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The potential use 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 than 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

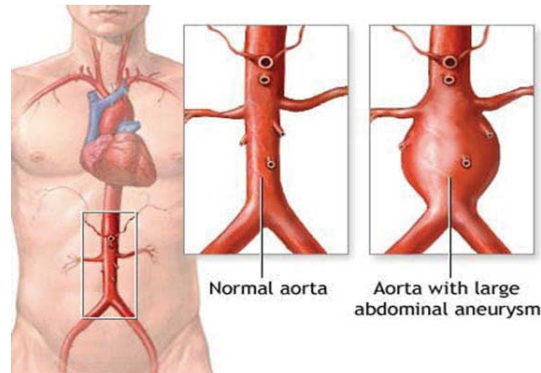


Figure 1: Abdominal Aortic Aneurysm (AAA)

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 chemoattractant 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 (M Φ).^{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).

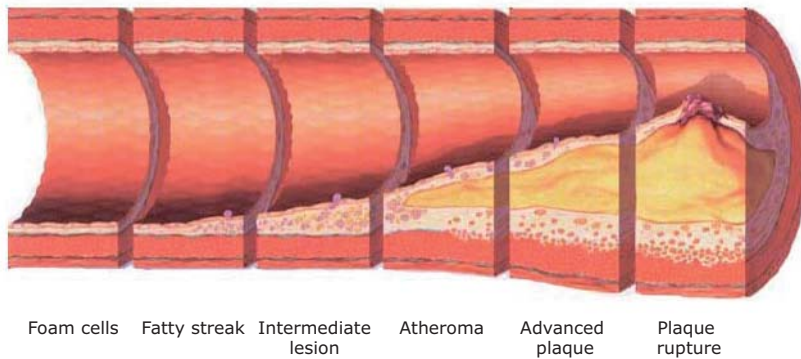


Figure 2: The different stages of atherosclerotic lesion development.

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 determined

Effective priming of naïve 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 CD1d³² 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.

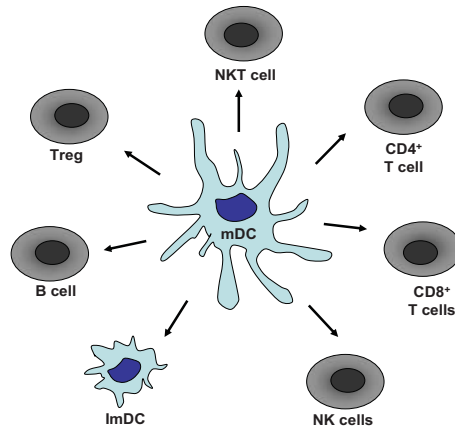


Figure 3: A central role for mature DCs in stimulation of cells of the innate and adaptive immune system

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 contribute 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 contributing 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 HSP60⁴⁷ 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 conventional T cells but also with NKT cells.^{38, 50} DC-induced T cell activation may further stimulate 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-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 and 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 (T β R_{II}) 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.

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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 administered 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 Va14 and Ja18 subunits paired with a restricted set of V β chains (V β 8, V β 7 and V β 2).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 α -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 (Va14Ja18 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 than 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 where 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 then 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 discover 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 trials (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

1. Kostis JB. From hypertension to heart failure: update on the management of systolic and diastolic dysfunction. *Am J Hypertens*. Sep 2003;16(9 Pt 2):18S-22S.
2. Metra M, Nodari S, Bordonali T, et al. Beta-blocker therapy of heart failure: an update. *Expert Opin Pharmacother*. Feb 2007;8(3):289-298.
3. Shimizu K, Mitchell RN, Libby P. Inflammation and cellular immune responses in abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. May 2006;26(5):987-994.
4. Weintraub NL. Understanding abdominal aortic aneurysm. *N Engl J Med*. Sep 10 2009;361(11):1114-1116.
5. Multicentre aneurysm screening study (MASS): cost effectiveness analysis of screening for abdominal aortic aneurysms based on four year results from randomised controlled trial. *Bmj*. Nov 16 2002;325(7373):1135.
6. Hackam DG, Thiruchelvam D, Redelmeier DA. Angiotensin-converting enzyme inhibitors and aortic rupture: a population-based case-control study. *Lancet*. Aug 19 2006;368(9536):659-665.
7. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev*. Apr 2006;86(2):515-581.
8. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol*. Jul 2006;6(7):508-519.
9. Robertson AK, Hansson GK. T cells in atherogenesis: for better or for worse? *Arterioscler Thromb Vasc Biol*. Nov 2006;26(11):2421-2432.
10. Kuller LH. Nutrition, lipids, and cardiovascular disease. *Nutr Rev*. Feb 2006;64(2 Pt 2):S15-26.
11. Glasser SP, Selwyn AP, Ganz P. Atherosclerosis: risk factors and the vascular endothelium. *Am Heart J*. Feb 1996;131(2):379-384.
12. Kritchevsky D. Diet and atherosclerosis. *Am Heart J*. Nov 1999;138(5 Pt 2):S426-430.
13. Lopez AD, Mathers CD, Ezzati M, et al. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet*. May 27 2006;367(9524):1747-1757.
14. Caro CG, Fitz-Gerald JM, Schroter RC. Arterial wall shear and distribution of early atheroma in man. *Nature*. Sep 13 1969;223(5211):1159-1160.
15. Zarins CK, Giddens DP, Bharadvaj BK, et al. Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. *Circ Res*. Oct 1983;53(4):502-514.
16. VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. *Arterioscler Thromb Vasc Biol*. Jan 2004;24(1):12-22.
17. Fan J, Watanabe T. Inflammatory reactions in the pathogenesis of atherosclerosis. *J Atheroscler Thromb*. 2003;10(2):63-71.
18. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. Apr 21 2005;352(16):1685-1695.
19. Boring L, Gosling J, Cleary M, et al. Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. Aug 27 1998;394(6696):894-897.
20. Watanabe Y, Inaba T, Gotoda T, et al. Role of macrophage colony-stimulating factor in the initial process of atherosclerosis. *Ann N Y Acad Sci*. Jan 17 1995;748:357-364; discussion 364-356.
21. Libby P, Galis ZS. Cytokines regulate genes involved in atherogenesis. *Ann N Y Acad Sci*. Jan 17 1995;748:158-168; discussion 168-170.
22. Hansson GK, Hellstrand M, Rymo L, et al. Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med*. Nov 1 1989;170(5):1595-1608.

- 23.** Amento EP, Ehsani N, Palmer H, et al. Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb Sep-Oct 1991*;11(5):1223-1230.
- 24.** Newby AC, Johnson JL. Genetic strategies to elucidate the roles of matrix metalloproteinases in atherosclerotic plaque growth and stability. *Circ Res*. Nov 11 2005;97(10):958-960.
- 25.** Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev*. Jan 2005;85(1):1-31.
- 26.** Bobryshev YV, Lord RS. Co-accumulation of dendritic cells and natural killer T cells within rupture-prone regions in human atherosclerotic plaques. *J Histochem Cytochem*. Jun 2005;53(6):781-785.
- 27.** Bot I, de Jager SC, Zernecke A, et al. Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation*. May 15 2007;115(19):2516-2525.
- 28.** Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. *Annu Rev Immunol*. 2000;18:767-811.
- 29.** Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. *Cell*. Aug 10 2001;106(3):263-266.
- 30.** Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell*. Aug 10 2001;106(3):255-258.
- 31.** Griffiths KL, O'Neill HC. Dendritic cells as immune regulators: the mouse model. *J Cell Mol Med*. Oct 2008;12(5B):1909-1914.
- 32.** Shimizu K, Fujii S. DC therapy induces long-term NK reactivity to tumors via host DC. *Eur J Immunol*. Feb 2009;39(2):457-468.
- 33.** Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. 1991;9:271-296.
- 34.** Kalinski P, Hilkens CM, Wierenga EA, et al. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today*. Dec 1999;20(12):561-567.
- 35.** Langenkamp A, Messi M, Lanzavecchia A, et al. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol*. Oct 2000;1(4):311-316.
- 36.** Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell*. Aug 10 2001;106(3):259-262.
- 37.** Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol*. 2003;21:685-711.
- 38.** Bobryshev YV, Lord RS. Mapping of vascular dendritic cells in atherosclerotic arteries suggests their involvement in local immune-inflammatory reactions. *Cardiovasc Res*. Mar 1998;37(3):799-810.
- 39.** Kawahara I, Kitagawa N, Tsutsumi K, et al. The expression of vascular dendritic cells in human atherosclerotic carotid plaques. *Hum Pathol*. Sep 2007;38(9):1378-1385.
- 40.** Liu P, Yu YR, Spencer JA, et al. CX3CR1 deficiency impairs dendritic cell accumulation in arterial intima and reduces atherosclerotic burden. *Arterioscler Thromb Vasc Biol*. Feb 2008;28(2):243-250.
- 41.** Angeli V, Llodra J, Rong JX, et al. Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity*. Oct 2004;21(4):561-574.
- 42.** Llodra J, Angeli V, Liu J, et al. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A*. Aug 10 2004;101(32):11779-11784.
- 43.** Niessner A, Weyand CM. Dendritic cells in atherosclerotic disease. *Clin Immunol*. Jun 9 2009.
- 44.** Aicher A, Heeschen C, Mohaupt M, et al. Nicotine strongly activates dendritic cell-mediated adaptive immunity: potential role for progression of atherosclerotic lesions. *Circulation*. Feb 4 2003;107(4):604-611.

- 45.** Alderman CJ, Bunyard PR, Chain BM, et al. Effects of oxidised low density lipoprotein on dendritic cells: a possible immunoregulatory component of the atherogenic micro-environment? *Cardiovasc Res.* Sep 2002;55(4):806-819.
- 46.** Perrin-Cocon L, Coutant F, Agaoglu S, et al. Oxidized low-density lipoprotein promotes mature dendritic cell transition from differentiating monocyte. *J Immunol.* Oct 1 2001;167(7):3785-3791.
- 47.** Flohe SB, Bruggemann J, Lendemans S, et al. Human heat shock protein 60 induces maturation of dendritic cells versus a Th1-promoting phenotype. *J Immunol.* Mar 1 2003;170(5):2340-2348.
- 48.** Ge J, Jia Q, Liang C, et al. Advanced glycosylation end products might promote atherosclerosis through inducing the immune maturation of dendritic cells. *Arterioscler Thromb Vasc Biol.* Oct 2005;25(10):2157-2163.
- 49.** Price CL, Sharp PS, North ME, et al. Advanced glycation end products modulate the maturation and function of peripheral blood dendritic cells. *Diabetes.* Jun 2004;53(6):1452-1458.
- 50.** Yilmaz A, Reiss C, Tantawi O, et al. HMG-CoA reductase inhibitors suppress maturation of human dendritic cells: new implications for atherosclerosis. *Atherosclerosis.* Jan 2004;172(1):85-93.
- 51.** Ranjit S, Dazhu L, Qiutang Z, et al. Differentiation of dendritic cells in monocyte cultures isolated from patients with unstable angina. *Int J Cardiol.* Dec 2004;97(3):551-555.
- 52.** Yilmaz A, Reiss C, Weng A, et al. Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. *J Leukoc Biol.* Mar 2006;79(3):529-538.
- 53.** Sun D, Fernandes G. Lovastatin inhibits bone marrow-derived dendritic cell maturation and upregulates proinflammatory cytokine production. *Cell Immunol.* May 2003;223(1):52-62.
- 54.** Jonasson L, Holm J, Skalli O, et al. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. *J Clin Invest.* Jul 1985;76(1):125-131.
- 55.** Stemme S, Holm J, Hansson GK. T lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and the integrin VLA-1. *Arterioscler Thromb.* Feb 1992;12(2):206-211.
- 56.** de Boer OJ, van der Wal AC, Houtkamp MA, et al. Unstable atherosclerotic plaques contain T-cells that respond to Chlamydia pneumoniae. *Cardiovasc Res.* Dec 2000;48(3):402-408.
- 57.** Stemme S, Faber B, Holm J, et al. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc Natl Acad Sci U S A.* Apr 25 1995;92(9):3893-3897.
- 58.** Xu Q. Role of heat shock proteins in atherosclerosis. *Arterioscler Thromb Vasc Biol.* Oct 1 2002;22(10):1547-1559.
- 59.** Elhage R, Gourdy P, Bouchet L, et al. Deleting TCR alpha beta+ or CD4+ T lymphocytes leads to opposite effects on site-specific atherosclerosis in female apolipoprotein E-deficient mice. *Am J Pathol.* Dec 2004;165(6):2013-2018.
- 60.** Ludewig B, Freigang S, Jaggi M, et al. Linking immune-mediated arterial inflammation and cholesterol-induced atherosclerosis in a transgenic mouse model. *Proc Natl Acad Sci U S A.* Nov 7 2000;97(23):12752-12757.
- 61.** Zhou X, Nicoletti A, Elhage R, et al. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation.* Dec 12 2000;102(24):2919-2922.
- 62.** Reardon CA, Blachowicz L, White T, et al. Effect of immune deficiency on lipoproteins and atherosclerosis in male apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* Jun 2001;21(6):1011-1016.
- 63.** Song L, Leung C, Schindler C. Lymphocytes are important in early atherosclerosis. *J Clin Invest.* Jul 2001;108(2):251-259.

- 64.** Frostegard J, Ulfgren AK, Nyberg P, et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis*. Jul 1999;145(1):33-43.
- 65.** Huber SA, Sakkinen P, David C, et al. T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia. *Circulation*. May 29 2001;103(21):2610-2616.
- 66.** Paigen B, Ishida BY, Verstuyft J, et al. Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. *Arteriosclerosis*. Mar-Apr 1990;10(2):316-323.
- 67.** Gupta S, Pablo AM, Jiang X, et al. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest*. Jun 1 1997;99(11):2752-2761.
- 68.** Lee TS, Yen HC, Pan CC, et al. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol*. Mar 1999;19(3):734-742.
- 69.** Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in apolipoprotein E(-/-) mice through release of interferon-gamma. *Circ Res*. Feb 8 2002;90(2):E34-38.
- 70.** Whitman SC, Ravisankar P, Elam H, et al. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice. *Am J Pathol*. Dec 2000;157(6):1819-1824.
- 71.** Hauer AD, Uyttenhove C, de Vos P, et al. Blockade of interleukin-12 function by protein vaccination attenuates atherosclerosis. *Circulation*. Aug 16 2005;112(7):1054-1062.
- 72.** Davenport P, Tipping PG. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. *Am J Pathol*. Sep 2003;163(3):1117-1125.
- 73.** King VL, Szilvassy SJ, Daugherty A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. *Arterioscler Thromb Vasc Biol*. Mar 1 2002;22(3):456-461.
- 74.** van Wanrooij EJ, van Puijvelde GH, de Vos P, et al. Interruption of the Tnfrsf4/Tnfsf4 (OX40/OX40L) pathway attenuates atherogenesis in low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. Jan 2007;27(1):204-210.
- 75.** Leskinen MJ, Kovanen PT, Lindstedt KA. Regulation of smooth muscle cell growth, function and death in vitro by activated mast cells—a potential mechanism for the weakening and rupture of atherosclerotic plaques. *Biochem Pharmacol*. Oct 15 2003;66(8):1493-1498.
- 76.** Shimizu K, Shichiri M, Libby P, et al. Th2-predominant inflammation and blockade of IFN-gamma signaling induce aneurysms in allografted aortas. *J Clin Invest*. Jul 2004;114(2):300-308.
- 77.** Ghiringhelli F, Menard C, Terme M, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J Exp Med*. Oct 17 2005;202(8):1075-1085.
- 78.** Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol*. Apr 2005;6(4):345-352.
- 79.** von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol*. Apr 2005;6(4):338-344.
- 80.** Asseman C, Mauze S, Leach MW, et al. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med*. Oct 4 1999;190(7):995-1004.
- 81.** Paust S, Lu L, McCarty N, et al. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci U S A*. Jul 13 2004;101(28):10398-10403.
- 82.** Shevach EM, Piccirillo CA, Thornton AM, et al. Control of T cell activation by CD4+CD25+ suppressor T cells. *Novartis Found Symp*. 2003;252:24-36; discussion 36-44, 106-114.

- 83.** Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med.* Jul 17 2000;192(2):303-310.
- 84.** Tang Q, Boden EK, Henriksen KJ, et al. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur J Immunol.* Nov 2004;34(11):2996-3005.
- 85.** Gondek DC, Lu LF, Quezada SA, et al. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol.* Feb 15 2005;174(4):1783-1786.
- 86.** Grossman WJ, Verbsky JW, Barchet W, et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity.* Oct 2004;21(4):589-601.
- 87.** Heller EA, Liu E, Tager AM, et al. Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. *Circulation.* May 16 2006;113(19):2301-2312.
- 88.** Veillard NR, Steffens S, Burger F, et al. Differential expression patterns of proinflammatory and antiinflammatory mediators during atherogenesis in mice. *Arterioscler Thromb Vasc Biol.* Dec 2004;24(12):2339-2344.
- 89.** Ait-Oufella H, Salomon BL, Potteaux S, et al. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med.* Feb 2006;12(2):178-180.
- 90.** Steffens S, Burger F, Pelli G, et al. Short-term treatment with anti-CD3 antibody reduces the development and progression of atherosclerosis in mice. *Circulation.* Oct 31 2006;114(18):1977-1984.
- 91.** van Puijvelde GH, Hauer AD, de Vos P, et al. Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis. *Circulation.* Oct 31 2006;114(18):1968-1976.
- 92.** van Puijvelde GH, van Es T, van Wanrooij EJ, et al. Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis. *Arterioscler Thromb Vasc Biol.* Dec 2007;27(12):2677-2683.
- 93.** Geissmann F, Revy P, Regnault A, et al. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol.* Apr 15 1999;162(8):4567-4575.
- 94.** Nandan D, Reiner NE. TGF-beta attenuates the class II transactivator and reveals an accessory pathway of IFN-gamma action. *J Immunol.* Feb 1 1997;158(3):1095-1101.
- 95.** Bobik A. Transforming growth factor-betas and vascular disorders. *Arterioscler Thromb Vasc Biol.* Aug 2006;26(8):1712-1720.
- 96.** Li MO, Wan YY, Sanjabi S, et al. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol.* 2006;24:99-146.
- 97.** Robertson AK, Rudling M, Zhou X, et al. Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *J Clin Invest.* Nov 2003;112(9):1342-1350.
- 98.** Li D, Liu Y, Chen J, et al. Suppression of atherogenesis by delivery of TGFbeta1ACT using adeno-associated virus type 2 in LDLR knockout mice. *Biochem Biophys Res Commun.* Jun 9 2006;344(3):701-707.
- 99.** Lutgens E, Gijbels M, Smook M, et al. Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression. *Arterioscler Thromb Vasc Biol.* Jun 1 2002;22(6):975-982.
- 100.** Mallat Z, Gojova A, Marchiol-Fournigault C, et al. Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ Res.* Nov 9 2001;89(10):930-934.
- 101.** Uyemura K, Demer LL, Castle SC, et al. Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis. *J Clin Invest.* May 1 1996;97(9):2130-2138.

- 102.** Caligiuri G, Rudling M, Ollivier V, et al. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med.* Jan-Feb 2003;9(1-2):10-17.
- 103.** Mallat Z, Besnard S, Duriez M, et al. Protective role of interleukin-10 in atherosclerosis. *Circ Res.* Oct 15 1999;85(8):e17-24.
- 104.** Pinderski LJ, Fischbein MP, Subbanagounder G, et al. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. *Circ Res.* May 31 2002;90(10):1064-1071.
- 105.** Von Der Thusen JH, Kuiper J, Fekkes ML, et al. Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr^{-/-} mice. *Faseb J.* Dec 2001;15(14):2730-2732.
- 106.** Godfrey DI, Hammond KJ, Poulton LD, et al. NKT cells: facts, functions and fallacies. *Immunol Today.* Nov 2000;21(11):573-583.
- 107.** Joyce S. CD1d and natural T cells: how their properties jump-start the immune system. *Cell Mol Life Sci.* Mar 2001;58(3):442-469.
- 108.** Taniguchi M, Seino K, Nakayama T. The NKT cell system: bridging innate and acquired immunity. *Nat Immunol.* Dec 2003;4(12):1164-1165.
- 109.** Yoshimoto T, Paul WE. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J Exp Med.* Apr 1 1994;179(4):1285-1295.
- 110.** Fujii S, Shimizu K, Smith C, et al. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med.* Jul 21 2003;198(2):267-279.
- 111.** Arase H, Arase N, Kobayashi Y, et al. Cytotoxicity of fresh NK1.1+ T cell receptor alpha/beta+ thymocytes against a CD4+8+ thymocyte population associated with intact Fas antigen expression on the target. *J Exp Med.* Aug 1 1994;180(2):423-432.
- 112.** Smyth MJ, Thia KY, Street SE, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med.* Feb 21 2000;191(4):661-668.
- 113.** Mattner J, Debord KL, Ismail N, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature.* Mar 24 2005;434(7032):525-529.
- 114.** Agea E, Russano A, Bistoni O, et al. Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med.* Jul 18 2005;202(2):295-308.
- 115.** Tsuji M. Glycolipids and phospholipids as natural CD1d-binding NKT cell ligands. *Cell Mol Life Sci.* Aug 2006;63(16):1889-1898.
- 116.** Hong S, Wilson MT, Serizawa I, et al. The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med.* Sep 2001;7(9):1052-1056.
- 117.** Jahng AW, Maricic I, Pedersen B, et al. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med.* Dec 17 2001;194(12):1789-1799.
- 118.** Saubermann LJ, Beck P, De Jong YP, et al. Activation of natural killer T cells by alpha-galactosylceramide in the presence of CD1d provides protection against colitis in mice. *Gastroenterology.* Jul 2000;119(1):119-128.
- 119.** Oki S, Chiba A, Yamamura T, et al. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J Clin Invest.* Jun 2004;113(11):1631-1640.
- 120.** Chiba A, Oki S, Miyamoto K, et al. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide. *Arthritis Rheum.* Jan 2004;50(1):305-313.
- 121.** Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature.* Oct 4 2001;413(6855):531-534.

- 122.** Mizuno M, Masumura M, Tomi C, et al. Synthetic glycolipid OCH prevents insulinitis and diabetes in NOD mice. *J Autoimmun.* Dec 2004;23(4):293-300.
- 123.** Tupin E, Nicoletti A, Elhage R, et al. CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J Exp Med.* Feb 2 2004;199(3):417-422.
- 124.** Aslanian AM, Chapman HA, Charo IF. Transient role for CD1d-restricted natural killer T cells in the formation of atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* Mar 2005;25(3):628-632.
- 125.** Nakai Y, Iwabuchi K, Fujii S, et al. Natural killer T cells accelerate atherogenesis in mice. *Blood.* Oct 1 2004;104(7):2051-2059.
- 126.** Major AS, Wilson MT, McCaleb JL, et al. Quantitative and qualitative differences in proatherogenic NKT cells in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* Dec 2004;24(12):2351-2357.
- 127.** VanderLaan PA, Reardon CA, Sagiv Y, et al. Characterization of the natural killer T-cell response in an adoptive transfer model of atherosclerosis. *Am J Pathol.* Mar 2007;170(3):1100-1107.
- 128.** Daugherty A. Mouse models of atherosclerosis. *Am J Med Sci.* Jan 2002;323(1):3-10.
- 129.** van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, et al. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol Chem.* May 15 1993;268(14):10540-10545.
- 130.** von der Thusen JH, van Berkel TJ, Biessen EA. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation.* Feb 27 2001;103(8):1164-1170.
- 131.** Dekker RJ, van Thienen JV, Rohlena J, et al. Endothelial KLF2 links local arterial shear stress levels to the expression of vascular tone-regulating genes. *Am J Pathol.* Aug 2005;167(2):609-618.
- 132.** Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest.* Jun 2000;105(11):1605-1612.
- 133.** Ameli S, Hultgardh-Nilsson A, Regnstrom J, et al. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol.* Aug 1996;16(8):1074-1079.
- 134.** Fredrikson GN, Andersson L, Soderberg I, et al. Atheroprotective immunization with MDA-modified apo B-100 peptide sequences is associated with activation of Th2 specific antibody expression. *Autoimmunity.* Mar 2005;38(2):171-179.
- 135.** Fredrikson GN, Soderberg I, Lindholm M, et al. Inhibition of atherosclerosis in apoE-null mice by immunization with apoB-100 peptide sequences. *Arterioscler Thromb Vasc Biol.* May 1 2003;23(5):879-884.
- 136.** Freigang S, Horkko S, Miller E, et al. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neopeptides. *Arterioscler Thromb Vasc Biol.* Dec 1998;18(12):1972-1982.
- 137.** Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci U S A.* Jan 31 1995;92(3):821-825.
- 138.** Palinski W, Witztum JL. Immune responses to oxidative neopeptides on LDL and phospholipids modulate the development of atherosclerosis. *J Intern Med.* Mar 2000;247(3):371-380.
- 139.** Zhou X, Caligiuri G, Hamsten A, et al. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. *Arterioscler Thromb Vasc Biol.* Jan 2001;21(1):108-114.

- 140.** Nilsson J, Calara F, Regnstrom J, et al. Immunization with homologous oxidized low density lipoprotein reduces neointimal formation after balloon injury in hypercholesterolemic rabbits. *J Am Coll Cardiol.* Dec 1997;30(7):1886-1891.
- 141.** Lo J, Peng RH, Barker T, et al. Peptide-pulsed immature dendritic cells reduce response to beta cell target antigens and protect NOD recipients from type I diabetes. *Ann N Y Acad Sci.* Oct 2006;1079:153-156.
- 142.** Mackensen A, Herbst B, Chen JL, et al. Phase I study in melanoma patients of a vaccine with peptide-pulsed dendritic cells generated in vitro from CD34(+) hematopoietic progenitor cells. *Int J Cancer.* May 1 2000;86(3):385-392.
- 143.** Mahnke K, Qian Y, Fondel S, et al. Targeting of antigens to activated dendritic cells in vivo cures metastatic melanoma in mice. *Cancer Res.* Aug 1 2005;65(15):7007-7012.
- 144.** Banchereau J, Palucka AK, Dhodapkar M, et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res.* Sep 1 2001;61(17):6451-6458.
- 145.** Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A.* Jul 8 2003;100(14):8372-8377.
- 146.** Zhang S, Huang W. Dendritic cell based genetic immunization stimulates potent tumor protection dependent on CD8 CTL cells in the absence of autoimmunity. *J Cancer Res Clin Oncol.* Sep 2008;134(9):987-994.
- 147.** Heiser A, Coleman D, Dannull J, et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. *J Clin Invest.* Feb 2002;109(3):409-417.
- 148.** van Duivenvoorde LM, Louis-Pence P, Apparailly F, et al. Antigen-specific immunomodulation of collagen-induced arthritis with tumor necrosis factor-stimulated dendritic cells. *Arthritis Rheum.* Oct 2004;50(10):3354-3364.
- 149.** Link H, Huang YM, Masterman T, et al. Vaccination with autologous dendritic cells: from experimental autoimmune encephalomyelitis to multiple sclerosis. *J Neuroimmunol.* Mar 1 2001;114(1-2):1-7.
- 150.** Adorini L. Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting autoimmune diabetes. *Ann N Y Acad Sci.* Apr 2003;987:258-261.
- 151.** Martin E, O'Sullivan B, Low P, et al. Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity.* Jan 2003;18(1):155-167.
- 152.** Wakkach A, Fournier N, Brun V, et al. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity.* May 2003;18(5):605-617.
- 153.** Grainger DJ. Transforming growth factor beta and atherosclerosis: so far, so good for the protective cytokine hypothesis. *Arterioscler Thromb Vasc Biol.* Mar 2004;24(3):399-404.
- 154.** Frutkin AD, Otsuka G, Stempien-Otero A, et al. TGF- β 1 limits plaque growth, stabilizes plaque structure, and prevents aortic dilation in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol.* Sep 2009;29(9):1251-1257.

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