC-treated mice (122,846±21,470 μm^2) with mDC-treated mice (Figure 6C; 293,181±36,193 μm^2; P<0.01). Representative Oil-red-O and hematoxylin stained examples of plaques at the aortic root of mDC-treated mice and mice treated with OCH-pulsed mDCs are shown in Figure 6A and 6B, respectively.

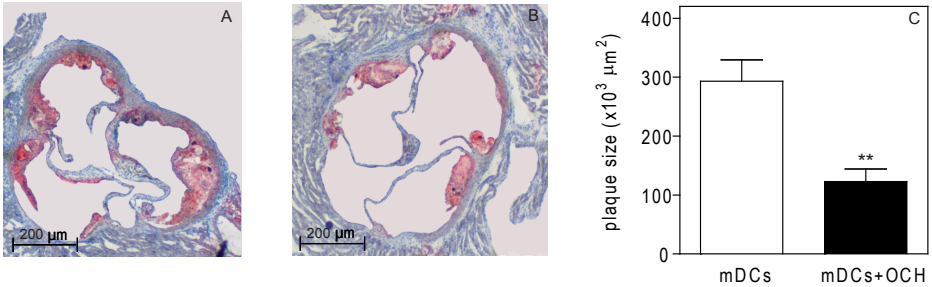


Figure 6. Effect of treatment on the lesion size in the aortic root.
LDLr$^{-/-}$ mice were treated with mDCs (A, n=11) or OCH-pulsed mDCs (B, n=9).Lesion size in aortic roots was determined after a total of ten weeks of WT diet feeding (C) (**P<0.01).

Plaque size at the aortic root of PBS-treated mice (228,667±32,747 μm^2) was again not significantly different from the plaque size in the mDC-treated mice (*P*=0.22, not shown). Both in plaques in the carotid arteries and at the aortic root no significant changes in plaque morphology were observed. Macrophages, smooth muscle cells and collagen were present to the same extent (data not shown). Because mDC-treatment and PBS-treatment gave similar results, only the mDC treatment was used as a control for further analysis.

To show that the effects of OCH pulsed mDCs are mediated by NKT cells, we performed a control experiment in mice lacking NKT cells ($J\alpha281^{-/-}$ mice) crossbred to a LDLr deficient background. Treatment of $J\alpha281^{-/-}/LDLr^{-/-}$ mice with OCH-pulsed mDCs had in contrast to the treatment of LDLr-/- mice no effect on plaque formation at the aortic root when compared with $J\alpha281^{-/-}LDLr^{-/-}$ mice treated with mDCs (Figure 7).

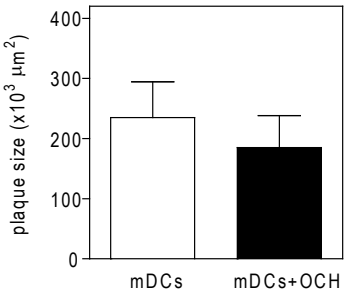


Figure 7. Effect of immunomodulation with OCH-pulsed mDCs on atherosclerosis in NKT cell deficient mice.
$J\alpha281^{-/-}LDLr^{-/-}$ mice were treated with mDCs or OCH-pulsed mDCs three times prior to atherosclerosis induction.

Increase in NKT cells due to vaccination with OCH-pulsed mDCs

During the experiment blood was withdrawn to determine the effect of vaccination with DCs on the number of NKT cells in blood. One day after the third vaccination with OCH-pulsed mDCs, which is 9 days after the start of the experiment, the percentage of NKT cells in the blood of mice treated with OCH-pulsed mDCs was significantly increased when compared with blood of mDC-treated mice (Figure 8A; 2.09±0.42% versus 0.94±0.24%, respectively; *P*<0.05). At the end of the experiment, the percentage of NKT cells in blood of mice treated with OCH-pulsed mDCs was still increased when compared to the mice treated with mDCs, but no significant difference was observed (1.18±0.14% versus 2.29±0.74%; P=0.20). To detect any changes in percentages of NKT cells in other organs we performed a new experiment, in which we repeated the treatment with the mDCs and OCH-pulsed mDCs as above but now sacrificed the mice one day after the last DC-injection. FACS analysis showed that treatment with OCH-pulsed mDCs significantly increases the percentage of NKT cells within the liver (6.62±0.79%) when compared with the mDC-treated mice (Figure 8B; 3.53±0.34%; *P*<0.05). No differences in NKT cell percentages were detected in other organs. The increase in NKT cell numbers in the circulation in mice treated with OCH-pulsed mDCs was not observed in mice treated with OCH intraperitoneally, indicating that treatment with DCs is more efficient in activating NKT cells.

The NKT cells in the liver of mice treated with OCH-pulsed mDCs also show a Th2-like cytokine profile. The percentage of IL-10 producing NKT cells increased 2.2-fold from 1.59±0.63% in mDC-treated mice to 3.42±0.33% in mice treated with OCH-pulsed mDCs (Figure 8C; $P<0.05$). In addition, the percentage of IFN-γ producing NKT cells in mice treated with OCH-pulsed mDCs (8.83±2.73%) was not significantly different from the percentage of IFN-γ producing NKT cells in mDC treated mice (6.71±2.52%; Figure 8D; $P=0.58$). No effect on the number of NKT cells and cytokine profile of NKT cells was observed immediately after the first and the second injection with OCH-pulsed mDCs when compared with mice treated with mDCS (data not shown).

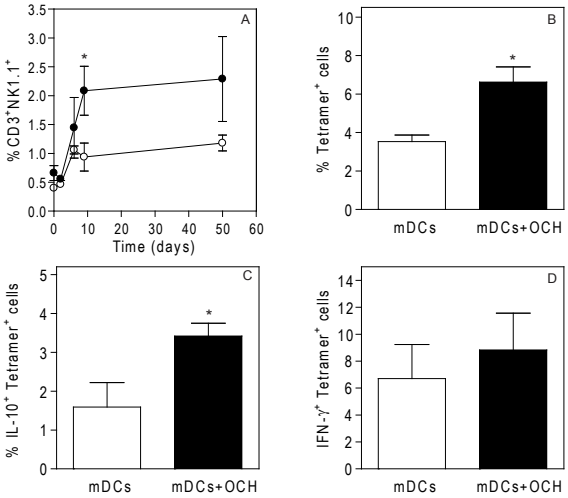


Figure 8. Effect of OCH-pulsed mDCs on the percentage and cytokine profile of NKT cells.
During the experiment, the number of NKT cells in blood was determined using FACS analysis (A). Grey line: mDC treated mice (n=3), black line: OCH-pulsed mDCs treated mice (n=3). In an independent experiment mice were sacrificed one day after the last injection with DCs and the percentage of CD1d-tetramer positive NKT cells in the liver was determined (B). The cytokine profiles of these NKT cells were determined using an intracellular FACS protocol. The production of IL-10 (C) and IFN-γ (D) was monitored (*$P<0.05$).

Vaccination with OCH-pulsed mDCs reduces cholesterol levels

During the *in vivo* DC vaccination experiment, body weight, serum cholesterol and serum triglyceride levels were measured at different time points. There was no significant difference in weight due to the treatment with OCH-pulsed mDCs (data not shown). Within the first 8 weeks of the DC-vaccination experiment, also no differences in total serum cholesterol levels were observed. However, between week 8 and 11 of the experiment a significant 23.7% lower cholesterol level was detected in mice vaccinated with OCH-pulsed mDCs (1,132±136 mg/dl) when compared with mDC-treated mice (Figure 9A; 1,483±67 mg/dl; $P<0.05$). To investigate whether this reduction in cholesterol is a reduction in cholesterol levels within a particular class of lipoproteins, a SMART analysis was performed. In serum of mice treated with OCH-pulsed mDCs, VLDL-cholesterol (111±21 mg/dl) and LDL-cholesterol (67±9 mg/dl) were significantly lower when compared with VLDL- and LDL-cholesterol levels in mDC-treated mice (Figure 9B; 188±17 mg/dl

and 104±9 mg/dl, respectively; *P*<0.05). HDL-cholesterol was also lowered but not significantly (46±12 mg/dl versus 25±2 mg/dl; *P*=0.14). No effects were observed on triglyceride levels in serum of these mice (data not shown).

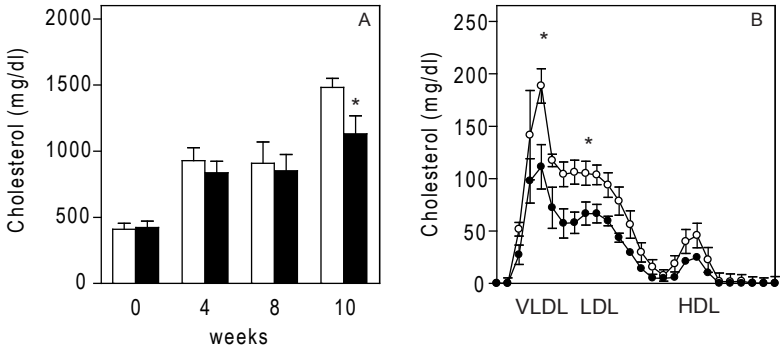


Figure 9. Effect of treatment with OCH-pulsed mDCs on cholesterol levels.
Serum cholesterol levels were measured at different time points (A). Sera were subsequently loaded onto a Sepharose 6 column and fractions were collected to obtain lipoprotein profiles. (white = mDC; black = OCH-pulsed mDCs) (*P<0.05).

Vaccination with OCH-pulsed mDCs has no effect on antibody production

To investigate a possible role of the humoral immune response in reducing plaque formation, titers of antibodies specific for anti-modified LDL were determined. Treatment with OCH-pulsed mDCs had no influence on either malondialdehyde modified (MDA)-LDL and oxLDL-specific IgM and IgG titers in serum of the mice (Figure 10A and B, respectively).

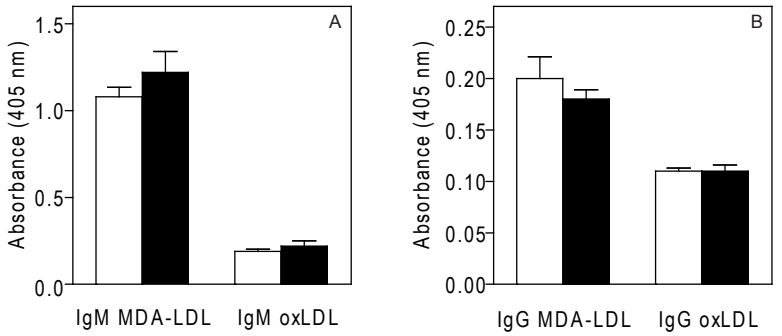


Figure 10. Influence of DC-treatment on MDA-LDL- and oxLDL-specific antibodies.
LDLr$^{-/-}$ mice were treated intravenously with mDCs (n=11) or OCH-pulsed mDCs (n=9) and at the end of the experiment serum levels of MDA-LDL- and oxLDL-specific IgM antibodies (A) and IgG antibodies (B) were measured using an ELISA. Values are mean OD 405nm ± SEM (white = mDCs; black = mDCs+OCH).

Discussion

The activation of antigen specific $CD4^+$ T cells is one of the main events in the inflammatory response of atherosclerosis. Within the atherosclerotic plaque, $CD4^+$ T cells are re-activated via the recognition of specific peptide-antigens presented by MHC class II molecules on APCs. Dendritic cells play a very important role in this process of antigen processing within the plaque. Normally, DCs are present in the intima of non-diseased arteries.[37] These DCs become either activated in early stages of atherosclerosis[10, 38, 39] while in more advanced stages, the DCs are recruited from the adventitia[40] and blood[41] into the lesion. Within the lesions, DCs are especially located in the rupture prone shoulder regions[11, 40] and are found to form cell clusters with T cells. Recently, co-localization of DCs with another T cell subtype, the NKT cell, was observed within the atherosclerotic plaque.[11] The colocalization of DCs and NKT cells in the shoulder regions of the plaque[38], and the observation of CD1d expression within the atherosclerotic plaque[10, 42] suggests that the DCs present a lipidic antigen via CD1d to the NKT cells resulting in their activation. These NKT cells, present in the atherosclerotic plaque[21, 22] were found to have an athero-promoting effect.[21-23]

The role of NKT cells in atherosclerosis has been studied by using the synthetic NKT cell ligand α-GalCer. Multiple intraperitoneal and/or intravenous injections of α-GalCer in atherosclerosis-prone apoE deficient mice resulted in a surprising aggravation of atherosclerosis due to the production of Th1 cytokines by NKT cells.[21-23] Especially, increased levels of IFN-γ were found in these treated $apoE^{-/-}$ mice.[21, 22] Studies on the effect of α-GalCer on other Th1-mediated diseases showed however that multiple injections with α-GalCer induced a more Th2-based cytokine profile of NKT cells.[14-20] Therefore it was surprising that treatment with α-GalCer resulted in an aggravation of atherosclerosis. Additionally, CD1d deficiency resulted in a reduction in atherosclerosis. $LDLr^{-/-}CD1d^{-/-}$ and $apoE^{-/-}CD1d^{-/-}$ mice showed a significant decline in plaque size when compared with $LDLr^{-/-}$ mice[22, 43] and $apoE^{-/-}$ mice[21, 23], respectively. From this we suggest that NKT cells in athero-prone mice are activated by certain endogenous ligands, which are still unknown and exert an athero-promoting effect. Stimulation with α-GalCer seems to worsen the situation. One explanation for this may be the increased production of IL-4, which is probably an athero-promoting Th2 cytokine.[44, 45]

In this study we used OCH, another synthetic NKT cell ligand. OCH has a lower affinity for CD1d because of a shorter lipid chain and due to this lower affinity, the TCR stimulation is shorter in time than with α-GalCer. Since IFN-γ production requires a longer TCR stimulation than IL-4 and IL-10, a more pronounced Th2 cytokine profile is induced after activation with OCH. Oki et al.[46] observed that the duration of NKT cell stimulation determines whether the NF-κB family member transcription factor c-Rel is transcribed effectively. c-Rel is identified as essential for IFN-γ production by NKT cells. They showed that c-Rel is transcribed in α-GalCer-stimulated, but not in OCH-stimulated NKT cells.[46]

In vivo administration of OCH in several mouse models is shown to abrogate Th1-mediated immune responses and to be protective against experimental autoimmune encephalomyelitis,[12] arthritis,[24] colitis[19] and diabetes.[25] In this study, OCH was administered intraperitoneally to $LDLr^{-/-}$ mice and an increased amount

of IL-10 producing cells was observed within the spleen. The increase in IL-10 was accompanied by a decrease in IFN-γ producing cells. This cytokine profile is in line with the studies on other Th1-mediated autoimmune diseases mentioned earlier. There was however no effect on the formation of atherosclerotic lesions induced by Western-type diet feeding and collar placement around the carotid arteries of $LDLr^{-/-}$ mice. Nakai et al.[22] showed before that injections of OCH accelerated the early phase of atherosclerosis. In that study, 8 weeks old $apoE^{-/-}$ mice, fed a regular chow diet were injected with OCH once within two weeks, three times in total. In this study they showed that the IFN-γ levels in serum of OCH-treated mice was lower than in serum of α-GalCer-treated mice while IL-4 levels were the same. This is however in contrast with other reports on OCH, because OCH is known for promoting Th2 cytokine production by NKT cells, especially when administered twice a week. Nakai et al[22], administered OCH once within two weeks and it is possible that this interval between two injections was too long to induce a Th2 profile. Additionally, data on IL-10 production are not presented. Another major difference between our study and the one of Nakai et al. is the used mouse model. Van den Elzen et al. showed that apoE is an important mediator in presentation of lipid antigens via CD1 molecules.[47] ApoE may bind exogenous lipid antigens and efficiently target them for receptor-mediated uptake by DCs. Furthermore, more important is apoE bound on the membrane to heparan sulfate proteoglycans, playing a role in the delivery of the lipid to compartments containing CD1d, leading to presentation of the lipid on CD1d. Since activation of NKT cells by OCH is CD1d dependent, apoE may have an important role in this process and the results obtained from $apoE^{-/-}$ mice may therefore be misleading. Additionally, our mice were fed a Western type diet instead of a regular chow diet. We hypothesize that this high fat diet may influence NKT cells before OCH was administered. This pre-activation of NKT cells may lead to another response to OCH, a more Th2 profile, than without this pre-activation. However, this pre-activation by a high fat diet needs further investigation.

Another explanation for the lack of an effect on atherosclerotic lesion development after injections with free OCH may be that the effect on cytokine levels we observed was very temporal. The mice were sacrificed within 48 hours after the last injection with OCH and the induced IL-10 production together with the decreased production of IFN-γ may have been not strong enough to abrogate the Th1 response against several autoantigens in atherosclerosis. A second explanation could be an ineffective and inefficient presentation of OCH by antigen presenting cells to the NKT cells in the liver after intraperitoneal injections.

We therefore used another way to administer OCH to $LDLr^{-/-}$ mice. In several studies, the usefulness of DCs as vaccination units to impair autoimmune diseases was shown. In our previous study, mDCs pulsed with oxLDL induced a protective oxLDL specific antibody response and a reduction in atherosclerotic plaque formation in $LDLr^{-/-}$ mice.[48] In a study by van Duivenvoorde et al. DCs were pulsed with collagen type II and this resulted in a reduction in arthritis due to a decrease in the collagen specific "Th1-associated" IgG2a response.[27] Additionally, Lo et al. showed that immature DCs pulsed with a peptide of glutamic acid decarboxylase protects NOD mice against type I diabetes.[28]

In our previous[48] and current study we show that mDCs injected intravenously,

particularly accumulate within the liver. Loading of dendritic cells with NKT cell ligands would therefore deliver the ligand directly to the largest pool of NKT cells in the body, *i.e.* the liver. In the liver, the DCs can present the ligand via CD1d and induce the activation of the NKT cells. When $LDLr^{-/-}$ mice were fed a Western-type diet, even more DCs accumulated within the liver, possibly due to the elevated inflammatory status in this organ.

It was shown before that NKT cells can be expanded *in vivo* via the administration of mature dendritic cells pulsed with α-GalCer.[30] This study, performed in humans, showed a more than 100-fold increase in circulating NKT cells in all patients treated with the pulsed mDCs. In two other studies a prolonged IFN-γ producing NKT cell response is induced after treatment with α-GalCer loaded mDCs.[29, 49]

Because OCH induces a Th2-cytokine production by NKT cells and DCs loaded with NKT cell ligands can induce a prolonged cytokine production by NKT cells, we used this technique to study the effect of OCH-pulsed mDCs on atherosclerosis. The maturation of DCs with LPS was not affected by the addition of OCH and the OCH-pulsed mDCs were injected in $LDLr^{-/-}$ mice. The treatment with OCH-pulsed mDCs resulted in a 70.6% decrease in atherosclerotic plaque size in the carotid arteries when compared with mDC-treated mice. The same extent of reduction was also observed at the aortic root of these mice (58.1% reduction). Additionally, this effect was absent in mice lacking NKT cells ($Jα281^{-/-}LDLr^{-/-}$ mice) showing that the observed effect is dependent on NKT cells. From these results we may suggest that the bad influence of NKT cells on atherosclerosis can be turned into an athero-protective effect, when the NKT cells are activated with OCH. The best way to deliver OCH is OCH loaded on DCs, because the drug itself was ineffective.

To investigate this athero-protective effect of NKT cells due to the DC treatment, the effects on NKT cell percentages and cytokine profile were determined. One day after the last injection with DCs, an increased percentage of NKT cells was observed in both blood and liver. In addition, more IL-10 producing NKT cells were found in the livers of mice treated with OCH-pulsed mDCs when compared with mDC-treated mice. The increased production of IL-10 and the unchanged production of IFN-γ could explain the reduced plaque size in these mice.[50] After activation within the liver, the NKT cells may possibly migrate out of the liver and become recruited to the atherosclerotic plaque. This migration to the plaque may explain the increased levels of NKT cells in blood after treatment with OCH-pulsed mDCs. Within the atherosclerotic plaque, the NKT cells may start excreting anti-atherogenic cytokines locally. These cytokines, such as IL-10, may influence the immune response in the vessel wall directly. It is shown before that IL-10 protects against atherosclerosis.[50-54] Another possibility is that the NKT cells may induce a bystander effect.[55, 56] It is known that the bystander effects induced by activation of NKT cells with α-GalCer and OCH are different. Upon stimulation with α-GalCer, NKT cells affect the functions of other cells such as T cells, B cells, NK cells and DCs in a direct or indirect manner. Little is known about the effect of OCH-activated NKT cells on neighbouring cells but recently Oki et al. demonstrated that OCH induces less production of IFN-γ and IL-12 by bystander cells due to a lower expression of CD40L on NKT cells.[57] A bystander effect on neighbouring cells such as macrophages and T cells within the plaque may contribute further to the abrogation of the Th1 inflammatory response of atherosclerosis.

Another bystander effect of NKT cell activation in the liver may also explain the observed reduction in cholesterol levels. During the experiment serum cholesterol levels were evaluated at several critical time points. Although there was no effect on the initial cholesterol levels, treatment with OCH-pulsed mDCs induced a significant 24% reduction in cholesterol levels after week 8 of the experiment. The reduced cholesterol levels were due to a lowering of VLDL- and LDL-cholesterol, while HDL-cholesterol was unaffected. This reduction can be caused by an effect on the activity of parenchymal cells in the liver. Von der Thüsen et al.[50] showed before that systemic IL-10 administration resulted in the lowering of VLDL and LDL cholesterol levels in $LDLr^{-/-}$ mice. In this study, the increased IL-10 production by the NKT cells in the liver may be responsible for the increased uptake of cholesterol from the blood and the subsequent secretion of cholesterol in the bile.[50]

To examine whether there are also some bystander effects of the activation of NKT cells via OCH-pulsed mDCs on the humoral immune response we determined the effect on oxLDL and MDA-LDL specific antibodies. There were however no effects on both IgM and IgG antibody titers.

In conclusion, the activation of NKT cells via the administration of OCH-pulsed mDCs reduces the atherosclerotic plaque formation in $LDLr^{-/-}$ mice. The reduction in atherosclerosis can be explained by an increased percentage of IL-10 producing NKT cells in the liver. This IL-10 in the liver can induce a bystander effect that may be responsible for the lowering of cholesterol levels in the treated mice. Altogether, this strategy of immunomodulation with mDCs loaded with OCH could be used as a new therapeutical approach to prevent atherosclerosis.

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Chapter 4

Adoptive transfer of immature dendritic cells prevents progression of established atherosclerosis in LDLr$^{-/-}$ mice

-Manuscript submitted-

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Abstract

Dendritic cells have the unique capacity to initiate and, more importantly, to regulate the immune response. Due to these properties, they have been used as an immunotherapy in many different models for cancer and autoimmune diseases. Over the past years, there has been accumulating data identifying an important role for the immune system and inflammation in atherosclerosis. Therefore, in this study we explored the possibility to use dendritic cells in a mouse model of established atherosclerosis to modulate disease progression. For this end male $LDLr^{-/-}$ mice were fed a Western-type diet for 20 weeks. After 20 weeks of diet, a baseline group of mice was sacrificed and others received three *i.v.* injections with either PBS or ImDCs (n=10; $1.5x10^6$ cells per injection). Subsequently, these mice were kept on diet for another 10 weeks. Upon sacrifice, lesion size was determined. Plaque area analysis at different sites throughout the vasculature showed that plaque progression during the additional 10 weeks on diet was inhibited in mice treated with ImDCs. During the same time, plaque size in the PBS-treated group continuously increased. The injection of ImDCs led to an increased peripheral/intrahepatic CD4/CD8 ratio and an induction of regulatory T cells. In addition, ImDCs treatment affected the cholesterol metabolism by reducing LIGHT and Lymphotoxin-α which resulted in lowered cholesterol and triglyceride levels. Furthermore, treatment resulted in a more beneficial lipoprotein profile with reduced VLDL and increased HDL levels. We also observed an increase in $CD4^+TCR\beta^+DX5^+$ cells after injection of ImDCs but adoptive transfer of these cells in our current model of established atherosclerosis did not affect lesion size. Our data demonstrate the immunomodulatory potential of ImDCs in a model for established atherosclerosis and further supports the possibility to use dendritic cell-based treatment in cardiovascular disease.

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells and have the unique ability to activate naïve $CD4^+$ and $CD8^+$ T cells during the primary phases of an immune response. Immature DCs (ImDCs) patrol the periphery and sample their micro-environment. In the steady state, ImDCs will take up self-antigens but remain in their immature or semi-mature state. These cells lack the appropriate expression of costimulatory molecules, such as CD80 and CD86. Therefore, presentation of self-antigens to T cell will lead to their anergy or to the induction of IL-10 producing regulatory T cells, hence, resulting in tolerance.[1] In the case of an infection, DCs sense "danger signals" and become fully matured and are able to activate T cells.[2] In healthy arteries, scattered DCs reside in the sub-endothelial space as well as in the media.[3] Arterial DCs may play an important role in monitoring the artery for antigens. During lesion development, lesions become enriched in DCs with a more mature phenotype. Lesional DCs may play an important role in activating T cells and thus sustaining plaque inflammation. In addition, DCs can also regulate atherosclerosis via secretion of various cytokines, such as IL-12 or IL-10.[4] These unique characteristics make DCs an interesting candidate for immunotherapies. Additionally, by modifying autologous DCs *ex vivo* in either tolerogenic or activating DCs, one can optimize their properties for further therapeutic applications. Indeed, DCs have been used in many animal models and in cancer therapy as an immunotherapy.[5, 6] Furthermore, DCs are already being used in clinical trials to activate tumor-specific $CD8^+$ cells.[7] Recently, we described the use of oxidized LDL (oxLDL)-pulsed DCs in a vaccination protocol in $LDLr^{-/-}$ mice. We showed that injection of oxLDL-pulsed DCs prior to the induction of atherosclerosis induced an oxLDL-specific humoral response, which leads to protection against atherosclerosis.[8] Because of the tolerogenic features of immature DCs (ImDCs), we hypothesized that the use of ImDCs could be a simple way to introduce a protective immunomodulation of established atherosclerosis. It has been shown that ImDCs can expand the regulatory T cell population (Tregs) by the production of IL-10[9, 10] and there is accumulating evidence suggesting a protective role of Tregs in atherosclerotic lesion development and progression.[11, 12] Furtermore, the injection of immature DCs has been shown to induce tolerogenic cells that play a protective role in a number of auto-immune diseases.[13-15] Therefore, we have explored the potential of immature DCs as an immunotherapy in a model of established atherosclerosis. In the present study we show that adoptive transfer of unloaded ImDCs *i.v.* into $LDLr^{-/-}$ mice with established lesions prevents further plaque progression at various sites of lesion development. The inhibited lesion progression may be explained by the induction of tolerance and the reduced mRNA levels of LIGHT and lymphotoxin-α which are correlated to the observed lowered levels of cholesterol. Consequently, our current data shows for the first time the beneficial effect of treatment of established atherosclerosis with immature dendritic cells, which further underlines the necessity to clinically explore immunomodulation by using dendritic cells.

Materials and methods

Media and reagents

Cell culture medium was IMDM (PAA, Germany) supplemented with 8 % FCS (PAA, Germany), 100 U/ml streptomycin/penicillin (PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 µM β-mercaptoethanol.

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male LDLr$^{-/-}$ mice were from Jackson Laboratories on a C57BL/6 background and bred in-house. Male C57BL/6J mice were from Charles River Laboratories. Mice were kept under standard laboratory conditions and were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Mice were 10-12 weeks old at the start of the experiment. Diet and water were administered *ad libitum*.

Generation and injection of BM-DCs

Bone-marrow cells of C57BL/6 mice were harvested and cultured during 10 days in complete IMDM in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) to obtain immature DCs. Purity was assessed by flow cytometry using CD11c as a specific DC marker (purity > 90%).

Induction of atherosclerosis

Male LDLr$^{-/-}$ mice were put on a western-type diet to induce atherosclerosis. After 20 weeks of diet, the baseline group was sacrificed (n=10). Remaining mice then received 3 *i.v*. injections (1 injection every other day) of ImDCs ($1.5x10^6$ cells in 100 µl; n=10) or 100 µl PBS (n=10) and were kept on western-type diet for an additional 10 weeks. After a total of 30 weeks on diet, mice were sacrificed. Tissues were then harvested after *in situ* perfusion using PBS and subsequent formalin perfusion. Fixated hearts and aortas were stored in formalin, while the brachiocephalic arteries were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use.

Histological analysis and morphometry

To determine plaque size, cryosections of the aortic root (10 µm), brachiocephalic artery (5 µm) and aortic arch (5 µm) were stained with Oil-Red-O and hematoxylin (Sigma Aldrich, The Netherlands). Corresponding sections were stained immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b, Research Diagnostics Inc, NJ) or were stained for collagen fibers using the Masson's Trichrome method (Sigma Aldrich, Zwijndrecht, The Netherlands). For the heart valves, the largest plaque and four flanking sections were used for assessment. The entire aorta was isolated and photographed. Relative plaque area was assessed *en face* by calculating the ratio of atherosclerotic lesions (white) to the surface of the entire aorta. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Cholesterol assay
Blood was collected at several time points during the experiment by tail vein bleeding. Plasma was obtained after centrifugation and total plasma cholesterol levels were measured using enzymatic procedures and a spectrophotometer (Roche Diagnostics, The Netherlands). Precipath was used as an internal standard (Boehringer, Mannheim, Germany). The cholesterol distribution over the different lipoproteins was analyzed by fractionation, using a Superose 6 column (3.2 x 30 mm, Smart-System, Pharmacia).

Flow Cytometry
After sacrifice, the blood, spleen, liver and mediastinal lymph nodes were isolated (n=5 per group). Single cell suspensions were obtained by using a 70 μm cell strainer (Falcon, The Netherlands). Blood and spleen cells were lysed using 0.83% NH_4Cl in 0.01 M Tris/HCl pH 7.2. Mononuclear cells from the liver were isolated from non-parenchymal cells using Lympholyte (Cederlane, Canada). Subsequently 300.000 cells were stained with antibodies (Ebioscience, Belgium). FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software.

ELISA for detecting IgG and IgM
Total and oxLDL-specific IgM, IgG, IgG1 and IgG2c levels were detected using the Mouse MonoAb ID kit (Zymed Laboratories Inc., South San Francisco, USA), according to manufacturer's instructions. Briefly, for the detection of total antibody levels serum samples were 1:1 diluted in PBS and absorbance was read at 405 nm. For the detection of oxLDL-specific Ab, high binding plates were first coated overnight with 50 μl Cuox-LDL (5 μg/ml). Plates were then washed and 50 μl of diluted serum samples were added. Absorbance was read at 405 nm.

ELISA for detecting cytokines
Spleens from mice treated with ImDCs (n=5) or PBS (n=5) were isolated and smashed through a 70 μm filter. Red blood cells were lysed using 0.83% NH_4Cl in 0.01 M tris/HCL, pH 7.2. Splenocytes were cultured in triplicates at $2x10^5$ cells/well in the presence or absence of oxLDL (5 μg/ml) in complete RPMI 1640 (cRPMI). Concanavalin A (ConA, Sigma-Diagnostics, MO) (2 μg/ml) served as positive control. After 24 hours, cytokine levels in the supernatant were determined, according to the manufacturer's protocol (Ebioscience, Belgium). Levels of TGF-β, IFN-γ, IL-10 and IL-4 were measured in the supernatants of the ConA stimulated spleen (Ebioscience, Belgium). In short, spleen culture triplicates treated with ConA (n=5 per group) were pooled and cytokine levels were determined in the undiluted supernatant following the manufacturer's instructions.

Adoptive transfer of $CD4^+TCR\beta^+DX5^+$ cells
8 male $LDLr^{-/-}$ mice on a western-type diet were injected with ImDCs as described above. Three days after the last injection, mice were sacrificed and lymphocytes were isolated from the spleen and liver using a density gradient (Lympholyte, Cederlane). $CD4^+DX5^+$ cells were isolated using CD4-FITC (Ebioscience, Belgium) and the Anti-FITC MultiSort Kit and subsequently CD49b (DX5) Microbeads,

following manufacturer's protocol (both from Miltenyi, The Netherlands). Purity of the isolated population was assessed by flow cytometry. A single *i.v.* injection of PBS, $CD4^+TCR\beta^+DX5^-$, or $CD4^+TCR\beta^+DX5^+$ cells (150.000 cells in 100 µl) was given to mice with established atherosclerosis. Mice were sacrificed after 10 more weeks of diet feeding.

Real-Time PCR

mRNA was isolated from livers using the guanidium isothiocyanate (GTC) method and reverse transcribed using RevertAid M-MiLV reverse transcriptase. Quantitative gene expression analysis was performed on the ABI7300 using sybergreen technology. The expression was determined relative to the average expression of household genes (36B4, 18S, β-actin and GAPDH). Expression levels of mice treated with PBS were set to 1.

Table 1: Primer sequencens

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
18s	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
β-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
GAPDH	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
Hepatic Lipase	CAGCCTGGGAGCGCAC	CAATCTTGTTCTTCCCGTCCA
ABCA1	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
ABCG1	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG
Lipoprotein Lipase	CCAGCAACATTATCCAGTGCTAG	CAGTTGATGAATCTGGCCACA
LIGHT	TGGAGCCCGGTTACTACTATGTG	TTCGGGTAGCGGGATGTG
Lymphotoxin-α	TGGAGCCTTTGCACTGTAGATTC	CGTGAGAGCTCCAGGTTATTTTAGT
Lymphotoxin-β receptor	AATTATGGATACTGACGTCAACTGTGT	CCCTGGATCTCACATCTGGTATG

Statistical analysis

Values are expressed as mean ± SEM. Data were analyzed with either a parametric or non-parametric ANOVA when comparing three groups followed with Turkey post T-testing. When comparing two groups, a two-tailed Student's T-test or Mann-Withey U test has been used. Statistical analysis was performed using the Instat3 software. Probability values of $P<0.05$ were considered significant.

Results

Injection of ImDCs prevents progression of atherosclerosis

To induce established atherosclerosis, LDLr$^{-/-}$ mice were put on a western-type diet for 20 weeks. At this time-point, the mice of the baseline group were sacrificed and the remaining mice received three *i.v*. injections of either ImDCs or PBS. After a total of 30 weeks of diet feeding, mice were sacrificed and plaque formation was analyzed at different sites in the vasculature. Figure 1A shows representative pictures of plaque size in the aortic root. Continuous feeding of Western-type diet resulted in a significant 1.5-fold increase in plaque size in mice that were treated with PBS compared to the baseline group ($4.06x10^5 \pm 7.22x10^4$ μm² vs $7.59x10^5 \pm 3.88x10^4$ μm²), while plaque progression in mice treated with ImDCs was completely prevented ($4.74x10^5 \pm 4.74x10^4$ μm²) (P=0.0118). Treatment with immature DCs had the same effect in the brachiocephalic artery (Baseline: $1.13x10^5 \pm 2.05x10^4$ μm²; PBS: $2.22x10^5 \pm 2.16x10^4$ μm²; ImDCs: $8.08x10^4 \pm 1.5x10^4$ μm²; P=0.0001) (Figure 1B). Plaque size in the aortic arch was also determined and we again observed an attenuation of plaque progression (Baseline: $4.91x10^4 \pm 8.84x10^3$ μm²; PBS: $7.42x10^4 \pm 5.01x10^3$ μm²; ImDCs: $5.06x10^4 \pm 6.01x10^3$ μm²) (P=0.0241) (Figure 1C). Representative pictures of the different sites are depicted in Figure 1D.

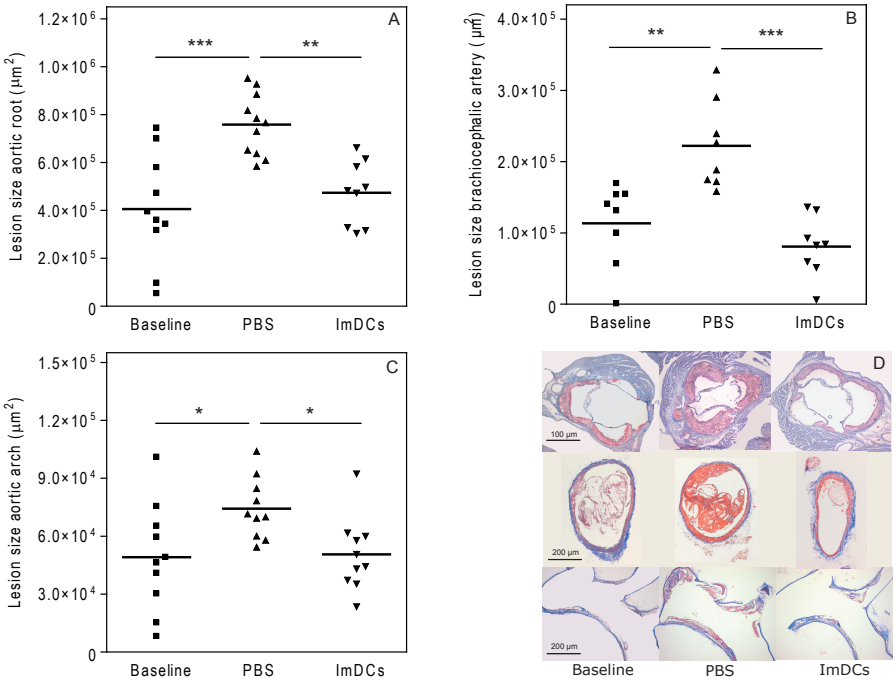


Figure 1: Adoptive transfer of ImDCs prevents further progression of plaque size throughout the vasculature

LDLr$^{-/-}$ mice were put on a western-type diet for 20 weeks. At this time point, the baseline group was sacrificed and other mice were given three *i.v* injections of PBS or ImDCs (1.5 x10^6 cells in 100 μl). After a total of 30 weeks of diet feeding, mice were sacrificed and lesion size was determined in the aortic root (A), the brachiocephalic artery (B) and the aortic arch (C). Representative pictures of lesion size at the different sites are shown in D (*P<0.05; **P<0.01; ***P<0.001).

In addition, when determining the relative plaque size in the entire aorta *en face,* we observed the same effect: mice that received PBS injections showed a 1.8-fold significant increase in plaque area after 30 weeks of diet compared to mice in the baseline group (0.283±0.045 vs 0.507±0.025). However, mice that received ImDCs showed no plaque progression (0.359±0.036) (*P*=0.0005) (Figure 2A and 2B). We then assessed plaque morphology by the number of macrophages and T cells in the aortic valves. Relative numbers of macrophage and T cell numbers were not affected (data not shown). Collagen content was assessed at all sites but was only significantly changed in the aortic arch of mice treated with ImDCs (Figure 3C).

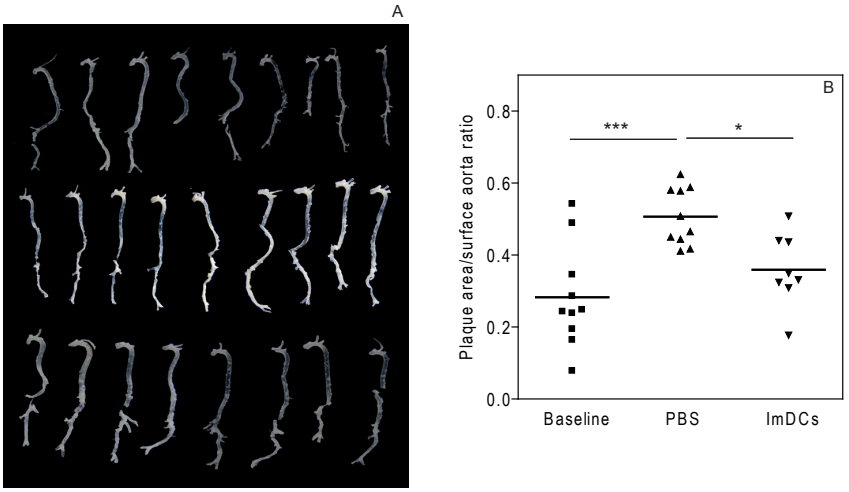


Figure 2: Plaque size growth in the entire aorta was prevented after ImDCs treatment
Entire aorta's were isolated and photographed after PBS and fixative perfusion. Representative pictures of the aorta of baseline, PBS and ImDCs treated mice are shown in A. The plaque area/surface aorta ratio was quantified (B) (*P<0.05; ***P<0.001).

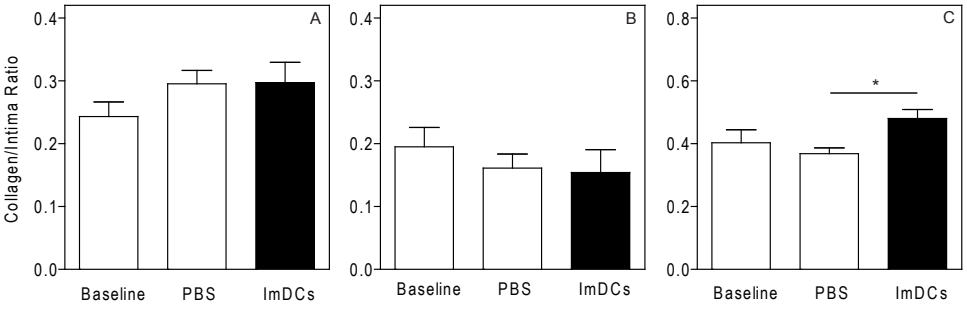


Figure 3: Collagen content was only affected in the aortic arch
Plaque composition was assessed in the aortic root (A), the brachiocephalic artery (B) and the aortic arch (C). The amount of collagen was determined by quantifying the ratio of collagen to intima size and was only increased in the aortic arch (*P<0.05).

Effect of adoptive transfer of ImDCs on lymphocyte subsets

To evaluate the effect of ImDCs injections *in vivo,* we analyzed at 3 days, 3 weeks and 10 weeks after injection the cellular composition of spleen, liver, lymph nodes and blood by flow cytometry. At three days after the last injection of ImDCs, we observed an increased percentage of $CD4^+TCR\beta^+DX5^+$ cells in both the liver (18.30±2.47% vs 42.15±16.12% $P<0.001$) and spleen (6.97±0.97% vs 16.12±0.71% $P<0.001$) compared to PBS treatment (Figure 4).

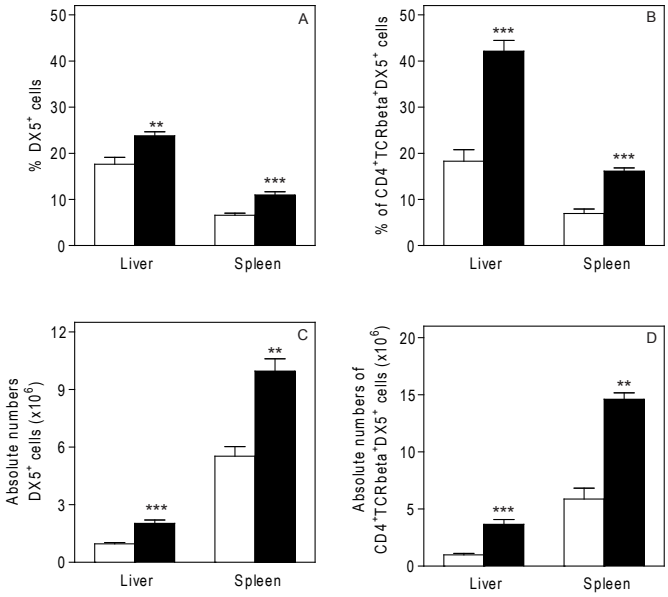


Figure 4: Induction of $CD4^+TCR\beta^+DX5^+$ cells 3 days after injection of ImDCs in both liver and spleen

$LDLr^{-/-}$ mice received three *i.v.* injections of PBS (white) or ImDCs ($1.5x10^6$ cells; black) and were sacrificed three days after the last injection. The percentage and absolute numbers of $DX5^+$ (A and C, respectively) and $CD4^+TCRbeta^+DX5^+$ (B and D, respectively) were determined in liver and spleen by flow cytometry (**=$P<0.01$; ***=$P<0.001$).

At this time point we also observed an increase in the numbers of $CD11c^+$ cells in the circulation (6.45±0.63% vs 10.77±0.87%; $P=0.0037$) and a decreased CD4/CD8 ratio in blood (0.89±0.04 vs 0.69±0.07; $P=0.047$), lymph nodes (1.35±0.03 vs 1.10 ±0.06; $P=0.006$) and liver (1.95±0.42 vs 0.86±0.12; $P=0.024$). In addition, we observed an increased peripheral/intrahepatic ratio of CD4/CD8 indicative of CD8 trapping in the liver (Figure 5). Three weeks after injection of the ImDCs, the early increase in $CD4^+TCR\beta^+DX5^+$ cells could no longer be observed in liver and spleen (data not shown). However, there was still an increase in the number of $CD4^+TCR\beta^+DX5^+$ cells (1.26±0.05% vs 2.41±0.39%; $P=0.049$) and $CD11c^+$ cells in the mediastinal lymph nodes (3.75±0.22% vs 4.73±0.31; $P=0.047$). Additionally, we also observed an increase in $CD4^+CD25^{high}$ regulatory T cells in blood (0.25±0.08% vs 0.71±0.12%; $P=0.021$), and this increase was still present at 10 weeks after injection (0.95±0.05% vs 1.55±0.24%; $P=0.049$).

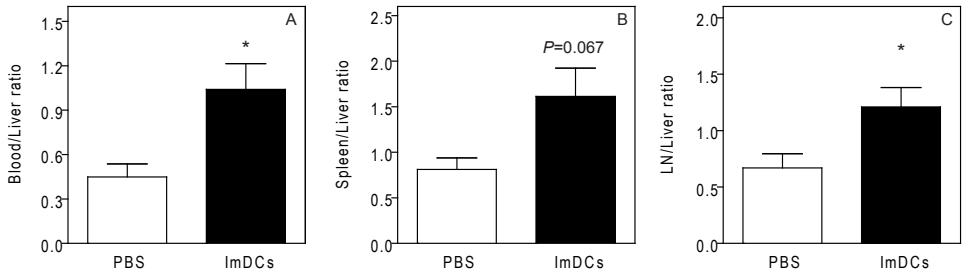


Figure 5: Increased peripheral/intrahepatic CD4/CD8 ratio after ImDCs treatment is indicative of tolerance induction
LDLr$^{-/-}$ mice on a WT-diet were given 3 *i.v* injections of ImDCs (1.5 x10^6 cells in 100 µl). Mice were sacrificed 3 days after injection and the percentage of CD4$^+$ and CD8$^+$ T cells was determined in different organs by means of flow-cytometry. Subsequently we calculated the peripheral/intrahepatic CD4/CD8 ratio for blood (A), spleen (B) and lymph nodes (C). The increased peripheral/intrahepatic ratio suggests CD8$^+$ T cell trapping in the liver, a key process in tolerance induction (*P<0.05).

To further test whether the adoptive transfer of ImDCs affected the humoral and T cell response, we checked for antibodies and T cell proliferation. Plasma samples from each mouse were obtained after sacrifice and total IgG, IgM and subclass specific antibodies were determined. Also, IgG, IgM and subclass specific antibodies against oxLDL were evaluated. We observed no differences in total IgM, IgG, and IgG1/IgG2c ratio (Figure 6A-C); and in oxLDL-specific IgM, IgG, and IgG1/IgG2c ratio (Figure 6D-F). Additionally, injection of ImDCs did not lead to altered proliferation or cytokine production after *ex vivo* stimulation of splenic T cells (Figure 7).

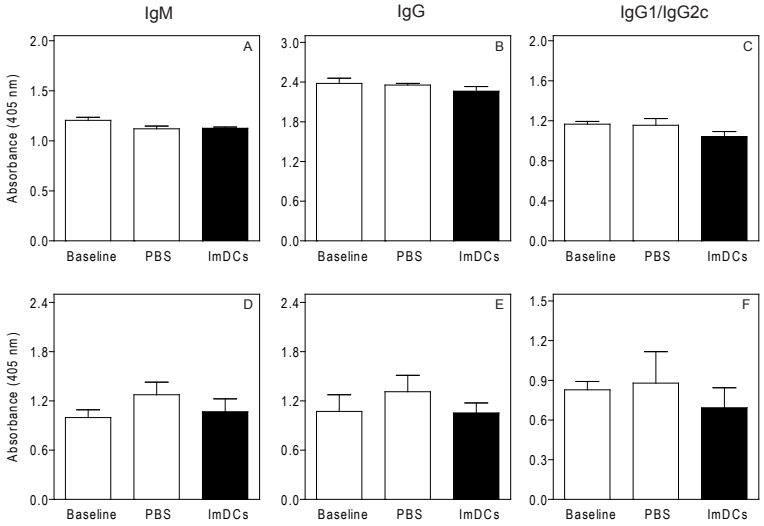


Figure 6: Humoral response was not affected after adaptive transfer of ImDCs
Serum of mice with established lesions was isolated 10 weeks after injections and the amount of antibodies was determined. Serum was 1:1 diluted in PBS and total IgM (A), IgG (B) and IgG1/IgG2c ratio (C) was calculated. Also the oxLDL-specific IgM (D), IgG (E) and IgG1/IgG2c ratio (F) was determined but no differences were observed. Absorbance was read at 405 nm.

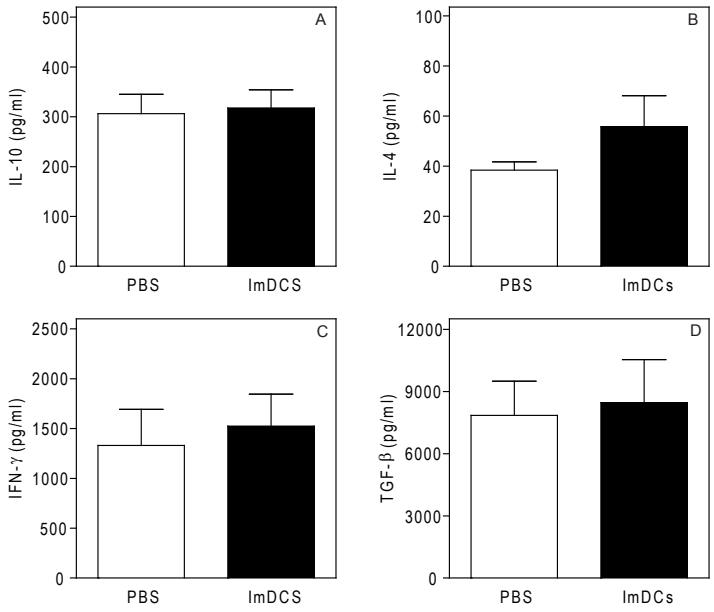


Figure 7: Cytokine production of splenocytes after *in vitro* stimulation was not affected
Supernatants of total splenocytes activated with ConA were collected and the level of IL-10 (A); IL-4 (B); IFN-γ (C) and TGF-β (D) were determined.

The inhibition of plaque progression after injection of ImDCs does not result from increased numbers of $CD4^+$ $TCR\beta^+DX5^+$ cells

Because of the therapeutic potential of tolerogenic DX5 cells in models for rheumatoid arthritis and multiple sclerosis, we determined whether the inhibition of plaque progression induced by ImDCs was mediated by $CD4^+TCR\beta^+DX5^+$ cells. To this end, we isolated $CD4^+TCR\beta^+DX5^+$ cells from the spleen and liver 3 days after ImDCs injections and transferred these cells into recipient $LDLr^{-/-}$ mice with established atherosclerosis. After 10 additional weeks on Western-type diet, mice were sacrificed and lesion progression was assessed. Plaque size in the aortic root did increase during the ten extra weeks of diet in the PBS treated group by a factor 1.74. Treatment with $CD4^+DX5^+$ cells did not affect lesion progression compared to the PBS treated group. Also collagen content was not affected after treatment (Figure 8). The additional control group treated with $CD4^+$DX5 negative cells did also demonstrate the same plaque progression as the PBS or $CD4^+DX5^+$ treated animals compared to baseline.

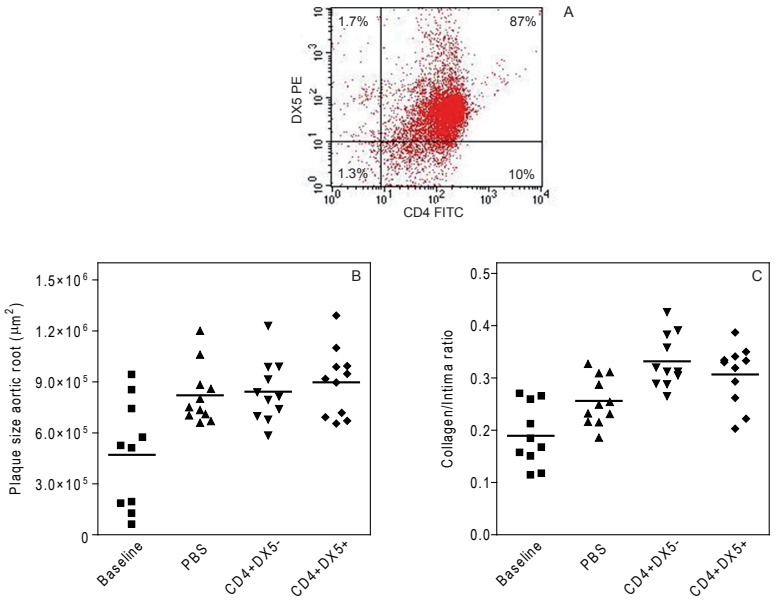


Figure 8: Adaptively transferring CD4$^+$TCRβ$^+$DX5$^+$ cells does not prevent plaque progression in a model of established atherosclerosis
8 LDLr$^{-/-}$ mice on a western-type diet were injected three times with ImDCs. Three days after the last injection, mice were sacrificed and spleen and liver were isolated. CD4$^+$ cells from both liver and spleen were isolated using CD4-FITC beads. After detaching the beads, DX5$^+$ cells were positively selected using DX5 beads. CD4$^+$TCRβ$^+$DX5$^+$ obtained from spleen and liver were pooled and purity was assessed with flow-cytometry (A). LDLr$^{-/-}$ mice with established atherosclerosis received a single *i.v.* injection of either CD4$^+$TCRβ$^+$DX5$^+$, CD4$^+$TCRβ$^+$DX5$^-$ cells or PBS (150.000 cells in 100 µl) and after 10 more weeks of western-type diet feeding, mice were sacrificed and lesion size and collagen content was determined. Lesion size (B) or collagen content (C) was not affected by adaptively transferring CD4$^+$TCRβ$^+$DX5$^+$ cells.

Treatment with ImDCs affects cholesterol metabolism

Both body weight and total cholesterol levels were determined at different time points during the experiment. Body weight did not vary between groups throughout the experiment. After 20 weeks of diet, mice were equally distributed according to their cholesterol levels prior to injection of the ImDCs (Figure 10A). Cholesterol levels in the mice treated with ImDCs showed a trend towards a reduction in cholesterol levels already three weeks after injection (P=0.08) (Figure 9B). At 10 weeks after treatment with ImDCs, a 41% reduction in plasma cholesterol level compared to the PBS treated mice was observed (P=0.0018; Figure 10B), while triglyceride levels were also lowered (P=0.0079; Figure 10C). In addition, mice treated with ImDCs presented a more beneficial cholesterol profile, with decreased VLDL levels and increased HDL levels (Figure 10D). When determining the expression levels of genes involved in lipid metabolism, we observed an initial increase in relative expression of hepatic lipase, lipoprotein lipase, ABCA1 and ABCG1 three days after the last injection in mice injected with ImDCs (Figure 9A), but these expression levels were normalized three weeks after injection (data not shown). However when we assessed gene expression levels at ten weeks after injection, we observed a lowered expression of LIGHT and Lymphotoxin-α (Figure 11).

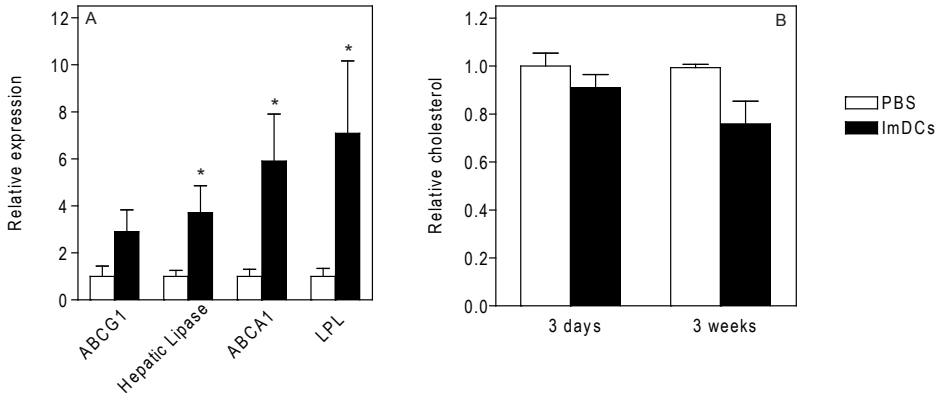


Figure 9: Short-term effect of ImDCs treatment on expression of genes involved in lipid metabolism in liver

Mice were given 3 injections of ImDCs and sacrificed after 3 days. mRNA was isolated from liver and the expression of hepatic lipase, lipoprotein lipase, ABCA1 and ABCG1 was determined relative to the average expression of household genes (18s, 36B4, β-actin and GAPDH) (A). Expression of genes from mice treated with PBS was set to 1. (B) Cholesterol levels were determined 3 days and 3 weeks after injection of ImDCs (B) (*P<0.05).

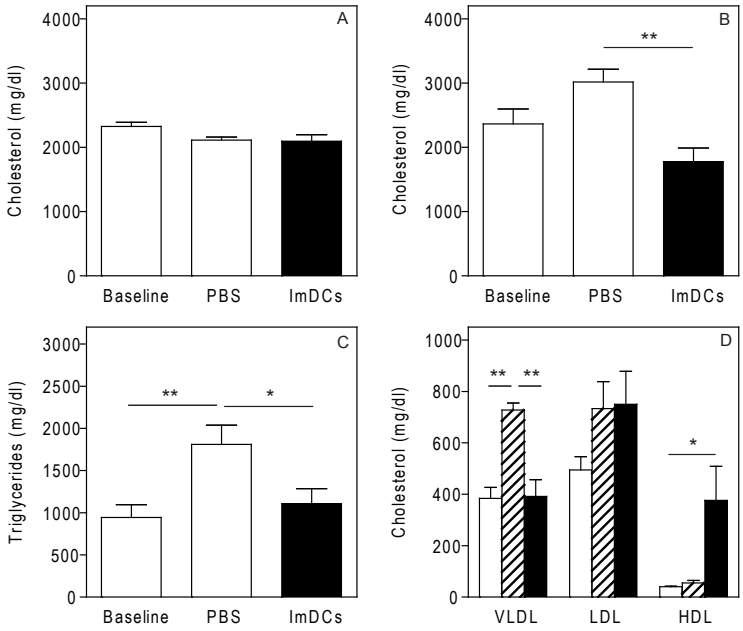


Figure 10: Treatment with ImDCs lowers serum cholesterol and triglyceride levels

Cholesterol levels in plasma were determined after 20 weeks of diet feeding to distribute the mice equally between groups before the injection of ImDCs or the sacrifice of the baseline group (A). At sacrifice, mice treated with ImDCs have lowered cholesterol levels (B) and triglyceride levels (C). Serum was separated on a Sepharose 6 column and fractions were collected to obtain lipoprotein profiles. The area under the curve was calculated. Mice treated with ImDCs had a more beneficial lipoprotein profile (D) (*P<0.05;**P<0.01).

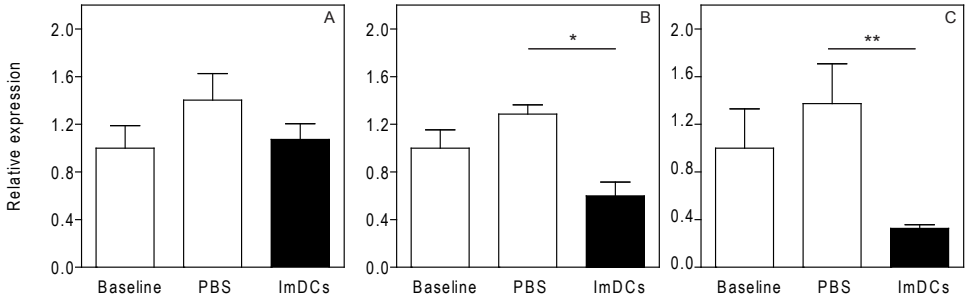


Figure 11: Expression of LIGHT and Lymphotoxin-α is reduced in mice treated with ImDCs
Liver mRNA from LDLR$^{-/-}$ mice with established lesions was isolated 10 weeks after injections. The expression of LTβ receptor (A), LIGHT (B) and LT-α (C) was determined relative to the average expression of household genes(18s, 36B4, β-actin and GAPDH). Expression of genes from mice treated with PBS was set to 1 (*=P<0.05; **=P<0.01).

Discussion

As the importance of inflammatory and immune responses in atherosclerosis becomes increasingly clear, it is fascinating to exploit this information in order to beneficially influence the outcome of atherosclerosis by immunotherapy. Especially dendritic cells (DCs) form an interesting target for immunotherapy. The therapeutic potential of immature DCs (ImDCs) has been shown in animal models for autoimmune diseases such as collagen-induced arthritis[13, 16], experimental autoimmune encephalomyelitis[17], and type 1 diabetes.[18] Dyslipidemia may impair the migration of antigen loaded DCs in the periphery to the lymph nodes.[19] In addition with the assumption that continuous presentation of self-antigens from the atherosclerotic lesions by DCs in the lymph nodes is essential for tolerance induction and maintenance, it may be hypothesized that the entrapment of DCs in peripheral tissues will play a major role in breaking tolerance and in the induction of inflammatory responses. Moreover, the maturation status of DCs will determine whether DCs will induce immunity or tolerance.[20] We hypothesized that the injection of ImDCs may overcome the impaired migration towards the lymph nodes and may reestablish tolerance towards atherosclerosis-related antigens. Immature DCs or semi-matured DCs will induce tolerance, whereas, mature DCs are fully functional to activate T cells.[21] *Krupa et al.* elegantly showed that in granulomatous arteritis DCs are trapped in the inflamed adventitia and are of a mature phenotype. A subset of these DCs expresses CD86, which is mandatory to stimulate T cells.[22] Therefore trapped DCs will play a major role in sustaining the inflammatory status of the atherosclerotic plaque. In the present study we show for the first time that ImDC-treatment prevents progression of plaque growth in a mouse model of established atherosclerosis. Lesion progression was stopped at all major sites of plaque development. To elude the mechanism, we intensively assessed the effect of ImDCs treatment on the cellular response after 3 days, 3 weeks and 10 weeks after injection. We observed an increase in the number of tolerogenic CD4$^+$TCRβ$^+$DX5$^+$ cells in liver and spleen in mice that received ImDCs as soon as three days after injection.

These $CD4^+TCR\beta^+DX5^+$ cells have been shown to have a protective activity in animal models of auto-immune diseases.[13-15, 23] Therefore, we isolated purified $CD4^+TCR\beta^+DX5^+$ cells from $LDLr^{-/-}$ mice after ImDCs treatment and adaptively transferred them into recipient $LDLr^{-/-}$ with established lesions. Unexpectedly, the transfer of the $CD4^+TCR\beta^+DX5^+$ cells did however not affect the progression of atherosclerosis. Neither the transfer of the $CD4^+TCR\beta^+DX5^+$ cells, nor the transfer of $CD4^+TCR\beta^+DX5^-$ cells did inhibit the progression of atherosclerosis, indicating that the effect of ImDCs treatment on atherosclerosis progression results from other effects than the induction of the immunoregulatory DX5 cells. Another important potential tolerogenic feature of ImDCs is their ability to induce regulatory T cells.[24] Puijvelde *et al.* showed that oral tolerance to oxLDL and HSP60 lead to an increase of Tregs and a subsequent reduction in lesion size.[11, 25] In accordance, Ait-Oufella *et al.* showed that depletion of Tregs with anti-CD25 treatment aggravated atherosclerosis in $ApoE^{-/-}$ mice.[12, 26] Taken together, these data imply an important protective role of Tregs in atherosclerosis. Also in our present study we observed that treatment with ImDCs induced Tregs in the circulation three weeks after injection which was continued until 10 weeks after treatment. This increase of Tregs could indicate a reduced immune activation after ImDCs treatment. Another frequently used measurement of immune activation is the CD4/CD8 ratio because it presents the ratio between $CD4^+$ T helper cells to cytotoxic/suppressor $CD8^+$ T cells. A decreased CD4/CD8 ratio is therefore an indication of tolerance and an increased intrasplenic/intrahepatic ratio indicates tolerance induced trapping of $CD8^+$ cells in the liver.[27, 28] Indeed we observed that the peripheral/intrahepatic ratio increased shortly after ImDCs injection. Overall, these data suggest that treatment with ImDCs reduced the immune activation as indicated by increased numbers of Tregs in the circulation and a decreased CD4/CD8 ratio. In addition we observed in the present study that the injection of ImDCs also affected cholesterol metabolism. The cholesterol lowering capacity of DCs has recently been described in a recent study by *Gautier et al.* who show that plasma cholesterol levels are lowered after transferring bone-marrow from transgenic mice expressing human Bcl-2 under the murine CD11c promoter into $LDLr^{-/-}$ and $ApoE^{-/-}$ mice. DCs from these mice have enhanced resistance to apoptosis which results in enhanced lifespan of DCs. In addition, diphtheria-toxin induced $CD11c^+$ cell depletion increased cholesterol levels but lesion size was not affected.[29]In contrast, *Stoneman et al.* showed that cholesterol levels were not affected after diphtheria-toxin induced $CD11b^+$ macrophage depletion, indicating that the cholesterol lowering capacity may be restricted to the function of DCs.[30] We now provide more detailed information as to how DCs influence the cholesterol metabolism. As early as three days after injection, the expression of ABCA1, ABCG1, hepatic lipase and lipoprotein lipase mRNA was increased. These genes play an important role in cholesterol transport and metabolism. Although, the expression level of these genes was normalized three weeks after injection, plasma cholesterol levels were already decreased. This decrease in cholesterol continued during the course of the experiment and after ten weeks, mice treated with ImDCs displayed lowered cholesterol and triglyceride levels and a more beneficial lipoprotein profile with decreased VLDL and increased HDL levels, which may contribute to the beneficial effect of ImDCs. It remains however unclear how exactly ImDCs injection affects

cholesterol metabolism. More research on gene expression of a more expanded cohort of genes involved in cholesterol metabolism in time is therefore needed. However, at present we observed a lower expression of LIGHT and lymphotoxin-α ten weeks after treatment. This decrease can also be correlated to the lowered cholesterol and triglyceride levels observed. *Lo et al.* described that LIGHT-overexpressing mice displayed hypercholesterolemia and hypertriglyceridemia even on a chow diet.[31] In another study it was shown that loss of Lymphotoxin-α (LT-α) led to reduced cholesterol levels but increased HDL in LT-α KO mice and a subsequent reduction of atherosclerosis in $ApoE^{-/-}$ mice.[32] Also in a clinical setting there is evidence that blockage of LIGHT and TNF has a beneficial effect on the lipoprotein profile. In a recent study lipoprotein profiles of patients treated with infliximab or etanercept were compared. The blockade of LT-α is a feature of etanercept. Higher HDL levels were observed in patients treated with etanercept, while patients treated with infliximab showed increased LDL levels and total cholesterol after one year of treatment.[33] In addition, rheumatoid arthritis patients have an improved atherogenic index after anti-TNF treatment with adalimumab.[34] Together these data support our findings of lowered cholesterol and a more beneficial lipoprotein profile and that this decrease can be correlated to the lowering of LIGHT and lymphotoxin-α. *Laxton et al.* showed that there is a strong association between susceptibly for myocardial infarction and a polymorphism in the Lymphotoxin-α gene.[35] More importantly, targeted depletion of LT-α expressing Th1 and Th17 cells inhibits autoimmune disease.[36] Therefore, reduced levels of LIGHT and lymphotoxin-α may indicate a reduced inflammatory T cell phenotype in our ImDCs treated mice. In conclusion, we show here for the first time that treatment with ImDCs impairs plaque growth in a mouse model for established atherosclerosis by inducing tolerance and affecting cholesterol metabolism. Therefore, our data provide strong evidence for the therapeutic potential of dendritic cells in a clinically relevant setting. In addition, the use of DCs in clinical trials has now provided proof of principle and has shown that the technique is safe with minimal side-effects, reviewed by *Tacken et al.*[37] Therefore, the use of DCs to shift immune responses towards an anti-atherogenic outcome could also prove to be benificial in the treatment of patients with cardiovascular disease.

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Chapter 5

Deficiency of TGF-β signaling in CD11c^{+} cells accelerates atherosclerosis by disrupting T-cell homeostasis

-Manuscript submitted-

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Abstract

TGF-β is an anti-inflammatory and pro-fibrotic cytokine that plays a bridging role between the innate and adaptive immune system. However, the exact liaison between TGF-β, the innate and the adaptive immune system in atherosclerosis is still unclear. Here we investigate the effect of deficiency of TGF-β signaling in cells of the innate immune system (ie $CD11c^+$ DCs, NK cells and macrophages) in atherosclerosis, by using transgenic mice with a targeted functional inactivation of TGF-βRII signaling in $CD11c^+$ cells ($ApoE^{-/-}$CD11cDNR). $ApoE^{-/-}$CD11cDNR mice were sacrificed at 20 wks of age and plaques in the aortic root were analyzed. $ApoE^{-/-}$CD11cDNR mice exhibited a 2-fold increase in plaque area and showed a substantial increase in $CD45^+$ leukocytes and $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, while macrophage content was not affected. Moreover, a significant reduction in α-smooth muscle cell (ASMA) and collagen content was observed in plaques of $ApoE^{-/-}$CD11cDNR mice. FACS analysis of lymphoid organs of $ApoE^{-/-}$CD11cDNR mice revealed an increase in both $CD4^+$ and $CD8^+$ $CD44^{high}CD62L^{low}$ effector memory T-cells, whereas the number of $CD4^+CD25^+FoxP3^+$ regulatory T-cells had increased. In the supernatant of CD3/CD28 stimulated splenocytes from $ApoE^{-/-}$CD11cDNR mice, elevated levels of Th1 (IFN-γ), Th2 (IL-4, IL-10) and Th17 (IL-17) cytokines were detected. In conclusion, deficient TGF-β signaling in $CD11c^+$ cells results in a disturbed T-cell homeostasis, thereby accelerating atherosclerosis and inducing a pro-inflammatory plaque phenotype.

Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall.[1-3] Immune cells from both the innate and the adaptive immune system orchestrate the formation and progression of atherosclerotic plaques by controlling their structure, composition and expansion.[3] This complex interplay between different cells and cell types of the immune system is coordinated by a broad array of pro- and anti-inflammatory chemokines and cytokines. The balance between pro- and anti-inflammatory modulators is decisive in the degree of atherosclerosis progression and the plaque phenotype that develops.[4-7]

One of the central regulators of the immune response is transforming growth factor beta (TGF-β).[8, 9] Three highly conserved isoforms of TGF-β have been described: TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is present at high levels in healthy blood vessels and interestingly, higher levels of TGF-β1 are present in human asymptomatic lesions than in lesions from symptomatic patients.[10] The closely related TGF-β2 and TGF-β3 isoforms are either absent or present at low levels.[11]

The classical signaling receptors for TGF-β are the TGF-β receptor I (TGF-βRI), also known as activin-like kinase (ALK), and the constitutively active type II receptor (TGF-βRII). Access of the ligands to the type I en type II receptors is regulated by soluble ligand binding proteins and by the accessory type III receptor, also known as betaglycan.[12, 13] TGF-β signals in most cells via TGF-β type II receptor and ALK5.[14-17]

A wide range of cell types, important in atherosclerosis, expresses TGF-β.[8, 11, 18] TGF-β exerts crucial anti-inflammatory functions by inhibiting the proliferation and differentiation of T-cells and the activation of macrophages.[11, 18] In endothelial cells, TGF-β inhibits the expression of adhesion molecules and chemokines required for the recruitment of leukocytes.[19, 20] Moreover, TGF-β is also known to stimulate collagen production in smooth muscle cells (SMC).[21, 22] In 2002 we showed that treatment of ApoE$^{-/-}$ mice with a soluble TGF-β-receptor II protein (TGF-βRII:Fc) that prevents TGF-β signaling, accelerated atherosclerosis. Plaques exhibited an unstable phenotype that contained low amounts of fibrosis, an increased amount of inflammatory cells, and even intraplaque hemorrhages.[23] Mallat *et al.* who injected neutralizing antibodies against TGF-β1 into ApoE-/- mice, obtained similar results.[24] Recently, Frutkin *et al.* showed that mice with cardiac over-expression of TGF-β reduced atherosclerosis formation in the aortic roots and prevented aortic dilatation.[25] These effects could be attributed to TGF-β signaling in T-cells. Mice, with deficient TGF-β signaling in CD4$^+$ T-cells (CD4-dnTGF-βRII), also showed accelerated plaque progression, and a shift towards an unstable atherosclerotic plaque phenotype.[26] This was accompanied by an increased differentiation of T-cells towards both Th1 and Th2 phenotype. Increased levels of IFN-γ, IL-10 and IL-4 were found in cultured splenocytes.[27]

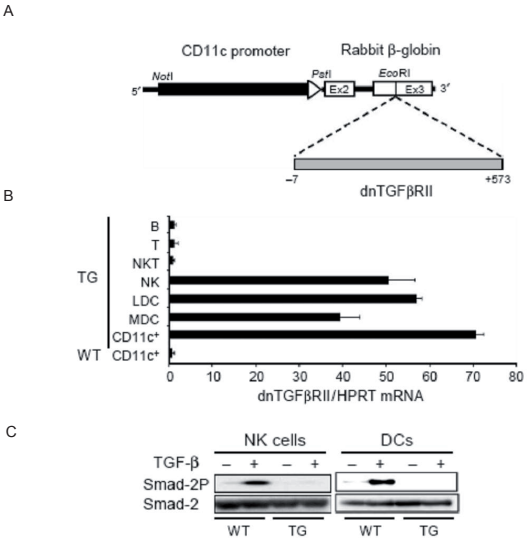
Although the effects of TGF-β signaling of the adaptive immune system in atherosclerosis have been described, limited data are available on the effect of TGF-β signaling in the innate immune system. In atherosclerosis, the principal effector cells of the innate immunity are the DCs, monocytes/macrophages, neutrophils and NK cells, which all express CD11c. In the present study, we investigated the effect of TGF-β signaling in CD11c$^+$ cells in atherosclerosis using ApoE$^{-/-}$ mice

that contained a dysfunctional TGF-β Receptor II (ApoE$^{-/-}$CD11cDNR). We found that blockade of TGF-β signaling in CD11c accelerated atherogenesis, enhanced the influx of both CD4^{+} and CD8^{+} T-cells into the plaques and decreased fibrosis. Systemically, an increased differentiation of T-cells towards effector memory T-cells (CD44^{high}CD62L^{low}) was found together with increased levels of Th1, Th2 and Th17 cytokines. Our data illustrate the important role of TGF-β as a regulator between innate and adaptive immunity in atherosclerosis.

Materials and methods

ApoE$^{-/-}$CD11c-dnTGF-βRII transgenic mice

CD11c-dnTGF-βRII (CD11cDNR) mice were generated as described previously and were backcrossed to ApoE$^{-/-}$ mice (both genotypes were on a C57BL/6 background). In short, the human TGF-β type II receptor sequence between nucleotides -7 and +573 that encodes for the extracellular and transmembrane regions of the TGF-β type II receptor was cloned into the EcoRI site of rabbit β-globin gene exon 3 of plasmid CD11c promoter vector pDOI-5. Expression of the transgene was determined by real-time PCR in freshly isolated cell types from spleen. Samd2 phosphorylation in isolated NK cells and DCs was used as a read-out for disturbed TGF-β signaling (Supplemental Figure 1).[28]



Supplemental Figure 1:Generation and characterization of CD11c-dnTGF-βRII transgene mice.
Transgene construct with in grey the dnTGF-βRII sequence, in white the rabbit β-globin exon gene 3 and in black the plasmid CD11c promoter (A). Normalized values to HRPT of dnTGF-βRII mRNA in cells isolated from spleen as determined by real-time PCR (B). Smad2 phosphorylation in DCs and NK cells after stimulation in the presence or absence of TGF-β with LPS or IL-12 and IL-18, respectively (C).

At the age of 20 weeks, mice were euthanized. Blood was obtained from the retro-orbital plexus and plasma cholesterol was measured using a colorimetric assay (CHOD-PAP, Roche). Spleen, liver and lymph nodes were harvested after in situ perfusion using PBS. Heart and aorta were isolated after subsequent perfusion using 1% parafomaldehyde. Hearts were frozen in Tissue-Tek (Shandon, Veldhoven, The Netherlands). Organs for autopsy were collected in 4% paraformaldehyde. All animal experiments were performed under approved Institutional Animal Care and Use Committee protocols (Yale University).

Histology and morphometry

Plaque area was analyzed using serial sections of 6 μm with 42 μm intervals, beginning from the onset of the aortic valves until the valves had disappeared. For histological analysis of atherosclerosis, sections were stained with hematoxylin and eosin (HE). The extent of atherosclerosis was assessed in aortic roots and plaque area was calculated by using a Leica DM3000 Light microscope (Leica Microsystems) coupled to a computerized morphometry system (Leica Qwin 3.5.1).

Immunohistochemistry

Corresponding sections were immunolabeled with CD45 rat monoclonal antibody (1:5000; Pharmingen) to detect all inflammatory cells, Moma-2 rat monoclonal antibody (1:50; Serotec) to detect macrophages and αSMA monoclonal mouse antibody (1:500; DAKO) as a marker for vascular smooth muscle cells and fibroblasts. To detect T lymfocytes CD3 rabbit monoclonal antibody (1:200;DAKO) was used. CD4 and CD8 rat monoclonal antibodies (undiluted, gift from W. Buurman) were used to distinguish between respectively T-helper cells and cytotoxic T-cells. Sirius red staining was used to detect collagen content. Morphometric analyses were performed using a Leica Quantimet with Qwin3.5.1 software (Leica Microsystems) Fluorescent immunohistochemistry was used to determine the presence of CD11c$^+$ cells in the aortic lesions. CD11c, CD11b, CD4 and CD8 antibodies (all BD Biosciences) were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin chlorophyll protein (PerCP). Sections were analyzed with a Leica TCS SP5 multi-photon microscope (Leica, Germany).

Flow Cytometry

Spleen and lymph nodes were harvested from donor mice and single cell suspensions were prepared and stained with anti-DX5, -TCR, -CD3, -CD4, -CD8, CD25, FoxP3, -CD44, -CD62L, -CD11c (BD Biosciences) or isotype control IgG (PharMingen). Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). Cells were analyzed using a FACS-Canto II (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

Cytokine production

Spleen and lymph nodes from ApoE$^{-/-}$ and DNR mice treated (n=5) or mDCs (n=6) were isolated and gently mashed through a 70 µm nylon cell strainer. Red blood cells were lysed using 0.83% NH_4Cl in 0.01 M tris/HCL, pH 7.2. Splenocytes and lymph node cells were cultured in triplicate at $3x10^5$ cells/well in the presence of soluble αCD3/CD28 (2 µg/ml) in cRPMI. 48 Hours later, supernatants were collected and TGF-β, IFN-γ, IL-10, IL-12p70, IL-4, IL-17 were measured. Cytokine levels were determined in undiluted supernatant following the manufacturer's instructions. (All ELISA's were purchased at Ebioscience, Belgium).

Statistical analysis

Values are expressed as mean ± SEM. Data were analyzed by either a two-tailed Student's T test or a non-parametric Mann-Withney U-test. Statistical analysis was performed using Prism software. Probability values of $P<0.05$ were considered significant.

Results

General

At 20 weeks of age, no significant differences were observed between ApoE$^{-/-}$ CD11cDNR mice and ApoE$^{-/-}$ mice with regard to body weight. Decreased levels of total plasma-cholesterol were detected in ApoE$^{-/-}$CD11cDNR animals compared to ApoE$^{-/-}$ controls (ApoE$^{-/-}$CD11cDNR 5.97±1.25 mg/dl vs ApoE$^{-/-}$ 10.10±0.78 mg/dl; $P<0.05$) while serum triglycerides (TG) did not differ between both groups (ApoE$^{-/-}$CD11cDNR 2.33±0.24 mg/dl vs ApoE$^{-/-}$ 2.57±0.29 mg/dl). In addition, autopsy of > 20 organs revealed no abnormalities or pathologies.

Localisation and subtype of CD11c^{+} cells in atherosclerotic plaques

To detect whether CD11c^{+} cells are present in atherosclerotic plaques, where they were localized and which plaque cell types express CD11c, aortic roots of both ApoE$^{-/-}$CD11cDNR and ApoE$^{-/-}$ mice were double labelled CD11c and cell-type specific markers. Using 2-photon microscopy we were able to clearly distinguish between the different CD11c^{+} expressing cell types (Figure 1).

CD11c positive cells were commonly present in the shoulder region of the plaque and in the adventitia. Interestingly, only a small percentage of CD11c^{+} cells expressed CD11b. This indicates that the majority of CD11c^{+} cells in the plaque were dendritic cells (DC), which was confirmed by double staining with CD11c, CD4 and/or CD8, or macrophage foam cells which are also known to be CD11c^{+}CD11b^{-} but not NK-cells (Figure 1).

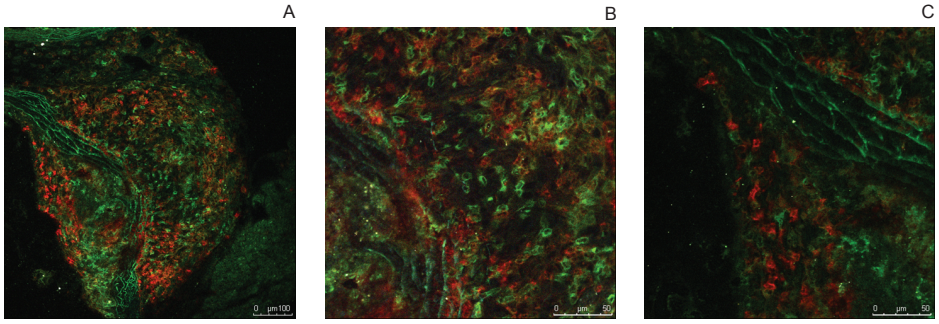


Figure 1: Multi-photon immunohistochemistry to determine the presence of CD11c^{+} cells in the aortic lesions.
(a) CD11c-FITC and CD11b-PE. Both cell types are clearly present in both lesion and adventitia. Only scarce double positive cells are present. (b) Detail of adventitia where massive infiltration of CD11c positive cells (FITC) is observed. (c) Detail of the atherosclerotic lesion. CD11c positive cells (FITC) together with CD11b positive cells (PE) are present in the shoulder region of the plaque.

Defective TGF-β signaling in CD11c$^+$ cells accelerates atherosclerosis and enhances the inflammatory state of the lesion

ApoE$^{-/-}$ mice with disrupted TGF-β signaling in CD11c$^+$ cells (ApoE$^{-/-}$CD11cDNR) exhibited a 2-fold increase in atherosclerotic plaque area in the aortic root (ApoE$^{-/-}$CD11cDNR 2.37x10^5±5.33x10^4 μm^2 vs ApoE$^{-/-}$ 1.11x10^5±2.09x10^4 μm^2, n=8/group, *P*<0.05) (Figure 2). Moreover, the percentage of CD45$^+$ leukocytes was drastically increased in plaques of ApoE$^{-/-}$CD11cDNR animals compared to controls (ApoE$^{-/-}$CD11cDNR 24.04±6.63% vs ApoE$^{-/-}$ 9.74±3.14% CD45+cells, *P*<0.05) (Figure 3 A-C).

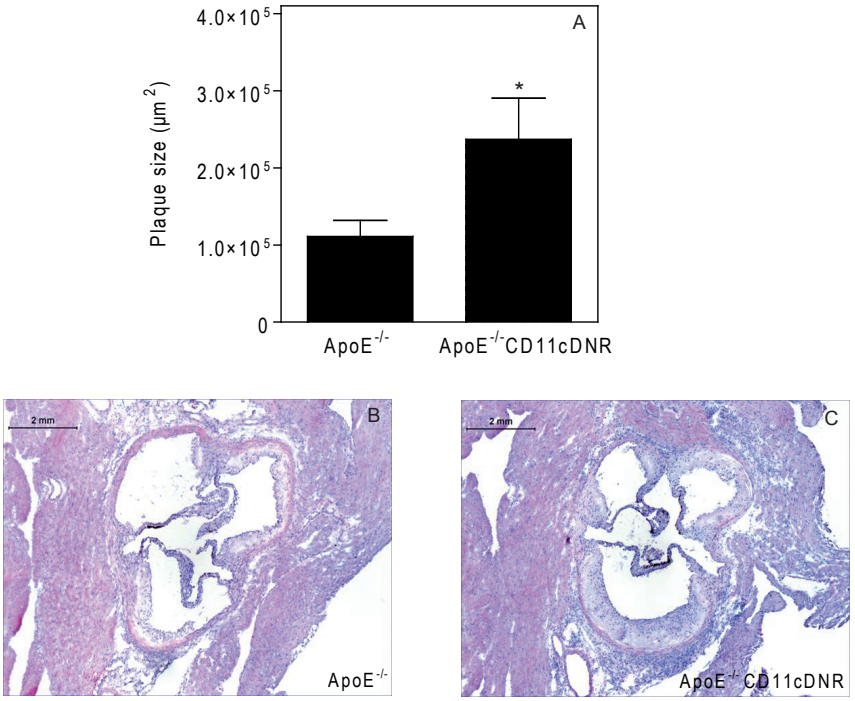


Figure 2: Defective TGF-β signaling in CD11c$^+$ cells accelerates atherosclerosis. Plaque area in μm^2 (A). Representative HE staining of atherosclerotic lesions of the aortic roots (B and C) (magnification 5x) (*P<0.05).

In order to have a more precise view on which leukocyte subsets were increased, we analyzed the lesions for the presence of different T-cell (CD3, CD4, CD8, FoxP3), and macrophage markers (Moma-2). Plaques of ApoE$^{-/-}$CD11cDNR mice contained significantly more CD3$^+$ T-cells than the controls (ApoE$^{-/-}$CD11cDNR 21.71±7% vs ApoE$^{-/-}$ 5.16±1% CD3$^+$cells, *P*<0.05) (Figure 3 D-F). Increased levels of CD4$^+$ T-helper cells were also found in ApoE$^{-/-}$CD11cDNR 12.14±2.73% vs ApoE$^{-/-}$ 5.73±1.25% CD4$^+$cells, *P*<0.05) (Figure 4 A-C). In addition, a substantial amount of CD8$^+$ cytotoxic T-cells was present in plaques of ApoE$^{-/-}$CD11cDNR mice while CD8$^+$ T-cells were almost absent in the control group (ApoE$^{-/-}$CD11cDNR 9.06±3.21% vs ApoE$^{-/-}$ 2.13±0.34% CD8$^+$cells, *P*<0.05) (Figure 4 D-F).

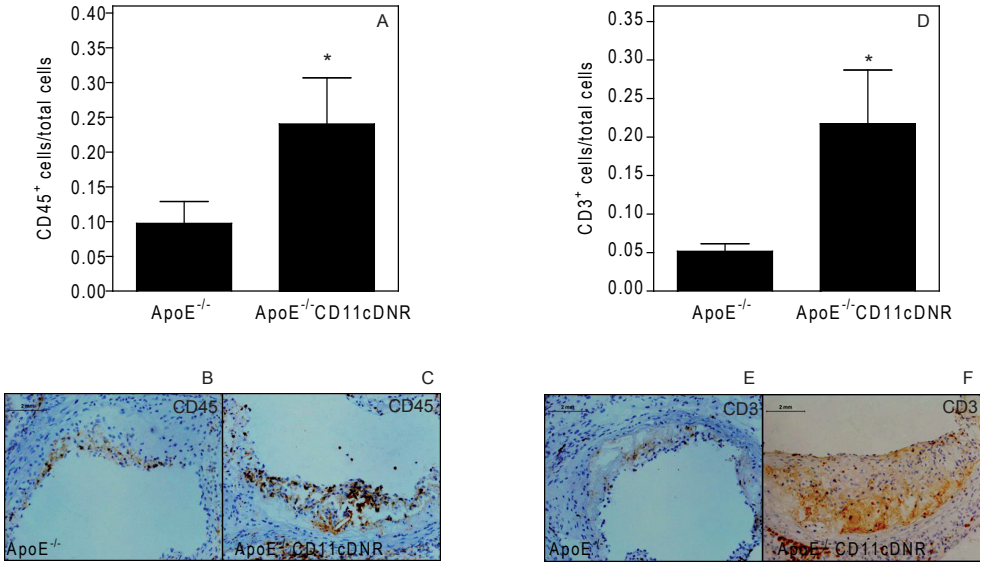


Figure 3: Disruption of TGF-β signaling in CD11c+ cells enhances plaque inflammation.
Number of CD45 positive cells per total cells number in the atherosclerotic plaque (A). Representative CD45 immuno staining (B and C) (magnification 20x). Number of CD3 positive cells per total cells number in the atherosclerotic plaque (D). Representative CD3 immuno staining (E and F) (magnification 20x) (*P<0.05).

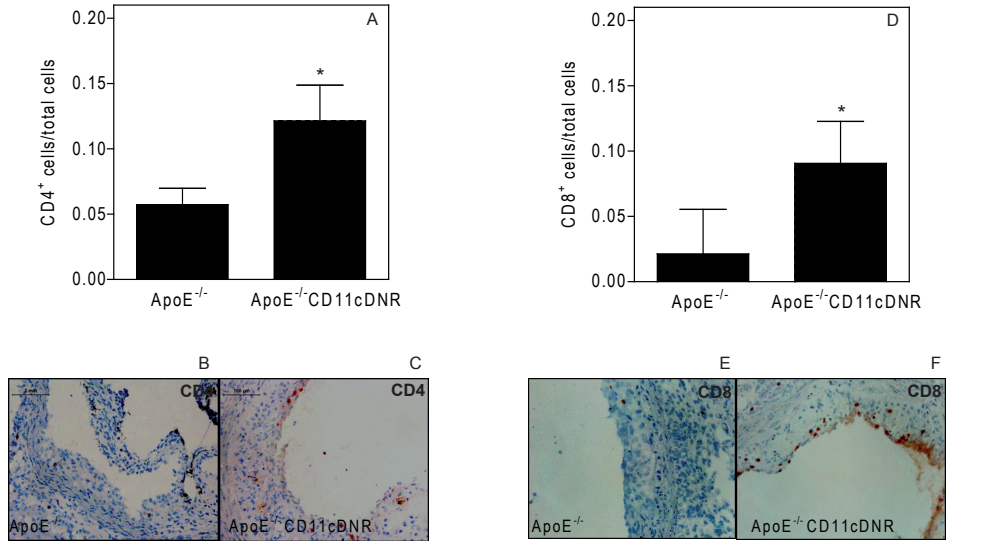


Figure 4: Defective TGF-β signaling in CD11c+ cells attracts both CD4+ and CD8+ T-cells into the lesions.
Number of CD4 positive cells per total cells number in the atherosclerotic plaque (A). Representative CD4 immuno staining (B and C) (magnification 20x). Number of CD8 positive cells per total cells number in the atherosclerotic plaque (D). Representative CD8 immuno staining (E and F) (magnification 20x) (*P<0.05).

However, no differences in FoxP3 staining could be observed between both groups (ApoE$^{-/-}$CD11cDNR 1.01x10^{-2} ±0.53x10^{-2} % vs ApoE$^{-/-}$ 0.87x10^{-2} ±0.37x10^{-2} % FoxP3^{+}cells). In addition macrophage (Moma-2) content did not differ between both groups (ApoE$^{-/-}$CD11cDNR 19.10±4.42% vs ApoE$^{-/-}$ 21.08±2.41% Moma-2^{+}cells).

Defective TGF-β signaling in CD11c^{+} cells inhibits plaque fibrosis

Collagen content on Sirius red-stained sections revealed significantly lower amounts of collagen in plaques of ApoE$^{-/-}$CD11cDNR mice than in ApoE$^{-/-}$ mice (ApoE$^{-/-}$CD11cDNR 30.21±2.15 % vs ApoE$^{-/-}$ 37.96±2.66 % Sirius Red $^{+}$ area, P<0.05) (Figure 5 A-C). The low plaque collagen content was accompanied by a decrease in α-smooth muscle cell^{+} (ASMA) content (ApoE$^{-/-}$CD11cDNR 4.02±0.71 % vs ApoE$^{-/-}$ 9.46±1.83 % ASMA^{+} area, P<0.05) (Figure 5 D-F). These results demonstrate that TGF-β receptor deficiency in CD11c^{+} cells reduces plaque fibrosis, indicative of plaque destabilization.

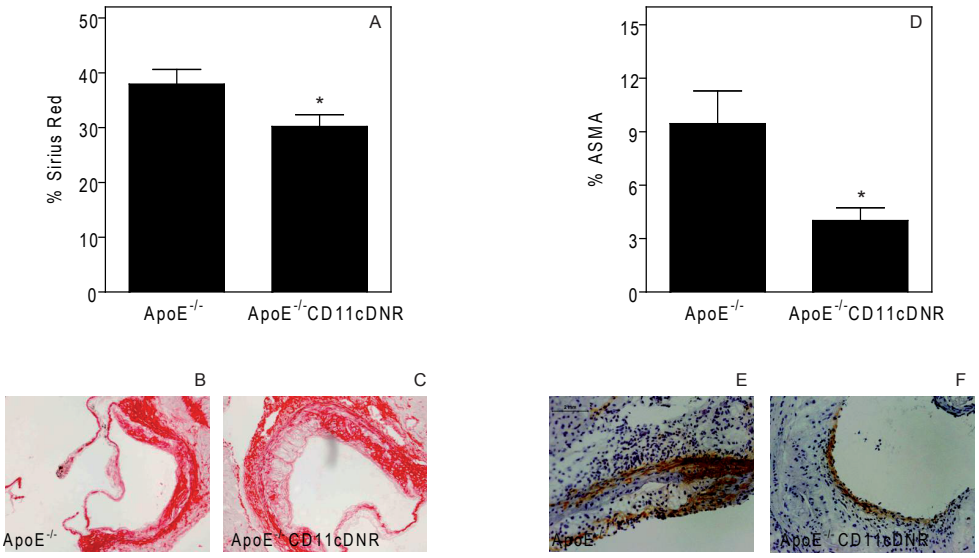


Figure 5: Defective TGF-β signaling in CD11c^{+} cells leads to an unstable plaque phenotype.
Percentage of Sirius red positive staining (A). Representative Sirius red staining (B and C) (magnification 20x). Amount of αSMC content in the atherosclerotic plaque (C). Representative ASMA staining (D and E) (magnification 20x) (*P<0.05).

Defective TGF-β signaling in CD11c^{+} cells alters T-cell homeostasis

To check whether the increased T-cell content in the ApoE$^{-/-}$CD11cDNR lesions was due to an altered T-cell homeostasis, we used FACS analysis to determine the expression of homing receptors CD44 and CD62L on CD4^{+} and CD8^{+} T-cells in the spleen to distinguish from naive (CD44^{low}CD62L^{high}) or effector-memory (CD44^{high}CD62^{Low}) phenotype (Figure 6). The CD4^{+} T-cell population of the ApoE$^{-/-}$CD11cDNR group had a significant decrease in the marker for naïve T-cells (CD4^{+} CD44^{low}CD62^{high}: ApoE$^{-/-}$ 52.96±4.46 % vs ApoE$^{-/-}$CD11cDNR 24.39±2.71 %; P<0.05) and an increase in the marker for effector memory T-cells (CD4^{+} CD44^{high}CD62^{low} population: ApoE$^{-/-}$ 45.85±4.40 % vs ApoE$^{-/-}$CD11cDNR 73.07±2.22 %; P<0.05). Similar effects were seen in the CD8 population (CD8^{+} CD44^{low}CD62^{high} population: ApoE$^{-/-}$ 81.66±2.50 % vs ApoE$^{-/-}$CD11cDNR 59.50±5.98 %; P<0.05; CD8^{+} CD44^{high}CD62^{low} population: ApoE$^{-/-}$ 17.21±2.43 % vs ApoE$^{-/-}$CD11cDNR 35.46±4.42 %; P<0.05). CD62L surface expression is lost when cells become activated. This means that both CD4^{+} T-helper cells and CD8^{+} cytotoxic T-cells from ApoE$^{-/-}$CD11cDNR have characteristics of differentiated T-cells. These effector T-cells, in contrast to naïve T-cells, are potent to enter inflamed tissues.

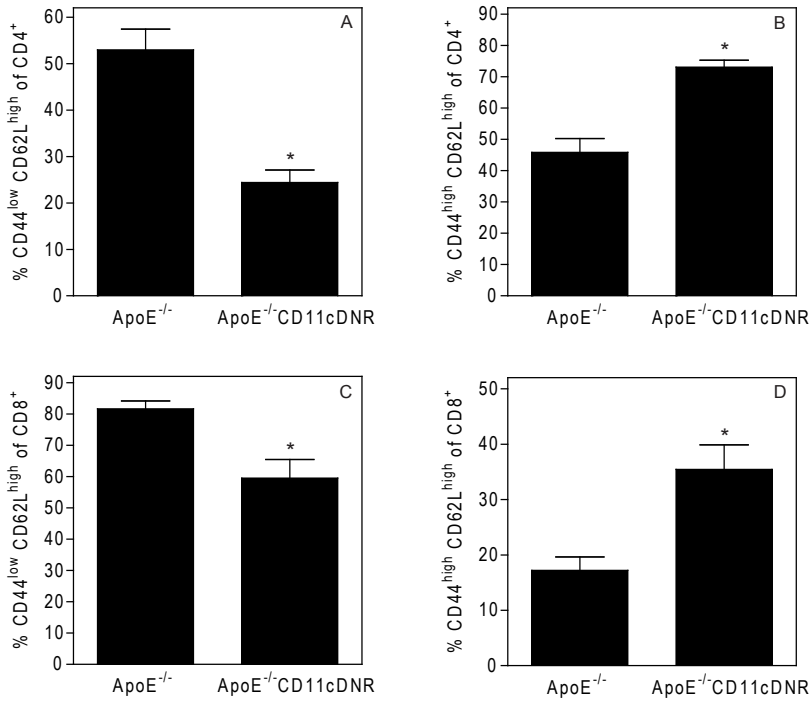


Figure 6: Loss of TGF-β signaling in CD11c^{+} cells leads to the generation of effector T-cells in spleen.
Percentage of naïve CD4^{+} T-cells (A). Percentage of naive CD8^{+} T-cells (B). Percentage of effector CD4^{+} T-cells (C). Percentage of effector CD8^{+} T-cells (D) (*P<0.05).

In addition to T-cell differentiation, an enhanced CD4/CD8 ratio was observed in the spleen of CD11cDNR animals (ApoE$^{-/-}$ 1.42±0.09 % vs ApoE$^{-/-}$CD11cDNR 1.826±0.14 % P<0.05) (Figure 7 A-C). Finally, a significant increase was observed in the amount of CD25^{+}FoxP3^{+} double positive cells within the CD4^{+} population (Tregs) in the spleen of ApoE$^{-/-}$CD11cDNR animals (ApoE$^{-/-}$ 9.16±0.65 % vs ApoE$^{-/-}$CD11cDNR 15.33±1.33 %; P<0.05) (Figure 7 D).

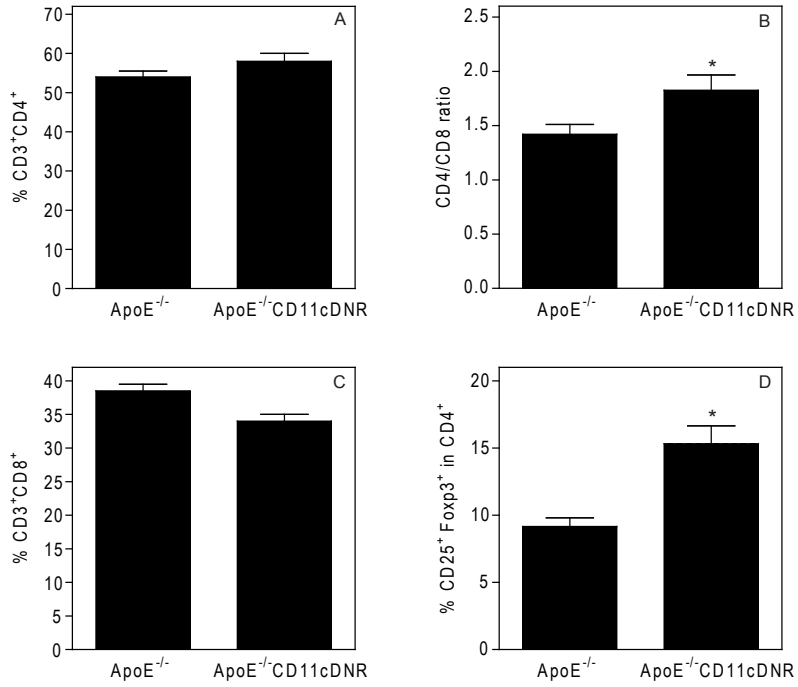


Figure 7: Inhibition of TGF-β signaling in CD11c^{+} cells disturbs T-cell homeostasis in spleen.
Percentage of CD3^{+}CD4^{+} T-cells (A). Percentage of CD3^{+}CD8^{+} T-cells (B). CD4/CD8 ratio T-cells (C). Percentage CD25^{+}FoxP3^{+} of CD4^{+}T-cells (D) (*P<0.05).

TGF-β deficiency in CD11c^{+} cells is associated with increased levels of Th1, Th2 and Th17 cytokines

To determine whether the observed T-cell phenotype correlated with enhanced cytokine levels, T-cell cytokines were determined in the spleen after re-stimulation in vitro with aCD3/CD28. An 8-fold increase of the Th1 cytokine IFN-γ was detected in the ApoE$^{-/-}$CD11cDNR group (ApoE$^{-/-}$CD11cDNR 1370.61±534.34 pg/ml vs ApoE$^{-/-}$ 171.17±70.5 pg/ml, P<0.05) (Figure 8 A). Furthermore, significant increases were found in Th2 cytokines IL-4 and IL-10 (IL-4: ApoE$^{-/-}$CD11cDNR 161.00±29.99 pg/ml vs ApoE$^{-/-}$ 71.00±10.53 pg/ml, P<0.05; IL-10: ApoE$^{-/-}$CD11cDNR 500.00±80.26 pg/ml vs ApoE$^{-/-}$ 346.67±13.14 pg/ml, P<0.05) (Figure 8 B and C). Finally, a 5-fold increase in IL-17 levels was detected (ApoE$^{-/-}$CD11cDNR 101.78±22.81 pg/ml vs ApoE$^{-/-}$ 20.93±1.73 pg/ml, P<0.05) (Figure 8D). In addition to cytokine levels, T-cell proliferation was measured but no significant differences could be observed in T-cell proliferation (data not shown).

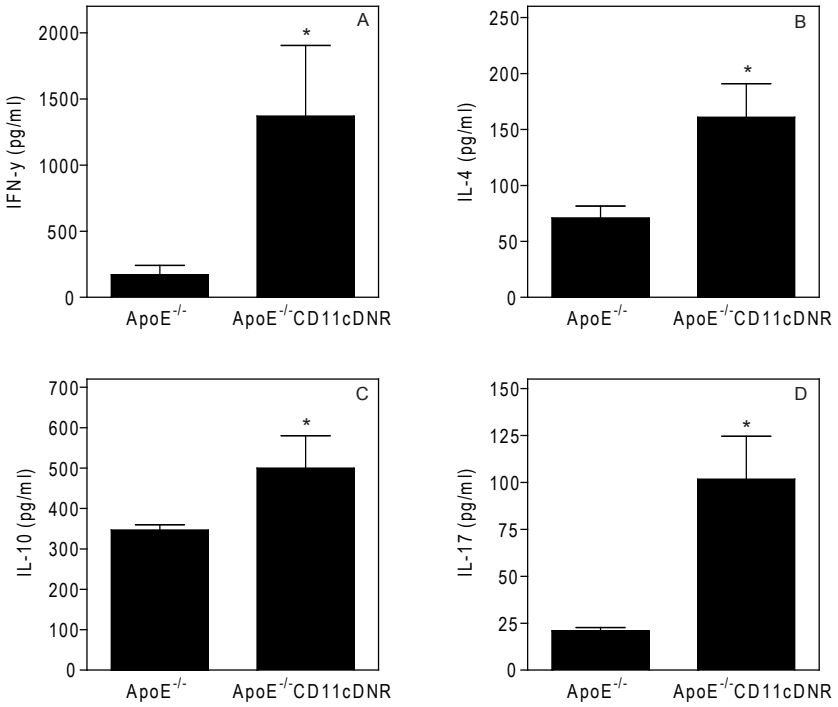


Figure 8: Disruption of TGF-β signaling in CD11c+ cells increases cytokine expression.
Concentrations (pg/ml) of IFN-γ (A), IL-10 (B), IL-4 (C), and IL-17 (D) in supernatants of cultured splenocytes, stimulated with aCD3/CD28 (*P<0.05).

Inhibition of TGF-β signaling does not affect DC maturation

Since the net outcome of T-cell immune responses is influenced by the activation stage of antigen-presenting cells,[30] we analyzed the maturation status of the CD11c+ DCs in spleen. TGF-β is known to inhibit critical co-stimulatory molecules on the surface of DCs[31] and therefore regulate DC maturation. However, analysis of cell surface markers of dendritic cells showed that DCs from ApoE-/-CD11cDNR animals had a differentiation process similar to ApoE-/- DCs. No differences could be observed in MHC-II, CD86 or CD40 expression (Figure 9).

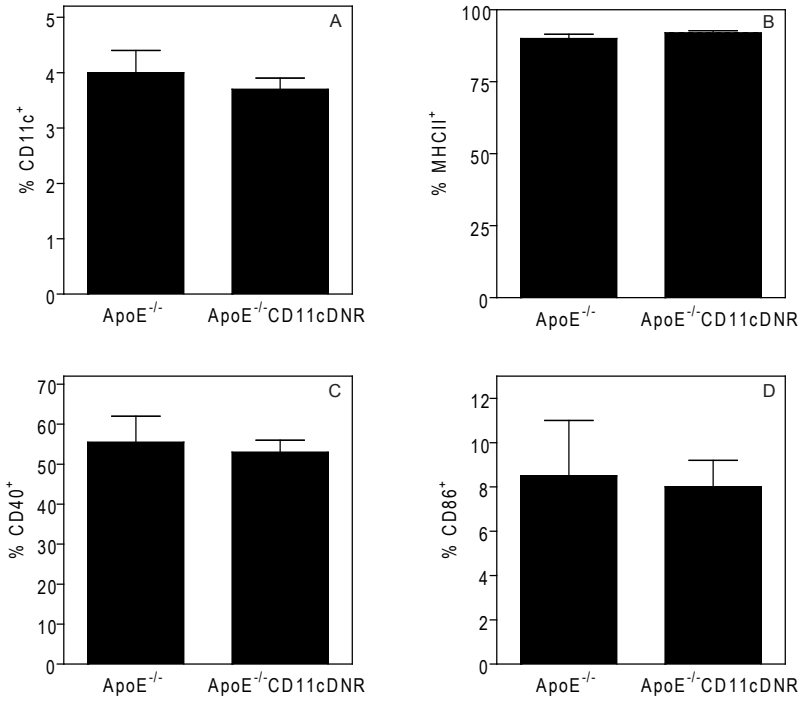


Figure 9: Disruption of TGF-β signaling in CD11c$^+$ cells does not alter DC maturation *in vivo*.
FACS analysis of maturation markers CD40 (B), MHC-II (C) and CD86 (D). Percentage of CD11c positive cells in spleen (A).

Discussion

In the present study, we highlighted the importance of TGF-β signaling in cells of the innate immune system in the progression of atherosclerosis. When TGF-β signaling in CD11c$^+$ cells (i.e. monocytes, dendritic cells, macrophages and NK-cells)[28, 32] was abrogated, atherosclerotic plaque size increased significantly. Moreover, plaques contained increased amounts of CD4$^+$ and CD8$^+$ T-cells and a low percentage of collagen, reminiscent of a rupture prone plaque in humans.
Several studies have provided evidence for an important role for TGF-β as an immune modulating cytokine in atherosclerosis. In patients, lowered serum levels of TGF-β are detected but can be increased with tamoxifen treatment with a consequent cardioprotective effect.[33] In addition, mutations and reduced expression of the type II receptor gene have both been detected in cells of human atherosclerotic lesions.[34-36] In mice, systemic inhibition of TGF-β signaling in ApoE$^{-/-}$ mice by using a recombinant soluble TGF-β type II receptor[23] or a blocking TGF-β1 antibody results in an unstable atherosclerotic plaque phenotype, whereas cardiac overexpression of TGF-β1 (resulting in increased plasma levels of TGF-β) limits plaque growth and induces plaque stabilization (Table 1).[24] However, the immune modulating effects of TGF-β are not restricted to T-cells. Although TGF-β producing regulatory T-cells

are well known to control T-cell activation and differentiation, and to play a crucial role in atherosclerosis,[37] the immune-regulatory capacity of other cell-types is also controlled via TGF-β. Interestingly, it has been reported that phagocytosis of apoptotic cells leads to TGF-β secretion, which inhibits the production of inflammatory cytokines and chemokines in macrophages including IL-1β and TNF-α.[18] In addition, several studies have shown that TGF-β inhibits in vitro activation and maturation of DCs.[38, 39] TGF-β inhibits upregulation of critical co-stimulatory molecules on the DC surface, thereby reducing its antigen-presenting capacity and DC mediated T-cell responses.[30] However these studies focused on Langerhans cell development, and might not be applicable in the present study, where we did not observe an effect on DC maturation in vivo. In this study we proved the importance of TGF-β signaling in CD11c positive cells in atherosclerosis using the CD11c-DNR mouse. In this mouse model, specific expression of dnTGFβRII was found in CD11c expressing cells, including DC subsets and NK cells, whereas no expression was found in NKT cells, T-cells or B-cells.[28]

In atherosclerotic plaques, an important local and systemic role for CD11c expressing cells has been reported recently.[29, 40, 41] In conditions of hypercholesterolemia, monocytes expressed high levels of CD11c, and show increased adherence to the arterial wall.[29] In $CD11c^{-/-}/ApoE^{-/-}$ mice, monocyte recruitment was impaired and only small atherosclerotic plaques developed.[29] This was also observed in the atherosclerotic plaque, where $CD11c^{+}$ cells with foam cell morphology also were positive for Moma-2, a macrophage marker. Cho *et al.* studied gene expression profiles during foam cell formation and confirmed that foam cells develop a dendritic cell-like phenotype accompanied by CD11c expression.[32] Moreover, CD11c plays a crucial, but yet unidentified role in cholesterol homeostasis.[41] When there is an expansion of $CD11c^{+}$ cells, cholesterol levels and the amount of atherosclerosis decreases, while a depletion of $CD11c^{+}$ cells results in hypercholesterolemia and an increase in atherosclerosis.[41] In the present study we observed decreased levels of cholesterol in the plasma of $ApoE^{-/-}$CD11cDNR mice. Recently it has been shown that depletion of DCs resulted in enhanced cholesteremia suggesting a close relationship between the DC population and plasma cholesterol levels.[41] Moreover, Frutkin *et al.* described that hypercholesterolemia induces TGF-β expression.[25] Since in our study we blocked TGF-β signalling in DCs, our data may suggest an interaction between TGF-β, DCs and the cholesterol metabolism. However, the reason why the reduced cholesterol levels did not result in reduced lesion size remains unclear.

In the present study, we found that TGF-β signaling in $CD11c^{+}$ cells is involved in the recruitment of inflammatory cells into the plaque, as well as in maintaining T-cell homeostasis. Lack of TGF-β signaling in $CD11c^{+}$ cells results in massive infiltration of $CD4^{+}$ and $CD8^{+}$ cells in atherosclerotic plaques and in a systemic expansion of $CD44^{high}CD62L^{low}$ T-cells. These data demonstrated that blockage of TGF-β signaling in CD11c cells lead to a shift from naïve T-cells towards effector ($CD44^{High}CD62L^{Low}$) T-cells. This effector-T-cell phenotype explained the influx of large amounts of $CD4^{+}$ and $CD8^{+}$ T-cells into the lesions, since naïve T-cells are not capable of infiltrating inflamed tissues. In addition, elevated levels of IFN-γ, TNF-α, IL-4, IL-10, IL-17 were detected. This broad array of expressed cytokines refers to both Th1 and Th2 subsets. The effect on atherosclerosis can therefore

not be explained by a shift in the Th1/Th2 balance. In addition, high levels of IL-17 were detected in the CD11cDNR animals, referring to an enhanced Th17 phenotype as well. These results are in concordance with Laouar *et al.* who studied the CD11cDNR mouse in experimental autoimmune encephalomyelitis (EAE) were an increase in inflammation in the CNS is accompanied with increased levels of Th1 and Th17 cytokines.[42] In addition abrogation of IL-17 receptor signaling in $LDLr^{-/-}$ mice attenuated atherosclerosis.[43] Moreover, Th17 cells have been identified in patients with acute coronay syndrome.[44] Taken together these data suggest a potential proatherogenic role. We therefore hypothesize that interruption of TGF-β signaling in $CD11c^{+}$ cells results in altered T-cell priming by DCs.

In conclusion, we state that TGF-β signaling in CD11c expressing cells is fundamental in the immunomodulation during atherogenesis. We showed that impaired TGF-β signalling in $CD11c^{+}$ cells resulted in an increase of activated effector T-cell which can more easily migrate into the atherosclerotic lesions with a consequential development of plaques, which are high in inflammation and low in fibrosis. We therefore provided a first inhibitory link between the innate and adaptive immune response with an important anti-inflammatory function for TGF-β signaling in $CD11c^{+}$ cells.

Table 1: Overview of studies on the effects of TGF-β signaling in atherosclerosis, with focus on plaque area, plaque phenotype and the induced cytokines.

Group (Year)	Reference	Experiment	Plaque area	Plaque inflammation	Plaque fibrosis	Induced cytokines
Mallat (2001)	24	Abs against TGF-β1, TGF-β2 and TGF-β3 $ApoE^{-/-}$ acceptor mice	+	+	-	
Lutgens (2002)	23	rs TGF-βRII $ApoE^{-/-}$ acceptor mice	+	+	-	
Robertson (2003)	26	CD4-dnTGF-βRII tg+ $ApoE^{-/-}$ background	+	+	-	increased IFN-γ, TNF-α IL-4,5, 10
Frutkin (2009)	25	Cardiac specific TGF-β overexpression $ApoE^{-/-}$ background	-	-	+	decreased IFN-γ TNF-α, MIP-1α

(Ref: Reference; BMT: Bone Marrow Transplantation; +: increase; - : decrease)

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Chapter 6

Foam cell formation affects MHC class I processing and presentation *in vitro* while hypercholesteremia induces a DC-like phenotype in macrophages *in vivo*

-Manuscript submitted-

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Abstract

Macrophage foam cell formation can induce a differentiation of macrophages into a dendritic cell-like phenotype. Because dendritic cells are the most potent antigen presenting cells, these changes could have implications in antigen presentation and subsequent T cell activation. To evaluate the effects of foam cell formation we have cultured bone-marrow derived macrophages in the presence of oxLDL (10 µg/ml) or acLDL (20 µg/ml). We observed a dendritic cell like phenotype with increased CD11c, MHC-I, MHC-II and costimulatory molecules suggesting an increased capacity to induce a specific T cell response. Consequently, we showed that MHC-I antigen presentation and processing of ovalbumin was increased while MHC-II induced T cell activation was not altered. Next we evaluated the effects of hypercholesteremia on peripheral macrophages *in vivo* in $LDLr^{-/-}$ mice. Again we observed an increase of $CD11c^{high}$ and MHC-I within the macrophage population. Furthermore, the activation status of both dendritic cells as macrophages was increased. In conclusion, both hypercholesteremic conditions as *in vitro* foam cell formation not only induced a dendritic-cell like phenotype in macrophages, but also provide macrophages with dendritic-cell like capacities. These data support the hypothesis that foam cell macrophages could play an important role in inducing an immune response during the early stages of atherosclerotic lesion development.

Introduction

Atherosclerosis is a multifactorial, chronic, auto-immune-like disease initiated by a combination of lipid accumulation and inflammatory processes.[1-3] Key processes in the development of atherosclerosis are the recruitment of inflammatory cells into the arterial wall and subsequent uptake of cholesterol by monocyte-derived macrophages. Cholesterol uptake is tightly regulated through the feedback regulation of the low-density lipoprotein (LDL) receptor. However oxidized LDL (oxLDL) is bound and internalized via a wide arrange of scavenger receptors which leads to the uncontrolled uptake of cholesterol. An ineffective cholesterol output leads to cholesterol accumulation and the formation of lipid-rich foam cells, which are characteristic of an atherosclerotic plaque.[4, 5]

Dendritic cells (DCs) are the most potent antigen presenting cells with the capacity to survey the body for pathogens and to induce and regulate the immune response.[6] As to their role in atherosclerosis, the presence of DCs has been reported in both healthy human and mouse aortic intima and their numbers increase during lesion progression.[7, 8] Evidence is accumulating that foam cell formation induces a dendritic cell-like phenotype in macrophages with enhanced CD11c and MHC-II expression.[9, 10] CD11c is characteristically expressed at high levels by DCs but not by monocytes/macrophages. Hypercholesteremic conditions induce CD11c expression in blood monocytes[11] and the increased CD11c (integrin) expression contributes to the migration of monocytes into the arterial wall of hyperlipidemic mice. In addition oxLDL enhances the adhesion and transmigration of DCs mediated by ICAM-1 and PECAM-1, respectively.[12] Recently, Wu *et al.* reported that under hypercholesteremic conditions, the uptake of modified LDL leads to the formation of $CD11c^+$ foamy macrophages which express high levels of VLA-4.[11] Together these data suggest that under hypercholesteremic conditions, monocytes become more activated, more dendritic cell-like and can migrate more easily into the sub-intimal space; which are all key processes in the progression of atherosclerosis.

However, at present it is unclear whether the induction of a dendritic cell-like phenotype in macrophage-derived foam cells affects their antigen presentation. The aim of this study was therefore to asses the effect of foam-cell formation in bone-marrow derived MΦ and DCs and in macrophages isolated from hypercholesteremic mice. We not only determined the changes in the expression of CD11c and costimulatory molecules upon foam-cell induction but also determined the effect thereof on antigen uptake and presentation and the subsequent capacity to activate T cell subsets.

Materials & methods

Animals

C57BL/6J mice were from Charles River Laboratories. OTII mice were a kind gift from J.M.M. den Haan (Dept. Molecular Cell Biology and Immunology, VUMC, Amsterdam). Mice were kept under standard laboratory conditions and were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Diet and water were administered *ad libitum*.

Cell lines and cell culture

Cell culture medium was IMDM (PAA, Germany) supplemented with 8 % FCS (PAA, Germany), 100 U/ml streptomycin/penicillin (PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μM β-mercaptoethanol (cIMDM). The murine T cell hybridoma B3Z is specific for the ovalbumin peptide SIINFEKL in the context of H-2K^{b} and harbors the β-galactosidase gene under the control of the IL2 promoter. B3Z cells were cultured in cIMDM supplemented with hydrochromycin B (500 μg/ml) to select LacZ expressing cells. The Ova-specific, class II-restricted murine T cell hybridoma D011.10 is cultured in cIMDM.

Generation and injection of BM-DC

Bone-marrow derived cells of C57BL/6 mice were harvested and cultured during 10 days in cIMDM in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) to obtain immature/non-foam cell DCs. Purity was assessed by flow cytometry using CD11c as a specific DC marker (CD11c^{high} > 90%).
To obtain macrophages, bone-marrow cells from C57BL/6 mice were resuspended in RPMI supplemented with 20% FCS and 30% of L929-conditioned medium (source for monocyte-colony-stimulating factor, M-CSF). After 7 days of culture, macrophages purity was tested by F4/80 expression (F4/80^{high}>90%).

Isolation of macrophages

Macrophages were isolated from male LDLr$^{-/-}$ mice that have been on a western-type diet for 10 weeks or a normal chow diet. Peritoneal macrophages were isolated by lavage of the peritoneal cavity without prior thioglycolate treatment to avoid activation. CD11b^{+} cells were positively isolated from spleen using magnetically labeled beads following manufacturer's protocol (BD Biosciences, Belgium). Purity of the isolated macrophages was analyzed using flow cytometry (CD11b^{+}>70%).

Antigen-presentation:
MHC class I and class II -restricted proliferation assay

Bone-marrow derived DCs or MΦ were cultured in the presence of medium (control), oxLDL (10 µg/ml) or acLDL (20 µg/ml) during 24 hours to obtain foam cells. APCs were than transferred to 96 well plates (50.000 cells/well) and cultured during 24 hours in the presence of various concentrations of SIINFEKL (0.001; 0.01; 0.1 ng/ml), OVA17 (0.001; 0.01; 0.1 µg/ml) or soluble ovalbumin (Grade V, Sigma-Aldrich, The Netherlands) (0.1; 1; 10 µg/ml) (n=5 per condition). After incubation, 100 µl medium was carefully removed and 100 µl B3Z cells or D011.10 cells were added ($1x10^6$ cells/ml). After 24 hours the medium of the B3Z cells was replaced with 100 µl lysis buffer (phosphate-buffered saline [PBS], 100 µM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40, and 0.15 mM chlorophenol red-ß-D-galactopyranoside) per well. After 3 hours absorbance was read at 590 nm. Values are corrected for the baseline proliferation of T cells in the presence of OVA peptides without APC. The supernatant of the D011.10 cells were collected and IL-2 concentrations were determined using ELISA. Because D011.10 cells are on a BALBc background we added an extra control in which D011.10 cells were co-cultured with non-foam cell APC to rule out the effect of an allogenic reaction. In some experiment proliferation was determined by ^3H thymidine-incorporation. Cultures were pulsed an additional 16 hours with 1 µCi/well of ^3H-thymidine (Amersham Biosciences, The Netherlands) and the amount of ^3H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R) as the number of disintegrations per minute (DPM).

CSFE-labeled T cell proliferation

T cells from OTII mice were isolated from spleen and lymph nodes. In short, lymphocytes were selected using a density gradient (Lympholyte, Cederlane). Subsequently, T cells were isolated using the T cell Enrichment Kit (BD Biosciences, Belgium) following manufacturer's instructions. Purity of the obtained T cells was measured with flow-cytometry (CD3>95%). T cells were labeled with CSFE (5 µM) for 15 minutes at 37°C. After washing, 10.000 CSFE-labeled T cells were added to the APCs and after 4 days proliferation was measured using flow-cytometry. The mean fluorescent intensity of CSFE was divided according the number of cell divisions and the percentage of cells within the MFI region for 5 cell cycles was analyzed.

Visualization of ovalbumin uptake by APCs using DQ-ovalbumin

DCs/MΦ were incubated with DQ-ovalbumin-immune complexes with concentrations ranging from 300 µg/ml-0.3 µg/ml (Molecular Probes, The Netherlands) during 1 hour at 37°C. After extensive washing, cells were treated with an acid wash (0.15 M NaCl, 50 mM glycine, 0.1% BSA, pH=2.2) to remove extracellular DQ-ovalbumin. Cytospins of the APCs were obtained by centrifuging cells for 5 min at 500 rpm (Thermo Shandon Cytospin 4). After centrifugation, slides were fixed for 30 minutes using zinc formal Fixx (Shandon, Pittsburgh, USA). Nuclei were counterstained with DAPI (0.2 µg/ml) for 20 minutes. Slides were embedded in Drako Fluorescent Mounting Medium (Dako, VS). Fluorescent microscopy was performed on the Nikon Eclipse E600 microscope. Uptake of the fluorescent DQ-ovalbumin was also checked with flow-cytometry.

Antigen uptake and presentation capacities of lipid-loaden APCs: Ovalbumin-specific proliferation

DCs/MΦ were incubated with various concentrations of ovalbumin ranging from 300 µg/ml-0.3 µg/ml. To further enhance the uptake, ovalbumin-immune complexes (OVA-IC) were generated by incubating soluble ovalbumin with 25 µg/ml polyclonal OVA-specific rabbit IgG (rIgGαOVA; Sigma-Aldrich, The Netherlands) during 30 minutes at 37° C. As a control, soluble ovalbumin was incubated with 25 µg/ml control rabbit IgG. APCs were incubated with OVA or OVA-IC during 24 hours after which class I/II specific proliferation was determined using B3Z/OTII cells or D011.10, respectively.

Statistical analyses

Values are expressed as mean ± SEM. Data were analyzed with either a parametric or non-parametric ANOVA when comparing three groups followed with Turkey post T-testing. When comparing two groups, a two-tailed Student's t-test or Mann-Withey U test was used. Statistical analyses were performed using the Instat3 software. Probability values of $P<0.05$ were considered significant.

Results

Lipid-loading induces a DC-like phenotype in MΦ

After culture, we checked the purity of the obtained bone-marrow derived MΦ and DCs. DCs were more than 90% positive for CD11c and showed an intermediate expression of F4/80. In contrast, MΦ expressed intermediate levels of CD11c but were more than 90% positive for F4/80 (data not shown). Foam cell formation was induced by culture in the presence of oxLDL (10 µg/ml) or acLDL (20 µg/ml). These concentrations induced foam cell formation with low levels of cell death as determined by flow cytometry (Figure 1).

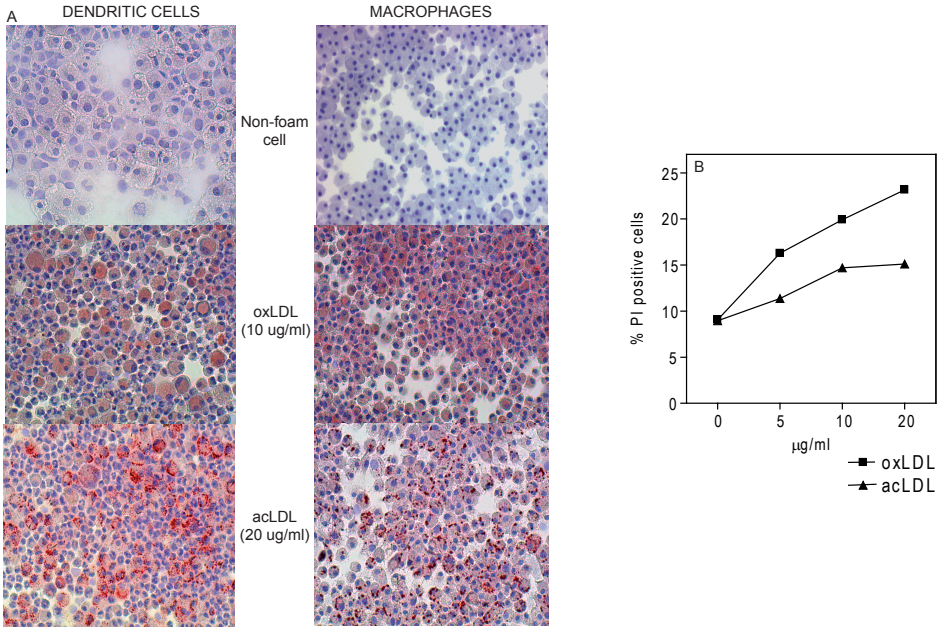


Figure 1: Addition of oxLDL or acLDL to bone-marrow derived DC and MΦ
Bone-marrow derived DCs and MΦ were incubated in the presence of various concentrations oxLDL or acLDL for 24 hours. A. Oil-Red-O staining visualises lipid accumulation. B. Cell death was determined using propidium iodide.

The level of CD11c was increased in MΦ by foam cell formation to reach the CD11c expression level of DCs (Figure 2A) while cholesterol loading in DCs did not affect their level of CD11c expression (Figure 2E). In addition, the level of MHC-I (Figure 2B) and MHC-II (Figure 2C) increased after cholesterol loading in MΦ while only MHC-II expression was higher in DC-derived foam cells (Figure 2F and 2G). Moreover, the expression of the maturation marker CD86 was increased in both MΦ- and DC-derived foam cells (Figure 2D and 2H). Although there was no difference in CD40 and CD80 expression between DCs and MΦ (data not shown), we did observe a higher expression of CD86 and MHC-II in DCs compared to MΦ suggesting that DCs are the better antigen-presenting cells (Figure 2D and 2H).

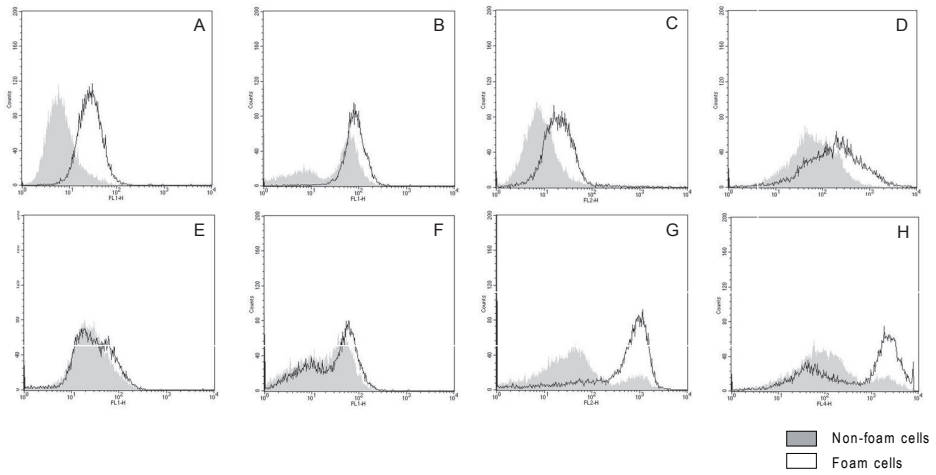


Figure 2: Foam cell formation in MΦ induces a DC-like phenotype
Expression level of several molecules was determined by flow cytometry in macrophages (A-D) and dendritic cells (E-H). CD11c expression (A) and MHC I (B) is increased in foam cell MΦ but is unchanged in foam cell DCs. In addition foam cell formation increased the expression of MHC II (C and G) and CD86 (D and H) in both MΦ and DCs.

Class I antigen presenting capacity of foam cells in vitro

Since lipid loading resulted in an increase in MHC expression, we determined the antigen presenting capacity of lipid loaden macrophages. We incubated MΦ with the MHC class I peptide SIINFEKL after cholesterol loading with either oxLDL or acLDL and determined OVA-specific proliferation. At low concentrations of SIINFEKL, there was no effect of acLDL loading. However, at higher concentrations there was a concentration dependent increase in proliferation of B3Z cells that recognize ovalbumin derived peptides presented by MHC-I (Figure 3A). The same dose response effects were observed after oxLDL loading (Figure 3B), which shows that MΦ-derived foam cells are better APCs than non-foam cell MΦ. The next question was whether lipid-loaded MΦ, which have increased costimulatory molecules, high expression of CD11c, higher expression of MHC-I, are equally potent APC as DCs. To this end, we cultured both DCs and MΦ in the presence of oxLDL (10 µg/ml) or acLDL (20 µg/ml) and determined the MHC-I dependent proliferation of B3Z cells. Again we observed an increased proliferation after lipid-loading of MΦ at the highest concentration of SIINFEKL (Figure 3C). DC-derived foam cells induced proliferation at lower concentrations of SIINFEKL than mΦ-derived foam cells(Figure 3D). Comparing DCs and MΦ we observed that DCs also after lipid loading are in all conditions more competent APCs then MΦ.

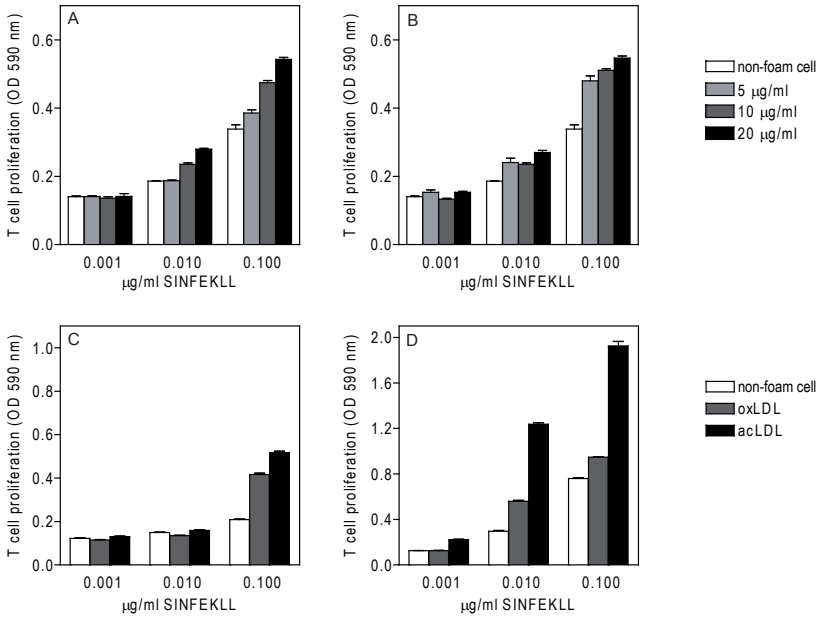


Figure 3: Incubation of APCs with oxLDL or acLDL enhances MHC-I presentation
Bone-marrow derived MΦ were incubated for 24 hours with various concentrations of oxLDL or acLDL. After foam cell formation, various concentrations of SIINFEKL were added and MHC-I specific antigen processing was determined using B3Z cells. We observed a concentration dependent effect of acLDL (A) or oxLDL (B) treatment on T cell proliferation. Next we compared MΦ (C) to DCs (D) after they have been cultured in the presence of oxLDL (10 µg/ml) or acLDL (20 µg/ml). Antigen-specific proliferation was increased after foam-cell formation in both cell types but DCs are better inducers then MΦ.

Antigen processing and MHC-I presentation capacity of foam cells *in vitro*

The previous experiments were performed with ovalbumin derived peptides that can directly bind to MHC-I and do not have to be taken up and processed by the antigen presenting cells. Since foam cell formation may affect antigen uptake and processing, we cultured APCs in different conditions with ovalbumin or with ovalbumin-Ab immune complexes (OVA-IC). Ovalbumin uptake by DCs and MΦ was tested determining the uptake of fluorescently-labeled OVA (DQ-OVA). APCs have taken up the DQ-OVA as shown with fluorescent imaging (Figure 4A) and by flow cytometry (Figure 4B) in a concentration dependent manner. Foam cell formation did not affect the uptake of DQ-OVA in both DCs (Figure 4B and 4C) and MΦ (Figure 4D and 4E).

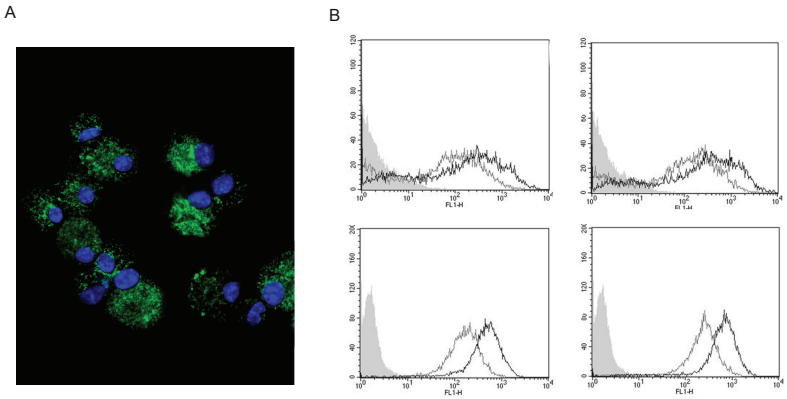


Figure 4: Foam-cell formation does not affect uptake of DQ-ovalbumin
DQ-ovalbumin uptake was visualized by fluorescent microscopy (A). Histograms showing the uptake of increasing concentrations of DQ-ovalbumin by non-foam cell DC (B), DC treated with oxLDL (10 µg/ml) (C); non-foam cell MΦ (D) or MΦ treated with oxLDL (10 µg/ml) (E). Grey curve: 0 ug/ml; grey line: 1 µg/ml; black line; 10 µg/ml DQ ovalbumin.

Next we determined the antigen processing capacities of non-foam cell, LPS-stimulated and MΦ- and DC-derived foam cells. Foam cell formation did not negatively affect ovalbumin antigen presentation in DCs (Figure 5A). In contrast, ovalbumin-specific proliferation was increased in MΦ-derived foam cells (Figure 5B).

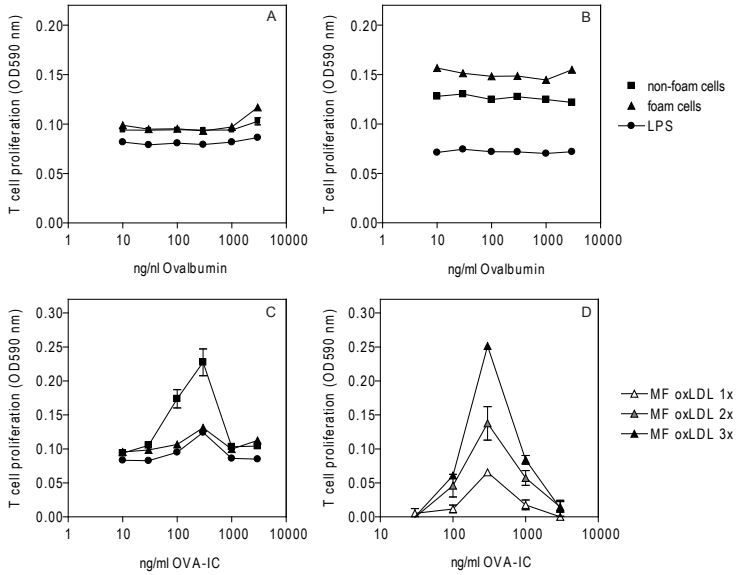


Figure 5: MHC-I specific antigen processing and presentation is enhanced after foam cell formation in macrophages
Bone-marrow derived DCs and MΦ were incubated with ovalbumin (A and B, respecively) or OVA-IC (C and D, respectively) after foam cell formation or LPS stimulation. T cell proliferation was not affected in DCs after foam-cell formation (A) while the activation of specific T cells was decreased after OVA-IC (C). In contrast, proliferation after ovalbumin processing was increased in foam-cell macrophages (B). Increasing amounts of immune-complexes enhanced MHC-I presentation in foam-cell macrophages (D). Values depicted in D are corrected for the amount of proliferation of corresponding non-foam cells.

To further study this observation, we generated ovalbumin-immune complexes (OVA-IC). At the optimal concentration for OVA-IC presentation (300 ng/ml) we analyzed the effect of foam cell formation on antigen processing and presentation. We observed a lowered proliferation of OVA-responsive classI dependent B3Z cells when they are stimulated with DC-derived foam-cells in comaprison to control DCs (Figure 5C). This is in contrast to MΦ-derived foam cells, which induced an increased ovalbumin specific proliferation compared to control MΦ with a optimal response at 300 ng/ml (Figure 6). Next we added larger amounts of immune complexes to further enhance this effect. We observed an increased proliferation of B3Z cells in mΦ-derived foam cells which was correlated to the amount of immune complexes added (Figure 5D). Values were corrected for the amount of proliferation of non-foam cells incubated with increasing amounts of immune-complexes.

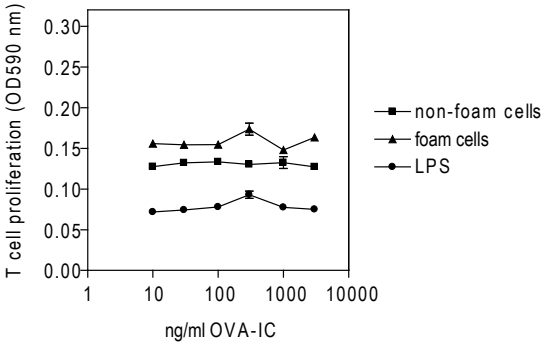


Figure 6: MHC-I specific proliferation is enhanced in macrophage-derived foam cells
Bone-marrow derived MΦ were incubated with OVA-IC after foam cell formation or LPS stimulation and proliferation of B3Z cells was assessed.

Class II antigen presenting and processing capacity of foam cells *in vitro*

To evaluate whether lipid-loading affected MHC-II presentation, we incubated MΦ and DCs with the class II peptide OVA17 and determined OVA-specific proliferation using ova-responsive D011.10 cells. In contrast to the MHC-I peptide SIINFEKL, OVA-17 induced proliferation was not altered after loading of MΦ or DCs with increasing concentrations of acLDL or oxLDL (Figure 7A and 7B). Again, DCs are the better APCs but as with macrophage-derived foam cells no effect of foam-cell formation on OVA-17 induced proliferation (Figure 7C and 7D).
When we assessed the MHC-II processing capacities of foam cells, we again did not observe an effect of foam cell formation on D011.10 proliferation in response to ovalbumin uptake (Figure 8A and 8B) or OVA-IC uptake (Figure 8C and 8D) as determined by IL-2 production.

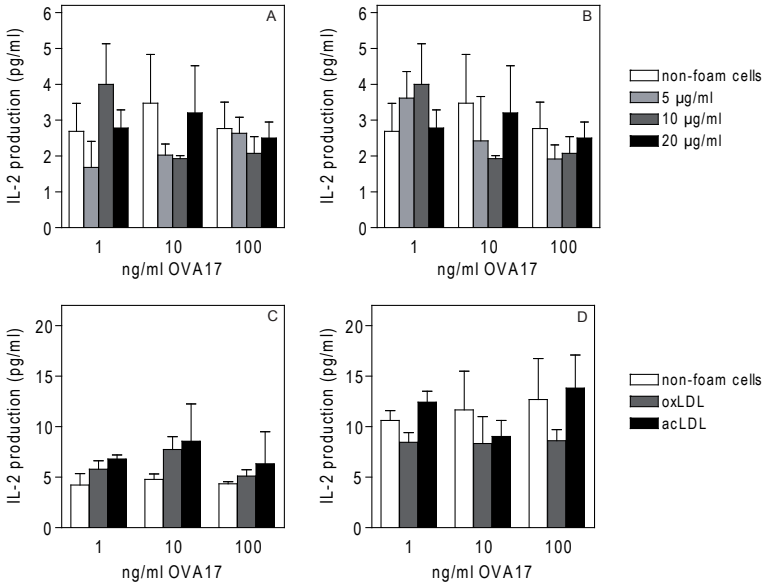


Figure 7: Foam cell fromation did not alter MHC-II antigen presentation
Bone-marrow derived MΦ were incubated for 24 hours with various concentrations of acLDL (A) or oxLDL (B). Also, we cultured MΦ (C) and DCs (D) in the presence of oxLDL (10 µg/ml) or acLDL (20 µg/ml). After foam cell formation, various concentrations of OVA-17 were added and MHC-II specific antigen processing was determined using D011.10 cells. Antigen-specific proliferation was not affected by foam cell formation in both MΦ and DCs.

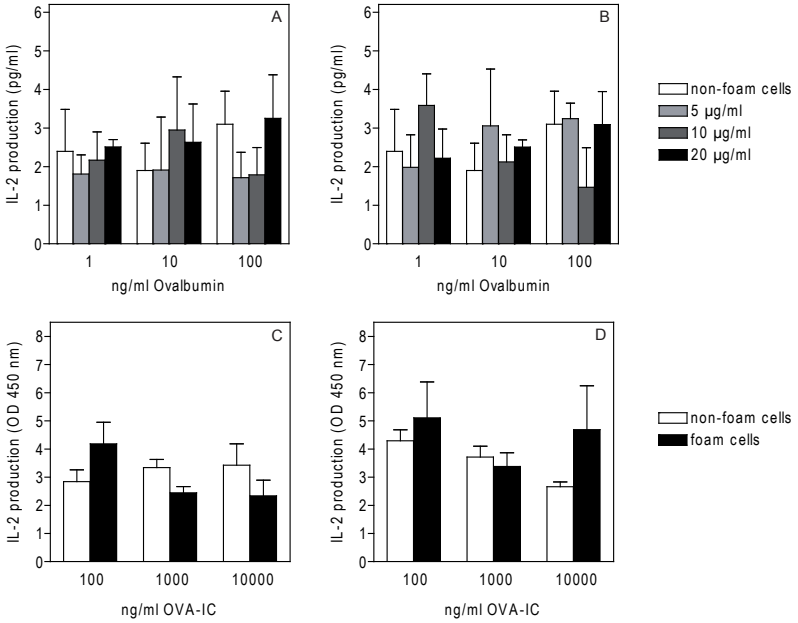


Figure 8: MHC-II processing was also not changed after foam cell formation
Bone-marrow derived MΦ were incubated for 24 hours with various concentrations of acLDL (A) or oxLDL (B) before incubation with ovalbumin. MHC-II processing was also determined with OVA-IC in MΦ and DCs after foam cell formation (C and D, respectively). MHC-II processing was not affected by foam cell formation in both MΦ and DCs.

These results were confirmed by using T cells derived from OTII mice. CSFE labeled T cells from OTII mice were stimulated with APCs that were incubated with OVA-17 or OVA-IC. Again, we did not observe an effect of foam-cell formation on MHC-II antigen presentation and processing. However, these data again confirmed that DCs are better APCs then MΦ with respect to MHC-II antigen presentation (Figure 9A and B, respectively) and processing in MHC-II (Figure 9C and D, respectively).

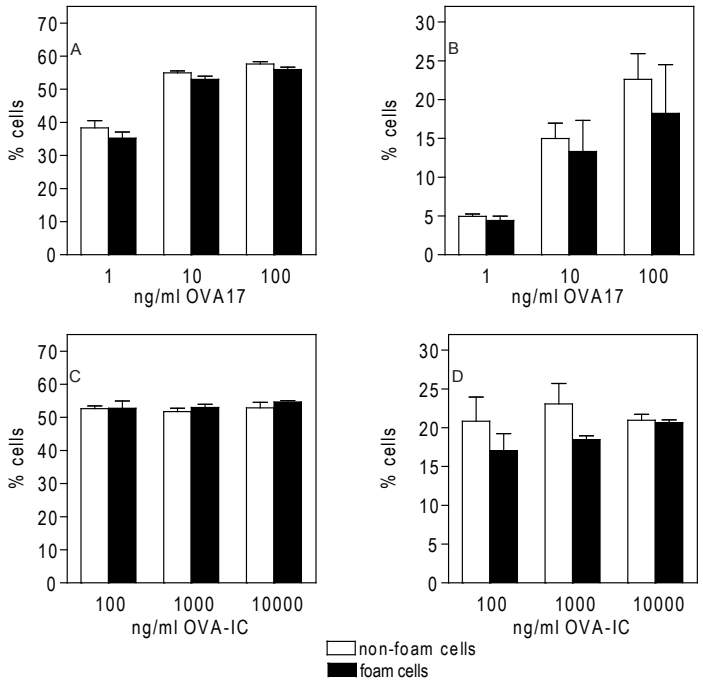


Figure 9: Proliferation of OTII cells was not affected by foam cell formation
T cells were isolated from OTII mice and labeled with CSFE. Next, OTII cells were cultured in the presence of MΦ or DCs that have been incubated with OVA-17 (A and B, respectively) or OVA-IC (C and D, respectively) and proliferation was measured using flow cytometry. The percentage of cells within the MFI region for 5 cell cycles was analyzed.

Hypercholesteremia increases CD11c expression in macrophages *in vivo*
Since foam cell formation affected the antigen presentation capacity of macrophages and their CD11c expression *in vitro*, we next assessed the effect of hyperlipidemia on the mΦ phenotype. Therefore, we compared the expression of DCs markers within the macrophage populations in LDLr$^{-/-}$ mice that were fed a western-type diet for 10 weeks to mice that were fed a chow diet. Western type-diet feeding increased the percentage of CD11c^{+}CD11b^{+} cells in blood, spleen and liver (Figure 10A). This increase was mainly due to an increase of CD11c^{high}CD11b^{+} cells (Figure 10B). Also the MHC-I expression was increased within CD11b^{+} cells after diet feeding (Figure 10C). More interstingly, the maturation status of both DCs and MΦ was increased in blood, spleen and liver of hypercholesteremic mice as indicated by the increased percentage of CD86^{+} cells (Figure 10D and E).

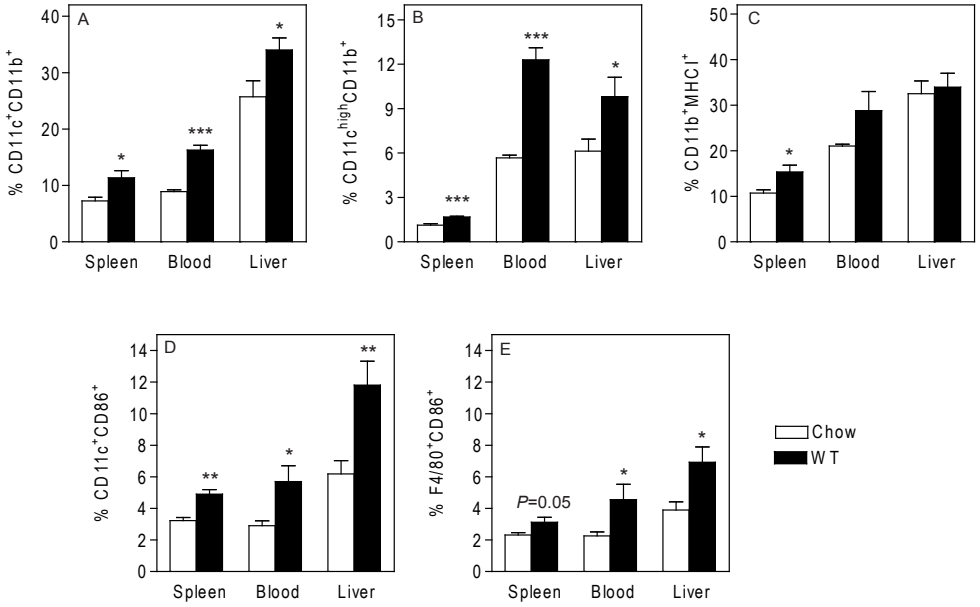


Figure 10: CD11c expression is enhanced in macrophages and APC are more activated under hypercholesteremic conditions
Percentage of $CD11c^{+}CD11b^{+}$ cells is increased in organs of $LDLr^{-/-}$ after western-type diet feeding of 10 weeks (A). This increase was mainly due to the increase of $CD11c^{high}$ cells within the MΦ population (B). Also MHC-I is upregulated in MΦ after diet feeding (C). Both DCs and MΦ are more activated after diet feeding as indicated by the higher % of CD86 double positive cells (D and E, respectively).

Discussion

Recent data suggest that foam cell formation has immunomodulatory properties affecting the capacity to present antigens by DCs and MΦ. The migration of DCs is impaired under hypercholesteremic conditions but on the other hand, recent studies show that CD11c expression is upregulated in MΦ upon foam cell formation. For instance, oxLDL induces the differentiation of the murine macrophage cell line RAW264.7 into dendritic-like cells with an increased expression of co-stimulatory molecules and MHC-II expression.[10] In addition, micro-array analysis of human monocyte derived macrophages isolated form blood also reveals a higher expression of CD11c and MHC-II after treatment with oxLDL.[9] Our present data on the effect of foam cell formation on antigen presentation by macrophages showed that the incubation of bone-marrow derived MΦ with oxLDL or acLDL increased CD11c expression to an expression level comparable to the level observed in DCs. More interestingly, the expression of MHC-I, MHC-II and costimulatory molecules was increased suggesting that foam cell formation may affect their antigen presenting capacities.

In order to test the antigen presenting capacity of the macrophage derived foam cells, we incubated foam cells with the MHC-I specific peptide SIINFEKL or the MHC-II-specific peptide OVA-17 and determined the antigen-specific proliferation using B3Z and D011.10 cells, respectively. We observed a significant increase in SIINFEKL induced proliferation of B3Z cells when we cultured bone-marrow derived MΦ with increasing concentrations of oxLDL or acLDL. DCs also demonstrated an enhanced MHC-I dependent proliferation upon foam cell formation. In addition, we also demonstrated that not only the direct presentation of peptides was enhanced, also uptake and subsequent processing of antigens was increased in MΦ-derived foam cells. This is in contrast to the effects seen in DC-derived foam cells. However, this may be explained by the fact that oxLDL uptake induces the maturation of DCs, which limits subsequent antigen uptake and presentation by the DCs. OxLDL is known to induce maturation but not to the same extent as LPS. During full maturation, DCs loose their capacity to phagocytose antigens. In our present study we showed an induction of CD86 and MHC-II in DC-derived foam cells, indicative of an induction of maturation while the expression of these markers remained low in MΦ. However, the uptake of fluorescently labeled ovalbumin was unaffected in DCs and MΦ suggesting that foam cell formation did not affect the uptake of the antigens but may affect the processing and subsequent antigen presentation.

In contrast to the enhanced MHC-I presentation by MΦ-derived foam cells, we did not observe any effects of foam cell formation on MHC-II presentation or processing as determined by proliferation assays using D011.10 cells or CSFE labeled OTII cells. This finding is in accordance with the study of Packard *et al.* who observe no effect of foam cell formation on isolated DCs on the MHC-II specific processing and presenting capacities of intact ovalbumin and ovalbumin-derived peptides by DCs. These effects are observed both *in vitro* after cholesterol loading with acLDL and *in vivo* in hypercholesteremic mice.[13]

Next we assessed the effect of hypercholesteremia on the MΦ population in LDLr$^{-/-}$ mice. We also observed *in vivo* an induction of a more DC-like phenotype of MΦ

in mice that are fed a high-cholesterol high-fat diet. The expression of CD11c on CD11b positive macrophages was increased in spleen, blood and liver. Moreover, both MΦ and DCs are more matured in mice being fed the western-type diet, although the macrophages and dendritic cells are not full foam cells under these conditions. These data are supported by the findings of Wu *et al.* who not only observes higher levels of CD11c on circulating mouse blood monocytes but also observes an increased granularity and increased expression of activation markers such as CD29.[11]

The observation of increased CD11c expression in MΦ in mice on a high fat diet may have major implications for atherosclerosis. Recently it has been shown that especially the firm arrest after rolling under shear flow conditions is enhanced in CD11c expressing cells.[11] In addition, patients with coronary artery disease have decreased numbers of both circulating plasmacytoid and myeloid dendritic cells which could be explained by an active recruitment of DCs into the lesions.[14] We saw an increase of CD11c expressing MΦ in blood suggesting more arrest to the endothelial wall and subsequent transmigration into the atherosclerotic plaques. Moreover, foam cell MΦ are abundantly present in early lesions. Our present data therefore suggests that the presence of foam cell MΦ with a DC-like phenotype can induce a local induction of a $CD8^+$ T cell response. Indeed, the inflammatory infiltrate of human plaques shows within the T cell population a slight predominance towards the $CD8^+$ phenotype.[15] In addition, the CD4/CD8 ratio in the plaque is lower than in the circulation which already indicates the shifted balance between $CD4^+$ and $CD8^+$ T cells.[15] Moreover, in the study of Rossmann *et al.* it is shown that the $CD8^+$ T derived from human carotid arteries cells have an increased IFN-γ secreting capacity compared to $CD4^+$ cells which consequently could activate resident T cells and MΦ.[15, 16] In addition, $CD8^+$ T cells have been found to play a predominant role in aneurysms formation and Kawasaki disease.[17, 18]

In conclusion, in the present study we show that foam-cell formation affects the antigen presentation and processing capacities of both MΦ and DCs. We showed for the first time that foam cell formation not only induces a DC-like phenotype in macrophages but it also enhances MHC-I antigen processing and presentation *in vitro*. Moreover, we verified our *in vitro* data by showing increased CD11c expression in the MΦ population and a more activated phenotype after western-type diet feeding. Taken together, our current findings indicate that foam cell formation may have major implications on the migration, activation and stimulating capacities of monocytes and consequently, in the initiation of atherosclerotic lesion formation. Furthermore our data suggests a possible role for $CD8^+$ T cell activation in early lesion development as *in vitro* MHC class I proliferation is increased in macrophage-derived foam cells.

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Chapter 7

Potential role of regulatory T cells in Aortic Aneurysms Formation

-Manuscript in preparation-

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Abstract

Regulatory T cells (Tregs) suppress autoimmune and inflammatory diseases by exerting suppressive effects on various immune cells such as effector CD4 T cells. We examined the importance and potential role of Tregs in a mouse model for abdominal aortic aneurysm formation. ApoE$^{-/-}$ mice were fed a western type diet during 4 weeks after which AngII infusion was started by means of an osmotic pump that was placed subcutaneously. Tregs were either depleted by injection of anti-CD25 or induced by the injection of anti-CD3. We observed a 40% death rate due to aortic rupture in mice in which the Tregs were depleted, suggesting a protective role of Tregs in preventing AAA rupture. In addition, we performed micro-array analysis on the aorta's after anti-CD25 treatment and observed a significant lower expression of genes involved in energy metabolism, more specifically the Krebs cycle, indicating a reduced energy production which could eventually lead to cell death. Treatment with anti-CD3 in ApoE$^{-/-}$ infused with AngII did not result in increased numbers of Tregs and had consequently no effect on the number or type of aneurysm formation. In conclusion our data demonstrate a possible protective role of Tregs in aneurysm formation but further research is needed in which the induction of Tregs is optimized to definitively show the protective effect of these cells on aneurysm formation.

Introduction

An aneurysm of the abdominal aortic (AAA) is a focal balloon-like dilation of the aorta. AAA is a common disease, which mainly affects men aged 60 or older. Mostly, aneurysms remain undiscovered and they grow approximately 0.3 cm each year and will, if untreated, rupture. Rupture of aneurysm has a high mortality rate and is the 13th leading cause of death in the United States.[1, 2] Development of AAA is associated with inflammation, tissue remodeling and the upregulation of matrix-degrading proteinases and it correlates with age, sex, pulmonary emphysema and high blood pressure.[3-5] Treatment of AAA mainly focuses on controlling the risk factors and/or the surgical removal of the aneurysm by techniques such as stent graft repair or complete replacement of the diseased part. Suitable pharmacological treatment is currently unavailable, although inhibitors of Angiotensin Converting Enzyme (ACE) and statins have been shown to benificially influence the progression or rupture of an aneurysm in patients.[6]

There is an increasing body of evidence supporting the role of the immune system in aneurysm formation where chronic inflammation of the aortic wall results in degradation of collagen and the elastic lamina. Inflammatory cells accumulate in AAA lesions with a predominance of $CD4^+$ T cells and to a lesser extent B cells and macrophages.[7-9] $CD4^+$ T cell depletion protects against AAA formation in a calcium-chloride induced model for AAA in mice[10] and $CD28^{null}$ T cells play an important role in the pathology of AAA[11] and CAD patients.[12] Therefore, the concept of T cell mediated inflammation is favored in AAA formation. $CD4^+CD25^+$ regulatory T cells (Tregs) play a critical role in the maintenance of immune homeostasis and tolerance to self-antigens. Tregs potently suppress the proliferation and cytokine production of effector $CD4^+$ and $CD8^+$ T cells and deficiency of Tregs leads to autoimmunity in both murine experimental models as in humans.[13, 14] In atherosclerosis, Tregs have been shown to play a protective role.[15-17] However, the exact role of Tregs in AAA has not been elucidated yet. Therefore we determined the role of Tregs in a mouse model of AAA formation.

Materials and methods

Mice

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male ApoE$^{-/-}$ mice on a C57Bl6 background were bred in-house. Mice were kept under standard laboratory conditions and were fed a normal chow diet or Western-type diet (WT-diet) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Mice were 6 months old at the start of the experiment. Diet and water were administered *ad libitum*.

Experimental protocol

Mice were put on WT-diet for 4 weeks prior to infusion with AngII and WT-diet feeding was continued during the rest of the experiment. Before placement of the osmotic pumps, mice were distributed into groups according to their cholesterol levels. 1.44 mg AngII/kg/day was infused using Alzet osmotic minipumps (Model 2004, Alzet, Charles River, The Netherlands) which were subcutaneously implanted in anesthetized mice. 24 hours later mice were *i.p.* injected with anti-CD25 or rat control IgG (100 µg/mouse). In the second experiment mice were *i.p.* treated with 50 µg/mouse anti-CD3 to induce Tregs prior to pump placement.

En face aorta

After perfusion with PBS and Zinc-Fix, heart and aorta were isolated and stored in zinc-Fix. Aorta's were prepared and photographed. Aneurysm formation was classified using the morphological grade of aneurysm (grade 0-IV) according to Daugherty *et al.* [18]

Micro-array analysis

Aorta's were crushed using liquid nitrogen and RNA was isolated using the GTC-method. aRNA was amplified using the NanoAmp RT-IVT Labeling Kit (Applied Biosystems, The Netherlands) and labeled with DIG-UTP. aRNA from 2 mice were pooled. The Applied Biosystems (AB) Mouse Genome Survey Micro-array was used which contains ~34,000 features including a set of ~1000 controls. Each micro-array uses 32,996 probes targeted to 32,381 curated genes representing 44,498 transcripts. Micro-array hybridization (using 10 µg of fragmented, DIG-labeled cRNA), processing, chemiluminescence detection, imaging, auto-gridding, and image analysis were performed according to AB protocols, using the 1700 Chemiluminescent Micro-array Analyzer Software v.1.0.3. For each group we performed 2 micro-array analyses. Panther Probe_ID annotations were updated to Mouse Micro-array V2 using the AB1700_MouseV2_MB_Updater. Transcriptome analysis based on raw intensity data was performed essentially as described[19] using the limma package[20] and in-house scripts in R/Bioconductor.[21] Intensities were log2-transformed and then normalized using quantile normalization.[22] Differential expression between the treatments of interest was assessed using a moderated t-test.[23] This test is similar to a standard t-test for each probe except that the standard errors are moderated across genes to ensure more stable inference for each gene. Genes were considered significant if the P values, adjusted for multiple

testing by using Benjamini and Hochberg's method were <0.05. The false discovery rate was thereby controlled to be <5%. Finally, we used Panther software for the analysis of enriched biological processes and molecular functions.[24]

Validation of candidate genes with Real-Time PCR

Total RNA was converted into cDNA using the RevertAid M-MuLV reverse transcriptase according to manufacturer's protocol. Relative gene expression was performed on the ABI7300 using SYBR Green technology. PCR primers were designed using Primer Express software (Applied Biosystems). 36B4, 18s ribosomal RNA, HPRT and GAPDH were used as housekeeping genes.

Flow cytometry

After sacrificing the mice, blood, spleen and draining lymph nodes (para-aortic and mediastinal) were isolated (n=6 per group). Single cell suspensions were obtained by smashing the cells through a 70 µm cell strainer (Falcon, The Netherlands). Blood and spleen cells were lysed using 0.83% NH_4CL in 0.01 M Tris/HCL pH 7.2. Subsequently 300.000 cells were stained with antibodies in the presence of 1 % mouse serum. FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software. All antibodies were obtained at Immunosource, Belgium.

Statistical analyses

Values are expressed as mean ± SEM. Data were analyzed with a two-tailed Student's t-test or Mann-Withey U test. Statistical analyses were performed using the Instat3 software. Probability values of $P<0.05$ were considered significant.

Results

Depletion of Tregs using anti-CD25 induces aortic rupture

To study the role of regulatory T cells in aneurysm formation, we depleted Tregs with anti-CD25 treatment in our aneurysm model. Six months old ApoE$^{-/-}$ mice were fed a WT-diet for 4 weeks, after which AngII (1.44 mg/kg/day) was infused using osmotic pumps and mice were kept on a WT-diet. Twenty-four hours after placement of osmotic pumps, mice were injected *i.p*. with a depleting anti-CD25 antibody (100 µg) or rat control IgG. Mice were sacrificed two days or two weeks after anti-CD25 treatment and the number of Tregs in the draining lymph nodes was determined by flow cytometry. The percentage of CD4^{+}CD25^{high} cells was almost completely depleted two days after anti-CD25 treatment (Figure 1A). However, the percentage of CD4^{+}CD25^{high} cells, although still significantly lowered in the anti-CD25 treated group, already started to recover two weeks after treatment (Figure 1B).

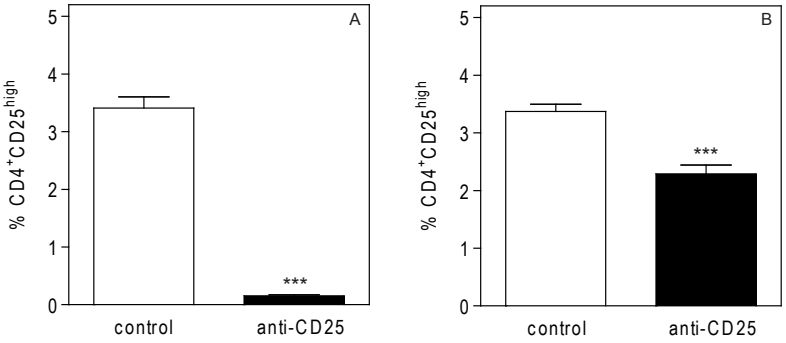


Figure 1: Depletion of Tregs after anti-CD25 treatment
ApoE$^{-/-}$ mice were fed a western type diet for 4 weeks after which osmotic pumps were placed and AngII infusion was started (1.44 mg/kg/day). The next day mice received a single *i.p.* injection of either control IgG or anti-CD25 (100 µg/mouse). Two days (A) or two weeks later (B), mice were sacrificed and the percentage of Tregs in draining lymph nodes was determined by flow cytometry (***P<0.001).

With regard to aneurysm formation, we observed an extreme decreased survival rate due to rupture of the abdominal aorta in mice treated with anti-CD25 with a 60% survival rate after nine days after injection. In contrast, there were no deaths in the control group (Figure 2). The number of aneurysms were equally distributed between groups when taking into account the dead mice due to rupture (control IgG: 8 of 15 mice had AAA; anti-CD25: 8 of 15 mice had AAA of which 6 mice died due to rupture).

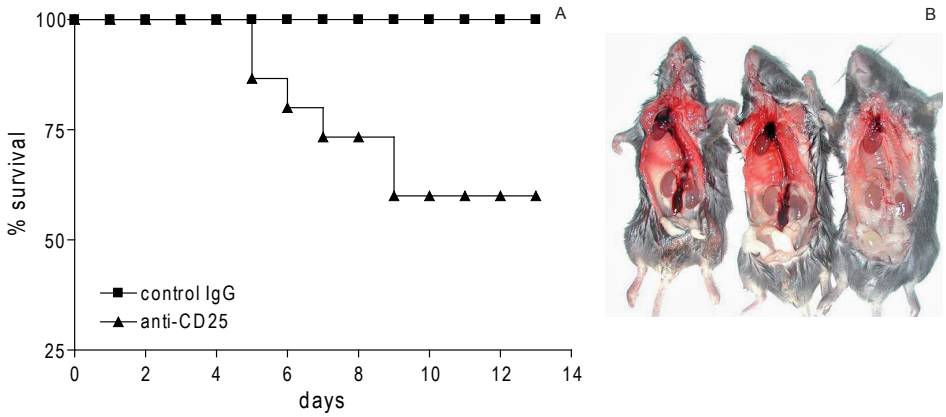


Figure 2: Kaplan-Meier survival curve of mice treated with control IgG or anti-CD25
Mice treated with anti-CD25 died due to rupture of the aorta. At day nine, the survival rate was reduced to 60% (A). Representative pictures of aortic rupture (B).

Micro-array analysis of dissected aorta's

Micro-array analysis was performed on dissected aorta's of mice treated with anti-CD25 or control IgG during AngII treatment. During analysis genes were rearranged according to disease pathology and we observed that genes involved in Aneurysm (*P*=2.09E-07) and Aortic Aneurysm (*P*=3.16E-06) were highly regulated in mice treated with anti-CD25. Interestingly, also Glucose Metabolism (*P*=5.05E-07) and Metabolic Disorders (*P*=4.6E-06) were in the top 5 of disease related genes (Table 1). Using a different approach to analyze the micro-array data we arranged the regulated genes according to the affected cellular processes and found that genes involved in the generation of intermediates in the biosynthetic pathway of glucose synthesis and energy metabolism were affected (*P*=1.08E-09). Further in depth analysis revealed that many of the genes involved in the Krebs cycle were significantly lowered in mice treated with anti-CD25 (Table 2). These results were confirmed with real-time PCR where we observed a reduction in the expression of pyruvate dehydrogenase beta (PDHB), pyruvate dehydrogenase alpha1 (PDHA1) and malate dehydrogenase 2, key enzymes involved in the Krebs cycle (Figure 3).

Table 1: Analysis of micro-array data of aorta's from anti-CD25 treated animals: gene rearrangement according to disease pathology

Disease	Ratio		P-value
Aneurysm	13	93	2,09E-7
Glucose Metabolism Disorders	45	901	5,05E-7
Aortic Aneurysm, Thoracic	6	19	3,16E-6
Metabolic Diseases	58	1405	4,60E-6
Cardiovascular Diseases	63	1670	2,99E-5

Table 2: Analysis of micro-array data of aorta's from anti-CD25 treated animals: genes involved in the Krebs cycle were negatively regulated

Gene	Fold	Bayes P
Malec enzym, supernatant	-20,4	0,021
Pyruvate carboxylase	-16,8	0,03
Pyruvate dehydrogenzase (lipoamide) beta	-9,8	0,019
Pyruvate dehydrogenase kinase, isoenzym 4	-5,82	0,011
Isocytrate dehydrogenase	-5,53	0,021
Lactate dehydrogenase 2, B chain	-4,51	0,016

Flow cytometry analysis: diet-dependent effects of AngII treatment

AngII induces a wide range of inflammatory responses. To study the effects of AngII on the immune system in ApoE$^{-/-}$ mice, we treated these mice with AngII for 4 consecutive days and used mice on a normal chow diet or mice fed a western-type diet for four weeks. In mice that were fed a chow diet, we observed an increased percentage of T cells and activated T cells in the blood and an increased percentage of CD4^{+}CD28^{null} T cells in the draining lymph nodes. However, these effects were not observed in mice on a WT diet (Table 3). In contrast, infusion of AngII in mice on a WT diet induced an increased percentage of CD11c^{+} and CD11b^{+} in blood and a decreased percentage of CD4^{+}CD28^{null} cells in lymph nodes.

Table 3: Flow cytometry data after subcutaneously AngII injections

Chow diet

BLOOD	PBS	AngII	
CD4	7,63 ± 1,02	12,68 ± 1,03	*P*=0.006
CD8	6,89 ± 1,08	9,38 ± 0,79	ns
CD4CD62L low	0,52 ± 0,05	1,24 ± 0,14	*P*=0.001
CD8CD62L low	1,05 ± 0,17	1,39 ± 0,18	ns
CD4CD25high	1,65 ± 0,20	2,15 ± 0,63	ns
CD4CD28null	2,35 ± 0,50	3,31 ± 0,45	ns
CD8CD28null	5,17 ± 0,83	6,68 ± 0,65	ns
CD11c	37,17 ± 6,30	31,35 ± 4,38	ns
CD11b	42,83 ± 0,42	37,26 ± 4,30	ns

Lymph Nodes	PBS	AngII	
CD4	23,70 ± 1,13	27,04 ± 1,90	ns
CD8	21,18 ± 1,97	21,62 ± 2,37	ns
CD4CD62L low	4,62 ± 0,31	5,47 ± 0,40	ns
CD8CD62L low	5,86 ± 1,16	3,87 ± 0,54	ns
CD4CD25high	4,73 ± 0,24	5,56 ± 0,25	ns
CD4CD28null	8,45 ± 0,59	12,47 ± 1,29	*P*=0.018
CD8CD28null	17,23 ± 1,72	18,08 ± 2,12	ns
CD11c	1,59 ± 0,30	1,50 ± 0,56	ns
CD11b	8,30 ± 1,11	6,41 ± 0,89	ns

Western type diet

BLOOD	PBS	AngII	
CD4	9,24 ± 0,57	7,220 ± 1,65	ns
CD8	7,82 ± 0,83	5,470 ± 1,55	ns
CD4CD62L low	0,84 ± 0,11	0,610 ± 0,16	ns
CD8CD62L low	1,11 ± 0,18	0,700 ± 0,24	ns
CD4CD25high	1,71 ± 0,46	2,360 ± 0,54	ns
CD4CD28null	2,67 ± 0,37	2,730 ± 0,56	ns
CD8CD28null	5,52 ± 0,56	4,170 ± 1,26	ns
CD11c	31,26 ± 1,95	52,780 ± 7,46	*P*=0.019
CD11b	35,47 ± 2,15	60,520 ± 9,74	*P*=0.02

Lymph Nodes	PBS	AngII	
CD4	22,58 ± 0,85	18,44 ± 1,58	0,044
CD8	19,97 ± 1,03	13,53 ± 1,25	ns
CD4CD62L low	4,73 ± 0,38	4,06 ± 0,39	ns
CD8CD62L low	4,67 ± 0,52	3,08 ± 0,59	ns
CD4CD25high	5,78 ± 0,27	5,01 ± 0,22	ns
CD4CD28null	9,18 ± 1,35	8,69 ± 1,10	ns
CD8CD28null	15,72 ± 1,17	10,97 ± 1,08	0,014
CD11c	1,83 ± 0,32	1,90 ± 0,26	ns
CD11b	9,60 ± 1,47	8,81 ± 1,02	ns

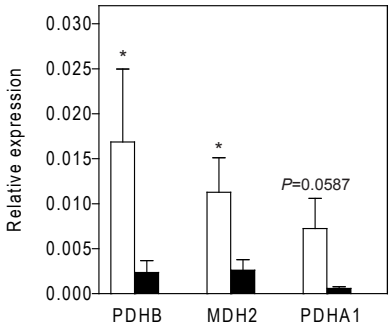


Figure 3: Expression of genes involved in Krebs cycle confirmed by real-time PCR
Gene expression of pyruvate dehydrogenase beta (PDHB), pyruvate dehydrogenase alpha1 (PDHA1) and malate dehydrogenase 2 (MDH2) were reduced in animals treated with anti-CD25 (*P<0.05).

Effect of anti-CD3 treatment on aneurysm formation

To further elucidate the importance of Tregs in aneurysm formation, we aimed to increase the number of Tregs by anti-CD3 treatment prior to AngII infusion. The working mechanism of this strategy is depicted in Figure 4A.

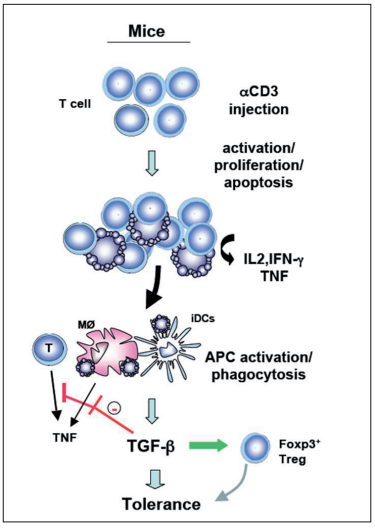


Figure 4: Mechanism of Treg induction by T cell induced apoptosis
Proposed model of T cell apoptosis induced tolerance. Anti-CD3 treatment results in T cell apoptosis. Macrophages and immature DCs digest apoptotic T cells and produce TGF-β. TGF-β down-regulated TNF-α induced immune responses but also induces Tregs. (Adapted from Perruche *et al.* 2008)

In short, injection of intact mitogenic CD3-specific antibody will result in apoptosis of T cells. Phagocytosis of these apoptotic cells by macrophages or dendritic cells will result in TGF-β production and a subsequent increase of Tregs. Treatment with anti-CD3 in our aneurysm model did not affect body weight (data not shown) and more importantly, mice did not die as a consequence of the induction of aneurysm formation. When we assessed the percentage of T cells in blood one week after anti-CD3 treatment, we observed a decreased percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in comparison to control treated mice (Figure 5A, B and C, respectively).

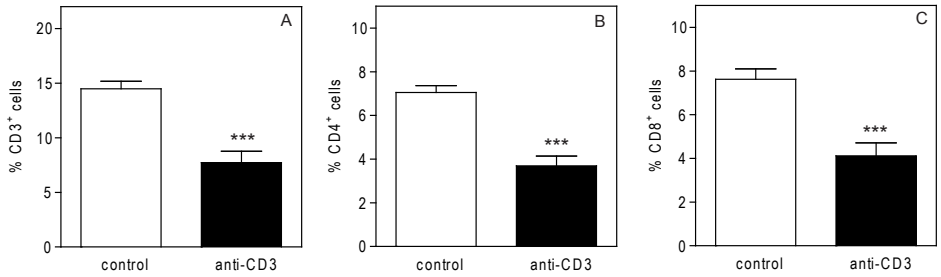


Figure 5: Flow cytometry analysis of blood samples one week after treatment
One week after anti-CD3 treatment blood was drawn by tail vein bleeding and the percentage of T cells was determined by flow cytometry. The percentage of $CD3^+$ T cells (A), $CD4^+$ T cells (B) and $CD8^+$ T cells (C) was reduced in animals treated with anti-CD3 (***P<0.001).

However, when we determined the percentage of Tregs in spleen, para-aortic lymph nodes and in mediastinal lymph nodes at four weeks of AngII treatment, we observed a non-significant reduction in the percentage of $CD4^+CD25^{high}$ cells (Figure 6). In addition, after isolation of the aorta's we observed that the number of different types of aneurysms was not affected. Both groups showed equal amounts of Type 0-IV AAA and TAA (Figure 7).

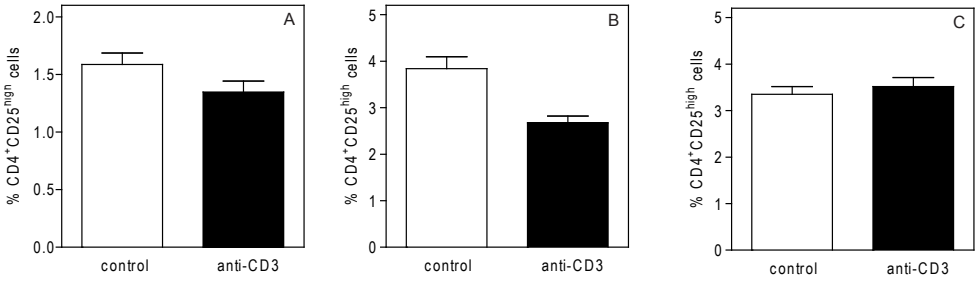
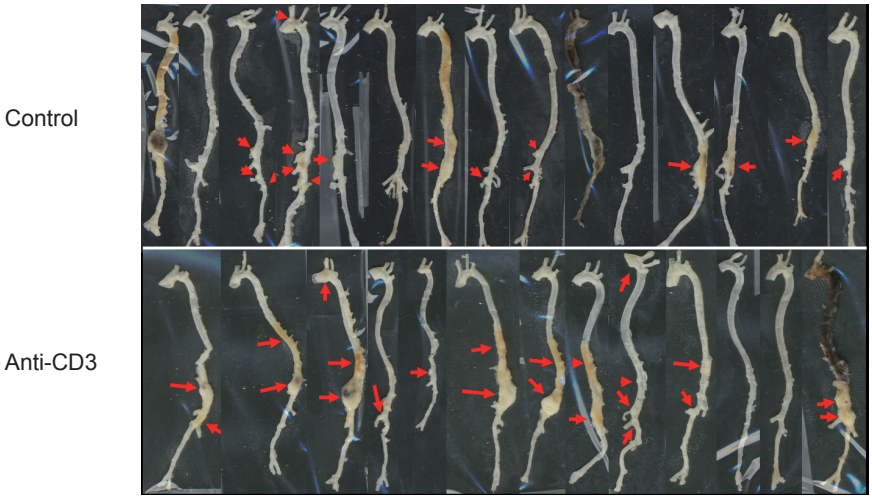


Figure 6: Treg number were not increased in mice treated with anti-CD3
Mice were treated with anti-CD3 or control IgG. AngII infusion was continued for 30 days after which mice were sacrificed. The percentage of Tregs was determined by flow cytometry in spleen, para-aortic lymph nodes and mediastinal lymph nodes (A, B and C, respectively).



Aneurysma	Type 0	Type I	Type II	Type III	Type IV	TAA
Control	3	5	3	2	2	3
Anti-CD3	2	4	3	2	2	5

Figure 7: Quantity and severity of aneurysm was not affected by anti-CD3 treatment
After 30 days of AngII infusion, mice were sacrificed and the entire aorta was isolated and photographed. Aneurysm formation was classified (AAA: Type 0-IV and TAA).

Discussion

In the present study we determined the role of Tregs in the formation of AAA. Within AAA there is a prominent role for inflammation in the initiation and progression of disease. Various types of immune cells have been found in AAA and in contrast to atherosclerosis, which is specifically marked by a Th1-type immune response, both Th1 and Th2 responses play an important role in AAA.[25, 26] It has been shown that AngII injections lead to inflammation and to AAA formation.[27] Therefore, we investigated the effect of AngII injection in mice on a normal chow diet compared to mice on a high-fat, high cholesterol, WT diet. We observed that in mice on chow, the number of $CD4^+$ T cells and moreover, the activated $CD4^+$ T cells are increased in circulation by infusion of AngII. Our findings are in line with prior studies, which demonstrated that AngII increases the number of circulating $CD4^+$ lymphocytes and furthermore, that the number of T cells in the aortic adventitia, and particularly in the peri-adventitial fat, is increased.[8] However, when comparing the two diets, we did not observe an increase in CD4 T lymphocytes in mice on the WT diet. In literature it is known that the number of circulating $CD4^+CD28^{null}$ and $CD8^+CD28^{null}$ cells are enriched in AAA patients compared to healthy controls.[11, 12, 28] $CD4^+CD28^{null}$ T cells are a subgroup of inflammatory T cells which lack the costimulatory molecule CD28. Functionally, these T cells are capable of producing

large amounts of interferon-γ, perforin and granzyme B providing them with the capacity to lyse cells.[29] Also in the present study we observed an increase in $CD4^{+}CD28^{null}$ T cells in the draining lymph nodes in mice on chow. Again, we did not observe these changes in mice on the WT diet. On the other hand, mice on a WT diet had higher percentages of macrophage and dendritic cells markers, CD11b and CD11c, respectively. These molecules are also integrins and increased expression may facilitate the transmigration of macrophages or dendritic cells into the arterial wall.[30] Taken together, our data showed that AngII injection in mice on chow diet indeed induced T cell proliferation/activation and the induction of $CD4^{+}CD28^{null}$ T cells. Moreover we showed that WT diet has a possible compromising role in this activation. Consequently, we have to conduct more research on the role of AngII infusion and the WT diet in aneurysm formation.

To investigate the potential role of Tregs in aneurysm formation we performed several experiments in which we depleted or induced Tregs. $CD4^{+}CD25^{+}$ regulatory T cells are implicated in the maintenance of self-tolerance and play a protective role in atherosclerosis. Recently, the adaptive transfer of Tregs has been shown to be effective in quenching autoimmune and allergic diseases.[31, 32] At present, we show that depletion of Tregs via anti-CD25 treatment resulted in increased death as a result of aortic rupture, suggestive of a protective role for Tregs in aneurysm rupture. It has recently been shown that $ApoE^{-/-}IFN\text{-}\gamma^{-/-}$ dKO mice experienced death with similar survival curves as the one observed after Treg depletion. Also in this experiment mice died due to rupture of the abdominal aorta.[33] Lack of IFN-γ is associated with reduced frequency and function of Treg and a subsequent heightened susceptibility to Collagen Induced Arthritis (CIA) and Experimental Autoimmune Encephalomyelitis (EAE).[34, 35] Therefore the study of King *et al.* could provide us with a direct link between IFN-γ production, Tregs and rupture of AAA.[33] Furthermore, when we assessed gene expression in aorta's of anti-CD25 treated mice we observed a high regulation of genes involved in aneurysms but also in glucose metabolism. Especially expression of genes involved in the Krebs cycle was negatively influenced by anti-CD25 as confirmed by real-time PCR. As a result, we can hypothesis that the reduced production of these essential products can increase cell death which could eventually affect the integrity of the aortic wall and result in rupture.

To further support the results obtained by anti-CD25 treatment, we attempted to increase Treg numbers. Treatment with non-mitogenic anti-CD3 has been shown to induce Tregs by induction of T cell apoptosis.[36] Although we induced the necessary T cell death by anti-CD3 treatment, we observed only a trend towards a reduced percentage of Tregs in spleen and draining lymph nodes. In line with the observation that Tregs were not affected, AAA was also not affected by anti-CD3 treatment.

In conclusion, we have observed a possible protective effect of Tregs in AAA formation and rupture but could not confirm these data because of failed induction of Tregs. Therefore, procedures to induce Tregs *in vivo* should be further optimized. For example: adoptive transfer of Tregs or injection of IL-2 murine antibody complexes could provide us with an effective method to induce Tregs *in vivo*[37] and to elucidate the precise role of Tregs in aneurysm formation or rupture.

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Chapter 8

General discussion & Perspectives

General discussion and perspectives

Introduction

Over the past decades, a tremendous effort has been put into the research to develop new therapeutic approaches for the treatment of atherosclerosis, the main underlying pathology of cardiovascular disease (CVD). However, standard treatment of CVD still focuses on the reduction of risk factors by lipid lowering or anti-thrombotic drugs in combination with instructions to improve life style and CVD. Therefore it is of the utmost importance that the processes that contribute to lesion progression are well understood in order to explore this knowledge to develop new, effective and preventive treatments. Atherosclerosis is a chronic inflammatory disease of the vasculature in which both a disturbed lipid metabolism and inflammatory immune responses against several self-antigens are involved. Because of the prominent role of the immune response, especially in the initial stages of lesion development, this thesis focused on the possibility to manipulate the immune response in murine models for both atherosclerosis as abdominal aortic aneurysm formation.
In **chapter two to four** dendritic cell (DCs) based therapies have been evaluated in different stages of atherosclerotic lesion development. In **chapter five** we assessed the importance of TGF-β producing DCs in atherosclerosis. TGF-β plays several atheroprotective roles such as reducing T cell and macrophage activation and stabilization of lesions through the induction of collagen production. Next, we analyzed the effects of foam cell formation on the antigen presenting and processing capacities of macrophages and DCs in **chapter six**. Finally we attempted to elucidate the role of regulatory T cells in aneurysm formation in **chapter seven**.

Dendritic Cells in atherosclerosis

Antigen presenting cells (APCs) play a prominent role in immune responses. APCs recognize both endogenous and foreign pathogens via their Pattern Recognition Receptors such as Toll Like Receptors (TLRs) and Nod Like Receptors (NLRs). After internalization of these pathogens, antigenic peptides will be presented to specific T and B cells to induce an immune response. Dendritic cells (DCs) are the most potent antigen presenting cells. They have the unique capacity to activate naïve cells and thereby form a bridge between the innate and adaptive immune response.[1, 2] Low numbers of DCs reside in the healthy vessels but their number increases during lesion progression.[3, 4] Moreover, hyperlipidemia can disturb the migration of DCs towards the lymphoid organs.[5] This may lead to a diminished presentation of atherosclerotic antigens and subsequently a disturbed immune response. Because of their capacity to activate all kinds of immune cells such as T cells, NKT cells and even B cells, DCs form an interesting opportunity to manipulate the immune response.

Vaccination strategy using oxLDL-pulsed mDCs

In **chapter two** we investigated the potential of pulsed mature DCs as a vaccination protocol. For this we treated LDLr$^{-/-}$ mice prior to atherosclerosis induction by means of collar placement and subsequent western type diet feeding. Oxidized Low-Density Lipoprotein (oxLDL) is a promising target for immunotherapy as several studies have shown the relevance of oxLDL-immunization leading to 40-70 % reduction in lesion size.[6-8] However, oxLDL is a very complex particle containing many different epitopes that can potentially act pro-atherogenic or atheroprotective. The vaccination strategies described in the literature focus on inducing an immune response to a single epitope or a limited number of epitopes found in (ox)LDL. In addition, most immunization protocols require the presence of adjuvants, which may cause undesired side-effects. In contrast, DC-based therapies do not require the addition of adjuvants and DCs will present a broad spectrum of epitopes after internalization and processing of oxLDL and can therefore activate a wide range of oxLDL-specific T cells. Treatment of LDLr$^{-/-}$ mice with oxLDL-pulsed mDCs resulted in attenuation of lesion development. In addition, a decreased number of macrophages and increased collagen content was observed, contributing to a more stable plaque phenotype. Vaccination with oxLDL-pulsed mDCs also resulted in the induction of oxLDL-specific IgG and moreover, the IgG2c/IgG1 ratio was reduced, suggestive of a reduced Th1 response. In addition, we observed an induction of oxLDL-specific T cells as determined by *ex vivo* proliferation which, when stimulated with ConA, produced less IFN-γ while the secretion of IL-4 and IL-10 was not affected. We hypothesize that in the current setup, injection of oxLDL-pulsed mDCs leads to a migration towards the lymphoid tissues where it can activate or generate oxLDL-specific T cells that provide help towards B cells. When hyperlipidemia becomes effective by western type diet feeding, oxLDL-specific antibodies and T cells can then be rapidly generated and can prevent the accumulation of oxLDL in the vasculature. At current, clinical studies showed that treatment with anti-ApoB100 antibodies may induce inhibition of plaque progression, indicating that the induction of specific antibodies may be very effective.[9]

DC-based immunotherapy to target NKT cells

Presentation of lipid antigens by antigen presenting cells (APCs) is not mediated by MHC class I or II molecules but by a MHC class I like molecule CD1d. Lipid antigens are therefore recognized by a specialized subset of T cells, the Natural Killer T (NKT) cells. In atherosclerotic lesions, NKT cells are found in close vicinity of DCs in the shoulder region and may have an important role in plaque destabilization.[10] The exact role of NKT cells in atherosclerosis remains however unclear. Data in literature are contradictive with an atherogenic function in ApoE$^{-/-}$ mice while in LDLr$^{-/-}$ mice the activation of NKT cells prior to atherosclerosis induction is protective. Because αGalCer mainly produces TH1 cytokines, we used an analogue of αGalCer, OCH which is known to induce a more TH2-phenotype in NKT cells.[11, 12] Subsequently, we investigated the potential of OCH as a therapeutic agent in atherosclerosis. In **chapter three** we observed that while *i.p.* injections

of OCH did not affect lesion development, adminstration of OCH-pulsed mDCs dramatically reduced lesion size. In addition, we showed that OCH-pulsed mDCs treatment in Jα281$^{-/-}$LDLr$^{-/-}$ KO mice had no effect on lesion progression, indicating that the observed effects are NKT cell dependent. We showed that treatment with OCH-pulsed mDCs resulted in an increase of NKT cells in blood and liver. Moreover, after restimulation *ex vivo*, NKT cells produced more IL-10 while IFN-γ production was not affected. The increased levels of IL-10 may explain the reduced cholesterol levels and the more beneficial lipoprotein profile as observed at sacrifice since systemic IL-10 administration has been shown to reduce VLDL and LDL cholesterol levels in LDLr$^{-/-}$ mice.[13]

DC-based therapy in established atherosclerosis

Clinical symptoms of atherosclerosis only appear at late stages of the disease. Also, vaccination strategies using DCs are likely to be used for the treatment of patients with ongoing atherosclerosis. In **chapter four** we investigated whether we could implement DC-based therapy in a model of established atherosclerosis. We have treated LDLr$^{-/-}$ with pre-formed lesions with immature, unpulsed DCs (ImDCs) because this type of DCs renders a higher protection in collagen induced arthritis then collagen-pulsed ImDCs.[14] We have observed an inhibition of plaque growth in mice treated with ImDCs. Moreover, we observed a clear induction of tolerogenic CD4^{+}DX5^{+} cells in liver and spleen but adoptive transfer of these cells in mice with established atherosclerosis, did not affect lesion size. ImDCs treatment also induced a decrease in cholesterol levels and a more beneficial lipoprotein profile which may be explained by a rapid induction of ABCA1, ABCG1, HL and LPL mRNA and a reduced expression of LIGHT and lymphotoxin-α mRNA expression in the liver.[15] Mice treated with ImDCs also exhibited higher numbers of regulatory T cells in blood three weeks after injection and this increase was still present ten weeks after injection. Induction of Tregs may reflect the induction of tolerance, which is confirmed by the increased peripheral to liver CD4/CD8 ratio which is also used as a measurement of tolerance. In addition we could not detect an increase in IgM or IgG levels suggesting that we have not induced a humoral response. Consequently, these data showed the beneficial effect of treatment with ImDCs in a mouse model of established atherosclerosis. Taking into account our previous work on the use of pulsed mDCs as a vaccination strategy, we thus provide additional evidence for the potential use of DC-based immunotherapies in cardiovascular diseases.

Deficiency of TGF-beta signaling in DCs

In **chapter five** we evaluated the effects of a disturbed TGF-β signaling in DCs and the subsequent effects on atherosclerosis. TGF-β exerts several crucial anti-inflammatory functions such as the inhibition of T cell proliferation and activation of macrophages and reduces the expression of adhesion molecules and chemokines required for the recruitment of leukocytes.[16, 17] Furthermore, TGF-β enhances plaque stability by stimulating collagen production by smooth muscle cells. Treatment with inhibitory antibodies for TGF-β has atherogenic effects while

overexpression of TGF-β inhibited lesion progression.[18-20] These effects could be attributed to TGF-β signaling in T-cells as mice with deficient TGF-β signaling in $CD4^+$ T-cells have larger and more unstable plaques.[21] There is however little known on the effect of a disturbed TGF-β signaling in DCs. Therefore we have used $ApoE^{-/-}$ mice which have a dysfunctional TGF-β Receptor II under the CD11c promoter. We observed an increased lesion progression with a more inflammatory phenotype as indicated by the enhanced influx of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the lesions. Also plaque stability was reduced as indicated by a lower collagen content. Splenic $CD4^+$ and $CD8^+$ T cells exhibited a more differentiated phenotype as indicated by an increase of activation markers in T cells of $ApoE^{-/-}$CD11cDNR mice. Moreover, T cells from these mice produced increased amounts of both TH1, TH2 cytokines as well as IL-17, while T cell proliferation was not altered. We also observed that deficiency in TGF-β signaling does not affect DC maturation *in vivo* but DC from $ApoE^{-/-}$CD11cDNR mice do produce lower amounts of TGF-β and IL-12 which may affect T cell differentiation and activation. In conclusion, we showed that TGF-β signaling in DCs is necessary to control T cell differentiation towards the effector T-cell phenotype. Loss of TGF-β signalling in DCs will increase the number of activated T cells that show an increased migration into atherosclerotic lesions and subsequently enhance the inflammatory state of the plaque. This, accompanied by a loss of collagen, may lead to a more vulnerable phenotype.

Effects of foam cell formation on APC functions

Key processes in the development of atherosclerosis are monocyte recruitment into the arterial wall, subsequent uptake of cholesterol and the formation of foam cells.[22] More recently there is accumulating evidence that macrophage foam cell formation induces a dendritic cell-like phenotype with increased CD11c and MHC-II expression.[23-25] However, at present it is unclear whether the induction of a dendritic cell-like phenotype in macrophages affects their antigen presentation or processing capacities. Therefore we assessed the effect of foam-cell formation on macrophages and DCs in **chapter six**. By means of flow cytometry we determined that bone-marrow derived macrophages (MΦ) express upon foam cell formation higher levels of CD11c, MHC class I and class II and co-stimulatory molecules suggesting that foam cell formation enhances the antigen presenting capacities of MΦ. Indeed we have observed higher MHC class I mediated presentation of ovalbumin peptide in both DCs and MΦ upon foam cell formation. In addition, we also demonstrated that not only presentation of peptides is enhanced, also MHC class I processing of ovalbumin was increased in foam-cell MΦ. In contrast MHC class II presentation and processing was not affected by foam cell formation. These data were confirmed *in vivo* were hypercholesteremia induced a DCs-like phenotype of MΦ with increased CD11c and CD86 expression. This may have major implications for the inflammatory process in atherosclerotic lesions as CD11c is an integrin and enhances the firm arrest of monocytes to the endothelial layer.[25] However, further extensive research on the exact role of hypercholesteremia on antigen presentation and processing by antigen-presenting cells still needs to be performed.

Potential role of regulatory T cells in Aortic Aneurysms

Regulatory T cells (Tregs) potently suppress the proliferation and cytokine production of effector $CD4^+$ (TH1 and TH2) and $CD8^+$ T cells and deficiency of Tregs leads to autoimmunity in both murine experimental models as in humans.[26, 27] In atherosclerosis, Tregs have been shown to play a protective role. In contrast, Tregs inhibit postischemic neovascularization by controlling the effector immune cell response.[28-31] However, the exact role of Tregs in AAA has not been elucidated yet. Therefore we sought out to determine the importance of Tregs in a mouse model of AAA formation in **chapter seven**. We used $ApoE^{-/-}$ mice that were infused with angiotensin II (AngII) to induce aneurysm formation. Depletion of Tregs using an anti-CD25 antibody immediately prior to AngII infusion reduced survival due to acute rupture of the aorta. Already after nine days, the survival rate dropped to 60% while in the control group no mice died. When we performed micro-array analysis on the aortas of the control group and the Treg depletion group we observed that genes related to the disease process aneurysm were highly regulated. Interestingly, the expression of genes involved in energy metabolism and especially genes of the Krebs cycle are down regulated in mice treated with anti-CD25. Next we attempted to induce Treg numbers by anti-CD3 treatment. Although we induced the required T cell death, we could not observe an increase in Tregs after treatment. In conclusion, the manipulation of the numbers of Tregs *in vivo* still needs further optimization. For example injection of IL-2 murine antibody complexes could provide us with an effective method to induce Tregs *in vivo*[35] and to elucidate the precise role of Tregs in aneurysm formation or rupture.

Perspectives

In this thesis we have described different strategies concerning the use of dendritic cells as an immunotherapy for atherosclerosis. It is tempting to speculate that we can translate the outcome of our preclinical studies into a clinical application. DC-based immunotherapies are already used in clinical trails for cancer research where DCs are pulsed with tumor antigens which induce a tumor specific $CD8^+$ T cell response. Yet, it has been observed that some patients are non-responsive to the treatment. This could partially be explained by the fact that these patients have induced numbers of Tregs that counteract the DC therapy.[36, 37] Therefore it is of the outmost importance that the immunological status of the patients is characterized prior to DC treatment and that the correct antigen is being used to pulse the DCs. Another possible way to use DC-based therapy in the future is the targeting of DCs *in vivo* through DC-specific receptors like for example CD205, the mannose receptor or DC-SIGN instead of culturing them *ex vivo*. By discrimination of markers like DC-SIGN, Langherin and MGL that are unique to certain types of DCs, it should be possible to target specific DC subsets. C-type lectins, like DC-SIGN or MGL recognize carbohydrate or glycan structures so glycosylation of antigens can therefore modify antigens for DC-targeting. Also the use of antigen-containing liposomes, which are coupled to antibodies recognizing DC specific subsets, may be used for *in vivo* targeting.

However the techniques described above rely on the use of a disease specific antigen. The choice of the correct antigen in the field of atherosclerosis is less evident as the specific antigen is still unknown. OxLDL forms an interesting target as we have shown that vaccination strategy, in which an oxLDL-specific immune is induced, is atheroprotective. However further activation of the immune system in patients could have adverse effects. As we have shown in chapter seven, defective TGF-β signaling in DCs (these DCs are not capable of becoming tolerogenic DCs) induced an enhanced lesion progression with a more inflammatory phenotype. Furthermore, an increasing number of preclinical studies are focusing on the use of immature DCs to silence immunity by either inducing T cell anergy or by expanding regulatory T cells. Therefore, the use of the unpulsed immature DCs (**chapter four**) forms an attractive approach to implement in patients. DCs are cultured from the patient's blood or bone marrow and can subsequently be transferred back to the patient without the need of adjuvants and further pulsing with an atherospecific antigen. Also the induction of tolerogenic $CD4^+DX5^+$ cells after treatment with unpulsed ImDCs remains an interesting finding as it has been shown that these cells are protective in other auto-immune diseases.[14, 41-43] Therefore we would like to implement $CD4^+DX5^+$ adoptive transfer at different stages of the disease and determine in which stage of atherosclerosis these cells could be protective.

We have also observed that foam cell formation or hyperlipidemia induces a DC-like phenotype in macrophages and moreover, influences the antigen presentation and processing capacities *in vitro*. Therefore we would like to investigate the role of foam cell formation *in vivo* on APCs function and the possible effect thereof

on local $CD8^+$ activation and lesion development. $LDLr^{-/-}$ mice that receive a bone-marrow transplantation of ABCA1/SRBI double knock-out mice exhibit an extreme foam cell phenotype in the periphery. Hence, these mice provide us with an ideal model to study the influences of foam cell formation on the activation of an immune response during hyperlipidemia and cardiovascular disease. We have shown that cholesterol loading affects APC function but on the other hand we have seen that DCs could also influence cholesterol levels. However, the underlying mechanism remains elusive. PPARγ is a transcriptional regulator of CD36 and the oxysterol receptor LXRα and is highly expressed on DCs where it is a regulator of DC function by altering antigen uptake, maturation, activation, migration, cytokine production and lipid antigen presentation.[38, 39] In addition, LXR decreases LDL and tissue cholesterol by facilitating cholesterol excretion in the gallbladder, reducing cholesterol absorption in the intestine and promoting cholesterol efflux from macrophages by increasing ABCA1, ABCG1 and ApoE.[40] Therefore we would like to extend our research about the potential LXR activating properties of DCs and the possible effect on cholesterol levels. With regards to our studies about the role of Tregs in aneurysm formation, we are planning to increase the number of Tregs by injection of mouse IL-2 immune complexes.

In conclusion, we have shown the high potential of DC-based therapy in atherosclerosis. Furthermore, the progress being made in the many clinical trails will further enhance our knowledge of DC-based therapy. Therefore, it seems likely that in the future it is save to implement DC-based therapy as a new strategy in the battle against atherosclerosis.

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Nederlandse samenvatting

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Pijn op de borst (*angina pectoris*), beroerte, aneurysma, trombose, etalagebenen, longembolie, aderverkalking, enz. al deze ziektes vallen onder de noemer hart-en vaatziekten en zijn vaak het gevolg van het dichtslibben van (middel) grote slagaderen, ook wel aderverkalking of atherosclerose genoemd. Door de vernauwing van het vat (plaque) stroomt er steeds minder zuurstofrijk bloed richting het orgaan. Wanneer de plaque echter scheurt ontstaat er een bloedstolsel (*trombose*) die de bloedtoevoer helemaal kan afsluiten en daardoor een infarct kan veroorzaken. De meest belangrijkste risicofactoren voor hart- en vaatziekten zijn verhoogde cholesterol waarden, hoge bloeddruk, roken, zwaarlijvigheid, diabetes en te weinig lichaamsbeweging. Het is daarom ook niet verwonderlijk dat hart- en vaatziekten de meest voorkomende doodsoorzaak is in de westerse wereld. Huidige therapieën focussen zich voornamelijk op het verminderen van de risicofactoren zoals het verlagen van de bloeddruk en cholesterol. Bij patiënten met een belemmerde bloedtoevoer kan men chirurgisch ingrijpen door het vat te dotteren of een stent te plaatsen om zo het vat open te houden. In het geval van een geblokkeerd vat kan er een bypass uitgevoerd waarbij de bloedtoevoer wordt omgeleid.

Atherosclerose wordt tegenwoordig beschouwd als een combinatie van cholesterolstapeling en een chronische ontsteking van de vaatwand. Algemeen wordt geaccepteerd dat de binnenste laag van de vaatwand (*endotheel*) wordt geïrriteerd door bijvoorbeeld roken of te hoge bloeddruk. Hierdoor wordt het endotheel geactiveerd en kunnen de cholesterol deeltjes uit de circulatie de vaatwand binnendringen. Deze deeltjes worden chemisch veranderd en zijn "vreemd" voor het lichaam. Als gevolg wordt het immuunsysteem geactiveerd en worden er ontstekingscellen (*leukocyten*) naar de vaatwand gestuurd om de deeltjes op te ruimen. Macrofagen zijn ontstekingscellen die gespecialiseerd zijn in het opruimen van gemodificeerde cholesterol deeltjes. Na het opnemen van cholesterol worden macrofagen schuimcellen ook wel foamcellen genoemd. De macrofagen worden door de opname van cholesterol deeltjes geactiveerd en produceren allerlei ontstekingsmediatoren waardoor andere leukocyten worden aangetrokken. Hierdoor ontstaat er een vicieuze cirkel die leidt tot het ontstaan van een plaque. Als beschermingsmechanisme wordt er een kapsel gevormd van bindweefsel en collageen. Dit kapsel zorgt ervoor dat de inhoud van de plaque niet in contact komt met het bloed. In dit stadium spreekt men van een stabiele plaque en zijn er weinig klinische manifestaties. Bij de verdere ontwikkeling zal het kapsel steeds dunner worden door allerlei enzymen die worden geproduceerd door de foamcellen. Als het kapsel uiteindelijk scheurt, komen stollingsfactoren uit de plaque vrij en ontstaat er een bloedprop. Deze bloedprop kan een vat volledig afsluiten waardoor het achterliggend orgaan geen zuurstof meer krijgt met een hart- of herseninfarct als gevolg.

In dit proefschrift hebben we onderzoek verricht naar de rol van antigeen presenterende cellen, namelijk dendritische cellen en macrofagen. Deze cellen herkennen "vreemde" deeltjes. Vreemde deeltjes zijn bacteriën en virussen maar dus ook gemodificeerd cholesterol. Antigeen presenterende cellen ruimen deze deeltjes (*antigenen*) op door ze op te eten (*fagocytose*) en ze te "verteren" (*processen*) waarna ze de kleine stukjes (*peptiden*) presenteren aan T cellen. Specifieke T cellen herkenen deze peptiden en gaan over tot de aanval en produceren cytokinen. Ook B cellen worden geactiveerd en gaan antilichamen maken. Dit proces van het activeren van T en B cellen noemt men een immuunreactie. Wanneer er een immuunreactie wordt opgewekt tegen lichaamseigen deeltjes, spreekt men van een auto-immuunziekte. Ook atherosclerose wordt beschouwd als een auto-immuunziekte omdat er een immuunreactie wordt opgewekt tegen het lichaamseigen, slechte cholesterol, de gemodificeerde lage-dichtheid lipoproteïnen (LDL).

De meest potente antigeen presenterende cel is de dendritische cel (DCs). DCs hebben de unieke capaciteit om zowel antigeen specifieke als naïeve T cellen te activeren. Vooral door hun eigenschap van het activeren van naïeve T cellen zijn DCs uitermate geschikt voor vaccinatie strategieën. In **hoofdstuk 2** gebruiken we DCs om muizen te vaccineren tegen geoxideerd LDL (oxLDL). In dit hoofdstuk hebben we DCs beladen met oxLDL en deze intraveneus toegediend aan LDLr deficiënte muizen voor de inductie van atherosclerose door middel van een vetrijk dieet en collar plaatsing. Deze therapie leidde tot 87% reductie van de plaque grootte en meer stabiele laesies. Het bewijs dat de vaccinatie strategie gewerkt heeft, is te vinden in de verhoogde concentratie van oxLDL-specifieke IgG en het verhoogd aantal oxLDL-specifieke T cellen. OxLDL-specifieke IgG antilichamen in het serum vormen immuuncomplexen met oxLDL. Deze immuuncomplexen worden dan herkend en opgeruimd door macrofagen. Daarnaast zullen oxLDL-specifieke T cellen in de aanval gaan in de aanwezigheid van oxLDL. Het schadelijke oxLDL wordt dus geklaard door het immuunsysteem voordat het de vaatwand kan binnen dringen en een mogelijk bewijs hiervoor is te vinden in het verlaagd cholesterol gehalte in de behandelde muizen.

DCs zijn ook in staat om via CD1d lipide antigenen te presenteren aan natural killer T cellen (NKT). Juist omdat NKT cellen een respons geven op lipide antigenen, kan de manipulatie van NKT cellen een interessante toepassing zijn in de behandeling van atherosclerosis. De precieze rol van NKT cellen in atherosclerose is afhankelijk van het stadium van de ziekte maar ook van het gebruikte muismodel met een beschermende rol in LDLr deficiënte muizen maar een atherogene rol in ApoE deficiënte muizen. Het endogene ligand voor NKT cellen is niet bekend maar er zijn 2 synthetische liganden: α-galactosylceramide (α-GalCer) en OCH waarbij α-GalCer leidt tot de productie van Th1 cytokinen (atherogeen) en OCH tot de productie van Th2 cytokinen (anti-atherogeen). In **hoofdstuk 3** tonen we aan dat LDLr deficiënte muizen die behandeld worden met OCH door middel van intra-peritoneale injecties verminderde Th1 cytokinen en meer Th2 cytokinen produceren maar dat het geen effect had op laesie ontwikkeling. Uit de literatuur blijkt dat NKT cellen langduriger worden geactiveerd door dendritische cellen. Daarom zijn

we overgestapt op het toedienen van OCH-beladen DCs wat resulteerde in 71% reductie in plaque grootte. Na injectie migreren DCs naar de lever waar een grote pool van NKT cellen aanwezig is. De behandeling leidde tot de inductie van NKT cellen in het bloed en deze produceerden meer Th2 cytokinen dan de NKT cellen in de controle dieren. Een verklaring voor de verlaagde cholesterol waarden kan gezocht worden in de verhoogde IL-10 productie die optrad in de behandelde muizen.

In de **hoofdstukken 2 en 3** hebben we ons gefocust op vaccinatiestrategieën. Dit zijn behandelingen die gestart werden voordat de muizen atherosclerose ontwikkelen. Aangezien de klinische manifestaties van atherosclerose zich voordoen bij een ver gevorderd stadium van de ziekte, zijn we in **hoofdstuk 4** gaan onderzoeken of we een DC therapie kunnen toepassen in dieren met reeds aanwezige atherosclerose. Omdat in atherosclerose het immuunsysteem reeds geactiveerd is, hebben we er voor gekozen om de muizen te behandelen met tolerogene DCs (immature DCs). Deze DCs presenteren wel de antigenen aan T cellen maar geven niet voldoende activatiesignalen. Hierdoor worden de antigeen specifieke T cellen anergisch en sterven af en wordt er op die manier tolerantie geïnduceerd. LDLr deficiënte muizen werden op een vetrijk dieet gezet gedurende 20 weken om een ver gevorderd stadium van atherosclerose te ontwikkelen. Daarna werden de muizen drie keer behandeld met immature DCs en werden de muizen verder het dieet gevoed gedurende tien weken. Het injecteren van tolerogene DCs leidde tot het stoppen van laesie groei in alle belangrijke plaatsen waar atherosclerose kan ontwikkelen. Een verhoogd aantal regulatoire T cellen werd gevonden in het bloed en er werd ook een verhoogde perifere/lever CD4/CD8 ratio vastgesteld. Beide processen zijn belangrijke onderdelen in tolerantie inductie. Ook zagen we dat de injectie van immature DCs leidt tot de inductie van tolerogene DX5 cellen in de milt en lever. Maar adoptieve transfer van deze cellen leidde niet tot een stop van plaque groei. Daarnaast hebben we vastgesteld dat injectie van immature DCs het cholesterol metabolisme beïnvloedt. Niet alleen waren de cholesterol waarden verlaagd, er werd ook een meer voordelig lipoproteïnenprofiel met lagere LDL en hogere HDL waarden gevonden. Ook werd er een verhoogde expressie van LIGHT en lymphotoxine mRNA vastgesteld wat dan weer gecorreleerd kan worden tot de verlaagde cholesterol waarden. In dit hoofdstuk tonen we aan dat DC-therapie ook toegepast kan worden in vergevorderde atherosclerose wat tot zeer interessante klinische applicaties kan leiden. Zeker omdat DCs al gebruikt worden in veel klinische studies in het veld van kanker.

In **hoofdstuk 5** onderzoeken we het effect van een verstoorde TGF-β signalisering in DCs. TGF-β heeft verschillende anti-atherogene werkingen. Zo ontwikkelen ApoE deficiënte muizen grotere laesies wanneer de TGF-β signalen worden geblokkeerd en omgekeerd, het injecteren van TGF-β in ApoE deficiënte muizen, vermindert de ontwikkeling van atherosclerose. TGF-β heeft dus een beschermende rol doordat het de activatie van T cellen en macrofagen vermindert maar ook door de aantrekking en migratie van leukocyten naar de vaatwand door de hoeveelheid adhesiemoleculen en de productie van chemokinen te verminderen. Daarnaast

bevordert TGF-β de productie van collageen en speelt het dus een belangrijke rol in het stabiliseren van de plaque. Het kweken van DCs in aanwezigheid van TGF-β creëert tolerogene DCs en DCs zelf produceren ook grote hoeveelheden TGF-β maar de precieze werking van TGF-β op cellen van het aangeboren (innate) immuunsysteem in de muis is nog niet onderzocht. Daarom hebben we de atherosclerose ontwikkeling onderzocht in ApoE deficiënte muizen die een defecte TGF-β signalisering hebben in CD11c positieve cellen. Deze $ApoE^{-/-}$CD11cDNR hebben grotere en onstabieler plaques dan gewone ApoE deficiënte muizen en de laesies bevatte meer T cellen. Dit is te verklaren door de verhoogde activatie status van T cellen in de milt. In $ApoE^{-/-}$CD11cDNR zijn de T cellen meer geactiveerd, produceren ze meer cytokinen en hebben meer CD44 expressie waardoor ze beter naar de plaats van ontsteking kunnen migreren, in dit geval de plaque. Dit leidt tot een verhoging van het aantal inflammatoire cellen en dit in combinatie met een vermindering van collageenproductie, kan de kans op plaque rupture aanzienlijk vergroten.

In **hoofdstuk 6** concentreren we ons op het effect van foamcelvorming op de functies van antigeen presenterende cellen (APCs). In de literatuur zijn er veel aanwijzingen te vinden dat macrofagen een DCs-achtig uiterlijk krijgen nadat ze foamcellen zijn geworden maar of ze daardoor ook DC-achtige functies verkregen was nog niet bekend. Daarom onderzochten we de antigeen presenterende en antigeen processing eigenschappen van DCs en macrofagen na foamcel vorming *in vitro*. Foamcel APCs werden geïncubeerd in de aanwezigheid van ovalbumine peptiden (om het effect op presentatie na te gaan) en ovalbumine zelf of ovalbumine-immuuncomplexen (om het effect op antigen processing na te gaan). Wij stelden een DC-achtig uiterlijk vast in macrofagen na foamcel vorming met een verhoogde CD11c, MHC klasse I, MHC klasse II en CD86 expressie. Ook vonden we een verhoogde MHC klasse I afhankelijke antigeen presenterende en processing capaciteit in macrofaag foamcellen. De MHC klasse II presentatie en processing was niet beïnvloed door foamcelvorming. In LDLr deficiënte muizen die een vetrijk dieet hebben gekregen, is de expressie van CD11c verhoogd binnen de macrofaag populatie. Ook zijn de DCs en macrofagen meer geactiveerd zoals aangetoond wordt door de verhoogde CD86 expressie. Al deze bevindingen samen, suggereren dat hoge cholesterolwaarden (hypercholestemie) een invloed kan hebben op de functies van macrofagen en DCs. Om dit te bevestigen is verder onderzoek vereist en moet de antigeen presenterende en processing eigenschappen van APCs worden onderzocht in muizen met een extreem foamcel fenotype in de organen, bijvoorbeeld in ABCA1/ABCG1 dubbel knock-out of ABCA1/SRB1 dubbel knock-out muizen.

In **hoofdstuk 7** stappen we over op de rol van regulatoire T cellen (Tregs) in aneurysma vorming. Een aneurysma is een plaatselijke verwijding van een slagader, bijvoorbeeld de aorta. Normaal gezien is de aorta twee tot drie centimeter breed maar als de doorsnede meer dan anderhalf keer groter is spreken we van een aneurysma. Het scheuren van een aneurysma (*rupture*) is levensgevaarlijk en is meestal fataal. Er is een groeiende hoeveelheid bewijs dat het immuunsysteem een belangrijke rol speelt in aneurysmavorming. In hoofdstuk 7 tonen we aan dat

het wegnemen (*depletie*) van Tregs, het scheuren van de aneursyma's bevordert. Met behulp van micro-array hebben we de genexpressie van duizenden genen in de aorta's kunnen bestuderen. We hebben vastgesteld dat de genexpressie van genen betrokken in aneurysma vorming en energie metabolisme sterk beïnvloedt zijn. Vooral de genexpressie van genen die een rol spelen in de citroenzuurcyclus is sterk verminderd in muizen behandeld met een depleteren antilichaam voor Tregs. Daarnaast hebben we, met behulp van verschillende technieken, geprobeerd de Treg populatie te vergroten en het effect hiervan op aneurysmavorming te bestuderen. Tot nu toe zijn we daar nog niet in geslaagd. Daarom is in de toekomst zeker verder onderzoek vereist waarin Tregs worden geïnduceerd door bijvoorbeeld een behandeling met muis IL-2 antilichaam immuuncomplexen.

Samenvattend, laat dit proefschrift zien dat DCs een belangrijk middel kunnen zijn bij het verhinderen maar ook het verminderen van atherosclerose. We hebben aangetoond dat vaccinatiestrategieën gebruikmakend van oxLDL-beladen DCs en OCH-beladen DCs, respectievelijk oxLDL-specifieke T en B cellen activeert of NKT cellen. Beide vaccinatiestrategieën, ook al maken ze gebruik van verschillende mechanismen, verminderen laesie groei in LDLr deficiënte muizen. Daarnaast hebben we aangetoond dat tolerogene DCs de verdere voortgang van laesieontwikkeling in muizen met een ver gevorderd stadium van atherosclerose, kunnen stoppen. Ook tonen we aan dat ApoE deficiënte muizen die niet meer in staat zijn om tolerogene DCs te maken, grotere en meer instabielere plaques hebben. Daarnaast tonen de studies in dit proefschrift aan dat hypercholestemie belangrijke effecten heeft op de functies van DCs en macrofagen maar ook dat er mogelijkheden zijn via DCs-gebaseerde therapieën het eigen immuunsysteem te activeren, of af te remmen, naargelang het stadium van de ziekte. Mede door de grote vooruitgang die wordt geboekt in het gebruik van DC-therapie in kanker, hebben we er hoop op dat deze therapieën mogelijk ook kunnen worden toegepast in de vermindering van het aantal hart -en vaatziekten bij de mens.

Curriculum Vitae

Kim Linda Liesje werd op 5 januari geboren in Maaseik, België. Zij behaalde in 1997 haar diploma van het hoger secundair onderwijs in het Koninklijk Atheneum te Maaseik. In 2000 behaalde ze haar diploma van Kandidaat in de Biologie aan de Universiteit van Diepenbeek. Hierna vervolgde ze haar opleiding tot Licentiaat in de Biologie, specialisatie Dierkunde Fysiologie aan de Katholieke Universiteit van Leuven waar ze in 2002 met *Grote Onderscheiding* afstudeerde. Tijdens haar opleiding heeft ze een wetenschappelijke stage gevolgd bij het Laboratorium voor Immunobiologie, Rega Instituut te Leuven onder de leiding van Prof. Dr. P. Matthys en Prof. Dr. F. Vandesande. Haar thesis was getiteld: "*Verhoogde gevoeligheid van interferon-γ receptor deficiënte muizen voor collageen geïnduceerde arthritis: Het belang van Granulocyt Chemotactisch Proteïne 2 (GCP-2)".*

Vervolgens heeft ze van 2002 tot 2003 als PhD student gewerkt op de afdeling Farmaceutische Biotechnologie onder de leiding van Prof. Dr. D. Deforce en Dr. D. Elewaut met als onderwerp: "*T cell receptor repertoire analysis of regulatory T cells: Generation of Ag-specific T cells after nasal tolerance induction in collagen induced arthritis".* Aansluitend is ze tot en met april 2005 gaan werken als Junior Scientist bij Barrier Therapeutics te Geel waar ze onderzoek verrichte naar inflammatie van de huid, meer bepaald atopische dermatitis.

Van april 2005 tot en met september 2009 heeft ze als assistent in opleiding het in dit proefschrift beschreven onderzoek uitgevoerd op de afdeling Biofarmacie van het Leiden/Amsterdam Center for Drug Research onder de leiding van Prof. Dr. Th.J.C. van Berkel en Prof. Dr. J. Kuiper. Van april 2005 tot heden volgde ze een opleiding tot medisch immunoloog bij het Leiden Institute for Immunology. Tijdens haar promotieonderzoek ontving ze in maart 2006 een fellowship van de Dutch Atherosclerosis Society voor best beoordeeld wetenschappelijk abstract en in maart 2007 ontving ze de Young Investigator Award tijdens de 13de Annual Scandinavian Atherosclerosis Conference. Daarnaast heeft ze in 2006 en 2008 de prijs ontvangen voor beste poster presentatie tijdens het LACDR Spring Symposium. Sinds 1 oktober is ze aangesteld als post-doctoraal onderzoeker bij de afdeling Biofarmacie van het Leiden/Amsterdam Center for Drug Research.

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Abbreviations

AAA	Aortic Abdominal Aneurysm
ABCA1/G1	ATP-Binding Cassette Transporter A1/G1
acLDL	acetylated Low Density Lipoprotein
ALK	Activin-Like Kinase
AngII	Angiotensin II
APC	Antigen Presenting Cell
ApoE	Apolipoprotein E
CAD	Coronary Artery Disease
CFSE	CarboxyFluorescein diacetate Succinimidyl Esther
CNS	Central Nervous System
ConA	Concanavalin A
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CVD	CardioVascular Disease
DC	Dendritic Cell
DMSO	DiMethylSulfOxide
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescent Activated Cell Sorting
FCS	Fetal Calf Serum
Foxp3	Forkhead Box p3
GAPDH	GlycerAldehyde-3-Phosphate DeHydrogenase
GalCer	GalactosylCeramide
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-Induced Tumor necrosis factor Receptor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HDL	High Density Lipoprotein
HL	Hepatic Lipase
HSP60	Heat-Shock Protein 60
IC	Immune Complex
ICAM	IntraCellular Adhesion Molecule
IFN	Interferon
IgG/M	Immunoglobulin G/M
IL	Interleukin
ImDC	Immature dendritic cell
IMDM	Iscove’s Modified Dulbecco’s Medium
KLF2	Kruppel Like Factor 2

LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein Receptor
LPL	Lipoprotein Lipase
LPS	LipoPolySaccharide
LT	Lymphotoxin
LXR	Liver X Receptor
MΦ	Macrophage
M-CSF	Macrophage Colony Stimulating Factor
MCP-1	Monocyte Chemotactic Protein-1
MDA	Malondialdehyde
mDC	Mature Dendritic Cell
MHC	Major Histocompatibility Complex
MIP	Macrophage Inhibitory Protein
MMP	Matrix MetalloProteinase
NK	Natural Killer cell
NKT	natural Killer T cell
NMR	Nuclear Magnetic Resonance
NLR	Notch Like Receptor
OVA	Ovalbumin
oxLDL	Oxidized Low Density Lipoprotein
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PPAR	Peroxisome Proliferator-Activated Receptor
PRR	Pattern Recognition Receptor
RPMI	Roswell Park Memorial Institute
S.I.	Stimulation Index
SMC	Smooth Muscle Cell
TCR	T cell Receptor
TGF-β	Tumor Growth Factor beta
TGF-βR	Tumor Growth Factor beta Receptor
Th	T helper
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor alpha
Treg	Regulatory T cell
Tr1	Regulatory T cell type 1
VALT	Vascular Associated Lymphoid Tissue
VCAM-1	Vascular Cell Adhesion Molecule 1
VLA-4	Very Late Antigen 4
VLDL	Very Low Density Lipoprotein
WT	Western Type