Novel immune cell-based therapies for atherosclerosis

Frodermann, V.

Novel Immune Cell-Based Therapies for Atherosclerosis

Vanessa Frodermann

NOVEL IMMUNE CELL-BASED THERAPIES FOR ATHEROSCLEROSIS
Vanessa Frodermann

Cover Art:	*The roots made me see, the soil made breathe.* by Nunzio Paci Courtesy Officine dell'Immagine, Milan (IT)
Layout:	Vanessa Frodermann
Printer:	Uitgeverij BOXPress, 's-Hertogenbosch

ISBN: 978-94-6295-176-1

Proefschrift Leiden
Met literatuur opgave - met samenvatting in het Nederlands

Novel Immune Cell-Based Therapies for Atherosclerosis

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 27 mei 2015
klokke 15.00 uur

door

Vanessa Frodermann

Geboren te Al-Jubail, Saoedi-Arabië
in 1984

PROMOTIECOMMISSIE:

Promotor:	Prof. Dr. J. Kuiper	
Co-Promotor:	Dr. S.C.A. de Jager	UMCU

Overige Commissieleden:

Prof. Dr. P.H. van der Graaf	
Dr. I. Höfer	UMCU
Prof. Dr. A.H. Lichtman	Harvard Medical School, USA
Prof. Dr. E. Lutgens	AMC

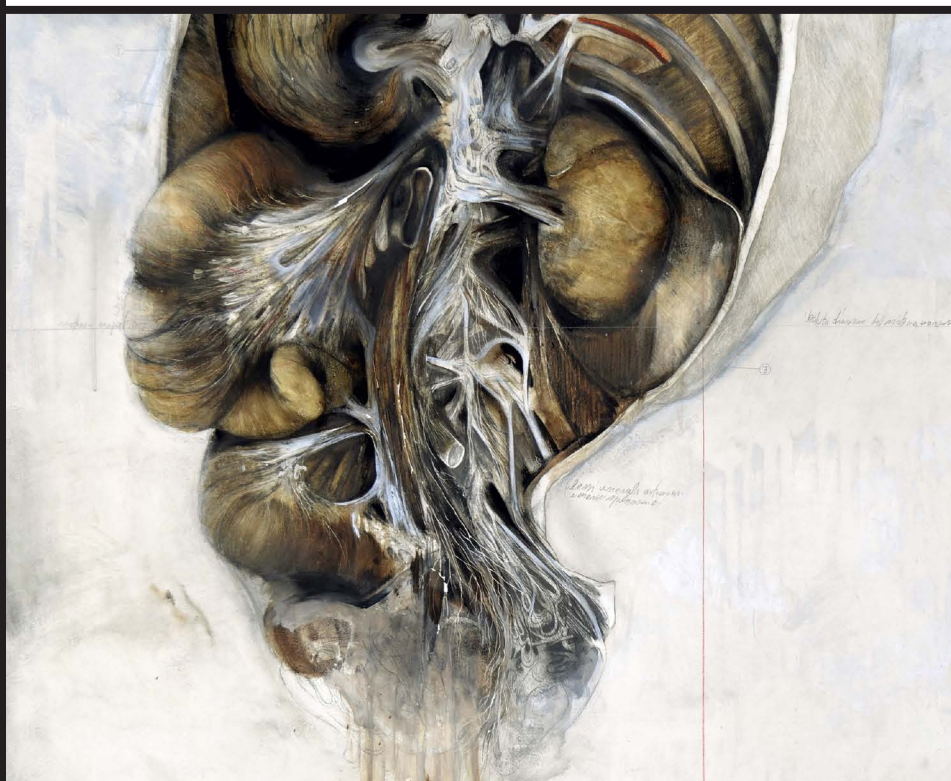
The research described in this thesis was supported by a grant of the Dutch Heart Foundation (DHF-2009B093) and was performed at the Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands. Financial Support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

The realization of this thesis was also financially supported by Peprotech, Greiner Bio-One, BD Biosciences and Leiden University.

TABLE OF CONTENTS

1

General Introduction



Contents

Cardiovascular diseases

Cardiovascular disease (CVD) includes all diseases that affect the heart and blood vessels. The main forms of CVD are coronary heart disease and cerebrovascular disease[1], of which atherosclerosis is the principal cause. The major risk factors for CVD are a high-fat diet, sedentary life-style, stress, excessive alcohol consumption and tobacco exposure[1,2]. Additionally, prior infections or underlying autoimmune diseases, e.g. rheumatoid arthritis, were shown to increase the risk for CVD[3]. CVD is the largest single cause of death in industrialized countries: Each year, CVD accounts for around 46% of deaths in the European Union[4] and 36% of deaths in the United States[1]. In the past 10 years, a large 30% decline has been observed[1,4]. This is largely due to improved prevention and treatment of CVD[5,6]. However, as a result of the improved treatment of acute coronary syndromes, overall heart failure incidence is increasing[1]. Additionally, many risk factors, such as obesity[7] and diabetes[8], are still increasing and could therefore negatively affect CVD incidence in the near future. Furthermore, it should be noted that while in Western countries overall disease mortality rates are declining, in the rest of the world mortality rates are dramatically increasing[9].

Together with secondary complications after survival, CVD results in an enormous burden on the global economy, estimated to cost the European Union about 196 billion Euro[10] and the United States about 396 billion Euro[1] annually. This clearly indicates a great need for new treatment possibilities (besides life-style changes, statins, and blood pressure lowering agents) to improve the prevention and treatment of the main underlying cause of CVD, atherosclerosis.

1. Atherosclerosis

Observations of atherosclerosis date back as far as Aristoteles, who observed that arteries of young individuals were straight and open vessels, while arteries of older individuals were tortuous and narrowed vessels[11]. Initially the process of arterial stiffening was seen as a result of aging and only in the 19th century was the process acknowledged as pathogenic. Early work by Nikolai Anichkov determined the role of cholesterol in the development of atherosclerosis[12]. Soon after, Virchow[13], von Rokitansky[13], Hodgson[14], and Hope[15] proposed a role for inflammation in atherosclerosis. Virchow incorporated the inflammatory characteristic of the disease into its name "Endarteritis deformans s. nodosa" and thereby changed the perception of the disease. However, this term sharply divided the fatty degenerative aspect from the inflammatory proliferative aspect and soon after the term "atherosclerosis" was proposed by Marchand[16]. This incorporates both the "atheromatous" (degenerative, gruel-like) and "sclerotic" (hardening) aspect of the disease and has been used since to describe the disease process. Today it has become widely accepted that atherosclerosis is a chronic inflammatory autoimmune-like disease of the large and medium-sized arteries. It already starts in early adolescence[17] and can either remain asymptomatic throughout an entire lifetime or can result in acute complications, such as myocardial infarction (MI). Interestingly, by means of ^{14}C content of lesions (which was released into the atmosphere during nuclear weapons tests in the 1950s-60s) it

was shown that remodeling of human atherosclerotic lesions is a very slow process. The biological age of components was found to vary from 6.5 years in the cap regions, to 10 years in the core, and 13 years in the shoulder region[18]. This minimal turnover already indicates the difficulty of modulating the atherosclerotic disease process. Endothelial dysfunction, vascular inflammation, and the accumulation of lipids and fibrous elements within the vessel wall are all characteristic for atherosclerosis. Eventually this will result in lesion formation, vascular remodeling, abnormal blood flow, stenosis, and possibly thrombus/emboli formation.

1.1 Initial Atherosclerotic Lesions

Atherosclerotic lesion development is closely linked to local hemodynamic factors. Dysfunction of vascular endothelial cells (ECs) preferentially occurs in areas, such as the inner curvatures of coronary arteries, where shear stress is low, or near bifurcations, where shear stress is oscillatory. This results in an altered gene expression and a related change in cell morphology of ECs, causing increased permeability for macromolecules, such as low-density lipoprotein (LDL), increased cell turnover, and increased expression of adhesion molecules for leukocytes, such as vascular adhesion molecule-1 (VCAM-1)[19].

LDL can passively diffuse through the disturbed EC layer and is retained in the intima by interactions with proteoglycans[20]. Trapped LDL can then undergo modifications, e.g. lipolysis, proteolysis, and oxidation. Evidence from animal models suggests that oxidation is a crucial step in the conversion of LDL into an atherogenic particle. Oxidation is likely facilitated by lipoxygenases, myeloperoxidases, inducible nitric oxide synthase (iNOS) and NADPH oxidases that are found within lesions[21]. Oxidized LDL (oxLDL) leads to the activation of ECs, which increase their expression of adhesion molecules, cytokines, and growth factors (e.g. macrophage colony-stimulating factor; M-CSF)[22]. These changes in the vascular environment result in an activation of resident lymphocytes, dendritic cells (DCs), macrophages, and smooth muscle cells (SMCs). Moreover, an enhanced recruitment and transmigration of leukocytes is mediated by three main chemokine receptor/chemokine pairs: CC chemokine receptor (CCR) 2/ CC chemokine ligand (CCL) 2, CX_3C-chemokine receptor 1 (CX_3CR1)/CX_3CL1 and CCR5/CCL5[23,24]. This culminates in a chronic low-grade inflammation of the vessel wall[25]. Recruited monocytes are exposed to growth factors within the vessel wall, which stimulate their differentiation to macrophages[26]. Moreover, M-CSF enhances the expression of scavenger receptors in macrophages, which recognize oxidation-specific epitopes and thereby enable uptake of cell debris and oxLDL[27]. By accumulating cholesterol esters, macrophages transform into lipid-rich foam cells, so called due to cytoplasmic lipid droplets giving them a 'foamy' appearance under electron microscopy. These cells are one of the most characteristic features of the atherosclerotic lesion. Eventually cholesterol accumulation induces cytotoxicity, resulting in foam cell apoptosis. The lipid-rich remnants of foam cells contribute to formation of a necrotic core in the lesion[28]. At this stage the lesions are referred to as fatty streaks (Figure 1A), which in humans can usually be found

in the aorta as early as the first decade of life[29]. In addition, lesional macrophages, as well as DCs, express Toll-like receptors (TLRs), which enable them to recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). TLR stimulation results in macrophage polarization and DC maturation, determined by upregulation of co-stimulatory molecules and release of pro-inflammatory mediators[30,31]. Released cytokines and chemokines promote further inflammation and recruitment of more leukocytes – either from the luminal side or via the adventitia through vasa vasorum[32,33]. Moreover, while only DCs can activate naïve T cells, both macrophages and DCs can present antigens and directly shape adaptive T cell responses.

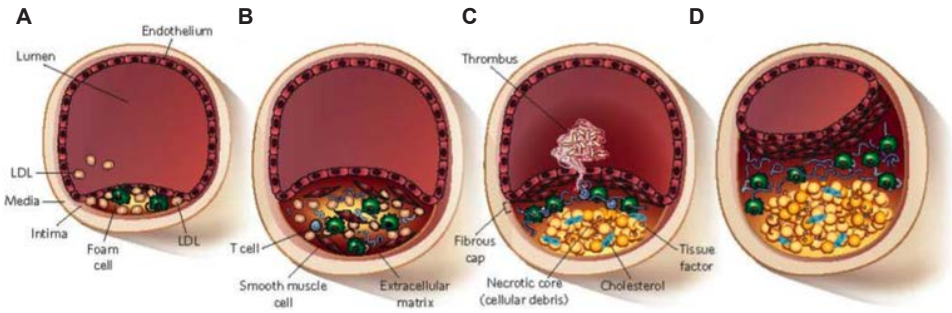


Figure 1. Initiation and Progression of Atherosclerosis. Atherosclerosis occurs at sites in the arterial tree where laminar flow is disrupted. **A.** Atherogenic lipoproteins such LDLs enter the intima, where they are modified and aggregate within the extracellular intimal space, thereby increasing their phagocytosis by macrophages. Unregulated uptake of atherogenic lipoproteins by macrophages leads to the generation of foam cells. Foam cell accumulation leads to fatty streaks formation. **B.** Smooth muscle cells secrete large amounts of extracellular-matrix components, such as collagen, increasing lesion formation. In addition to monocytes, other types of leukocytes are recruited and help to perpetuate a state of chronic inflammation. **C.** Foam cells eventually die, resulting in the release of cellular debris and crystalline cholesterol, contributing to necrotic core formation. In addition, smooth muscle cells form a fibrous cap. This non-obstructive lesion can rupture or the endothelium can erode, resulting in the formation of a thrombus in the lumen. If the thrombus is large enough, it blocks the artery, which causes an acute coronary syndrome or myocardial infarction. **D.** Ultimately, if the lesion does not rupture and the lesion continues to grow, the lesion can encroach on the lumen and result in clinically obstructive disease. Reproduced with permission from Nature Publishing Group & Palgrave Macmillan. Rader DJ and Daugherty A. Translating molecular discoveries into new therapies for atherosclerosis. Nature. 2008;21;451(7181):904-13.

Damaged and modified self-structures, such as dying cells or oxLDL, are potentially cytotoxic and should be removed by innate immune cells before they cause injury to the surrounding tissue[34]. When complete neutralization of the injurious agents does not occur and the inflammation advances, the immune response will switch from a protective to a damaging process. The amount of (activated) lesional leukocytes in early atherosclerosis steadily increases due to ongoing recruitment and further activation of the cells (Figure 1B). This results in a vicious progressive inflammatory cycle and fatty streaks then become more advanced atherosclerotic lesions, also called 'fibrous lesions'[29].

1.2 Advanced Atherosclerotic Lesions

Advanced atherosclerotic lesions are characterized by a lipid-rich necrotic core and by the presence of a SMC-rich fibrous cap. ECs and T cells produce growth factors and cytokines, which result in the migration of SMCs from the media into the intima and their local proliferation. SMCs secrete extracellular matrix proteins, which results in the formation of a fibrous cap. Moreover, they can also accumulate cholesterol and become SMC-derived foam cells[35]. The lesion initially expands towards the adventitia, but after a critical point expands into the lumen. The fibrous lesions continue to expand due to infiltration (and proliferation) of leukocytes, continuous production of extracellular matrix and accumulation of extracellular lipids[29].

The lesion stability appears to be critically dependent on its composition. Stable lesions usually have a uniformly dense fibrous cap and can eventually result in partial occlusion of the arteries. Consequently there is reduced blood flow and oxygen deprivation in target tissue. Vulnerable lesions usually have a thin fibrous cap with low numbers of SMCs and reduced collagen, an increased number of macrophages, and a large lipid-rich necrotic core[36]. Production of matrix metalloproteinases (MMPs) by macrophages has been shown to result in thinning of the fibrous cap. Therefore, rupture of the fibrous cap often occurs at the shoulder regions where monocytes enter and accumulate[37]. Degradation processes may be accompanied by the production of tissue factor by macrophages, which can accumulate in lesions. Upon rupture, this thrombogenic material will result in initiation of the coagulation cascade and thrombus formation (Figure 1C). The thrombus can either cause blockage of that particular artery or it can travel with the blood flow and lead to blockage of smaller arteries resulting in ischemia of the heart, brain, or extremities with the consequence of unstable angina, infarction or stroke[29]. Thrombi can also result from endothelial erosions exposing collagen and von Willebrand factor to the blood flow, resulting in platelet adhesion and activation. Other factors, such as calcification and neovascularization, both common features of advanced lesions, further influence the stability of the atherosclerotic lesion[29]. Interestingly, lesion composition has been found to correlate with the type of angina pectoris in patients: Unstable angina patients demonstrate complex lesions and thrombus formation, while stable angina patients demonstrate more non-complex stable lesions[38,39]. Additionally, increased tissue factor and macrophage content have been associated with unstable angina[40]. Patients with outward remodeling into the vessel wall have a much higher risk to develop unstable angina, whereas inward remodeling, i.e. obstruction of the vessel lumen, is more common in stable angina[41]. Surprisingly, a recent study by van Lammeren *et al.* found that atherosclerotic lesions in patients undergoing carotid surgery (from the Athero-Express study) were characterized by low numbers of macrophages and SMCs, and low lesional thrombosis[42]. While these characteristics are associated with stable lesions, these patients were included in the study due to their occurrence of major cardiovascular events. Therefore, future studies might have to readdress the actual relationship between lesion histology and lesion stability.

1.3 Experimental Models of Atherosclerosis

As with all diseases, preclinical research largely depends on appropriate in vitro and in vivo models for predictions of treatment effectivity. In vitro modulation of cells, either cell lines or primary cultures, can identify specific responses or genes involved in disease processes. Moreover, co-cultures of various cells can identify specific interactions between cells. Nonetheless, these experiments lack the complexity of human atherosclerotic lesions. Ex vivo models using vessel explants from mice[43] and rabbits[44], as well as human atheroma cultures[45] have been used. These enable the study of more complex cellular interactions of cells present in lesions. Additionally, artificial blood vessels have been generated by seeding SMCs, fibroblasts, and endothelial cells on a scaffold[46], and 3D printing is currently being investigated to achieve bio-printing of blood vessels[47]. Nevertheless, all these models do not represent the actual in vivo situation, as e.g. changes of cellular emigration/immigration as well as changes in blood cholesterol levels are absent.

Animal models therefore still provide the best opportunity to evaluate complex interactions in atherosclerosis. After initial in vitro testing, they offer the best opportunity for preclinical screening of therapeutic strategies. Numerous species have been used to investigate mechanisms of atherosclerosis, such as non-human primates[48], swines[49], rabbits[50], rats[51] and mice. The mouse has become the model of choice for atherosclerosis research for simple reasons: they are easily genetically modified, easy to house and breed, and relatively low cost to purchase. Several mouse models for atherosclerosis have been developed as wild type mice are relatively resistant to atherosclerosis and C57BL/6 mice only develop small fatty streak-like lesions when fed a high cholesterol diet[52]. The most commonly used transgenic strains to study atherosclerosis are the ApoE$^{-/-}$ mice and LDLr$^{-/-}$ mice[53].

Apolipoprotein E (ApoE) and the LDL receptor (LDLr) are both crucially involved in the clearance of chylomicrons and very low-density lipoprotein (VLDL) from the circulation. While ApoE is a lipoprotein found in chylomicrons and VLDL, the LDLr is expressed especially on liver cells and binds ApoE as well as apolipoprotein B (ApoB; lipoprotein found on LDL) to clear chylomicrons, VLDL and LDL from the circulation. Deficiencies in either ApoE or the LDLr thus result in an increase in cholesterol and triglyceride-rich lipoproteins in the circulation. In humans familial dysbetalipoproteinemia and familial hypercholesterolemia are caused by defects in ApoE and the LDLr, respectively.

ApoE$^{-/-}$ mice develop lesions already on a normal chow diet due to very high total cholesterol levels (400-500 mg/dL)[54,55]. Most of the cholesterol is carried in VLDL and chylomicron remnants, while most humans have high levels of LDL[56]. Furthermore, ApoE$^{-/-}$ mice develop more severe leukocytosis, neutrophilia and monocytosis than LDLr$^{-/-}$ mice[57]. They form more complex atherosclerotic lesions, which are characterized by excessive foam cell formation, a large lipid-rich necrotic core, and a high smooth muscle cell and collage content.

LDLr$^{-/-}$ mice have much lower total cholesterol levels (175-225 mg/dL) on a normal chow diet than ApoE$^{-/-}$ mice. Additionally, they need to be fed a high cholesterol (Western-type) diet to induce lesion development[58]. Lesions develop slower than those

in ApoE$^{-/-}$ mice and are rich in macrophages, which resembles more the slow process observed in humans. However, lesions are less severe and show less complexity.

2. The Immune System in Atherosclerosis

As mentioned, chronic inflammatory processes, besides dyslipidemia, are responsible for the atherosclerotic disease process. Early indications for this came from the observation of inflammatory infiltrates in atherosclerotic lesions[59]. Moreover, in humans markers of inflammation, e.g. C-reactive protein (CRP), have been found to correlate with cardiovascular syndroms[60]. Indeed, almost all immune cells have been found to either play a pro- or anti-atherogenic role. The role of monocytes, macrophages, DCs, T cells and their progenitors will be further discussed here. However, it should be noted that B cells[61], mast cells[62], neutrophils[63], eosinophils[64], NKT cells[65], NK cells[66], and γδ T cells[67–69] have all been shown to be involved in the disease process as well. The role of other cell types, such as myeloid-derived suppressor cells[70] and innate lymphoid cells[64], still needs to be established.

2.1 Stem Cells

Stem cells are undifferentiated multipotent progenitor cells that can differentiate to specific cell subsets upon various stimuli, but can also divide and self-renew. In general three different types of stem cells exist: embryonic stem cells, induced pluripotent stem (iPS) cells and adult stem cells. Embryonic stem cells originate from the pre-implantation stage of embryos and are pluripotent. iPS cells are reprogrammed adult somatic cells with similar properties as embryonic stem cells. Adult stem cells can be isolated from almost any tissue or organ and function in maintenance and repair. In general two populations have been described: the hematopoietic stem and progenitor cells (HSPCs), which can differentiate into all types of blood cells, and the mesenchymal stem cells (MSCs; also called bone marrow stromal cells or mesenchymal stromal cells) that can generate bone, cartilage, and fat cells.

HSPCs reside within the bone marrow and function to maintain hematopoietic homeostasis[71]. They have been extensively used to restore bone marrow function in cancer patients after treatment with cytotoxic drugs and/or whole body irradiation. Recently phase I clinical trials have established a positive effect of HSPCs on cardiac repair upon MI, as shown by improved left ventricular function[72,73]. In atherosclerosis, HSPC proliferation and mobilization might underlie the observed monocytosis. Studies by the Tall laboratory found that cholesterol trapped inside HSPCs increases their proliferation[57,74]. In addition, it has been recognized that chronic psychosocial stress is a risk factor for atherosclerosis. Indeed, a study by the Nahrendorf laboratory has shown that chronic stress in mice as well as humans (medical residents) results in mobilization of leukocytes. In mice it was shown that chronic stress and thus increased sympathetic nervous system activity induces HSPC proliferation and thereby increases monocyte and neutrophil numbers, which promotes atherosclerosis[75]. Acute tissue injury after MI was also shown to induce proliferation and mobilization of HSPCs[76,77]. HSPC recruitment was found to increase circulating monocyte numbers, resulting

in accelerated atherosclerotic lesion progression[77]. The effect was to a large degree dependent on sympathetic nervous system signaling indicating that acute anxiety and pain might affect HSPC mobilization from the bone marrow. These initial studies point towards a role of known risks factors for atherosclerosis, such as a high-fat diet and stress, in increasing HSPC production and output by the bone marrow, resulting in monocytosis.

MSCs were first identified in the bone marrow, but can also be isolated from other tissues such as umbilical cord, placenta and adipose tissue[78]. They can be easily expanded in vitro without loss of their multipotency, rendering them interesting tools for therapeutic strategies[79]. MSCs were shown to migrate to sites of tissue damage or inflammation where they can extravasate[80]. Originally, MSCs were investigated for their ability to repair injured tissues, e.g. after MI[81], but recently have also been investigated for their immunomodulatory capacities. Many elaborate studies have shown that MSCs can interfere with DC and T cell function and are immunosuppressive[78]. Interestingly, IFN-γ enhances the immunosuppressive capacities of MSCs[82]. Preclinical studies have shown that MSCs can prevent allograft rejection[83,84] and alleviate autoimmune diseases[85–87]. Moreover, in a phase II clinical trial, it was found that MSCs can reduce graft-versus-host disease[88]. Overall, MSC treatment has been established as safe and effective[80]. Due to these properties, MSC therapy could prove beneficial in atherosclerosis.

2.2 Monocytes

Monocytes play a critical role in atherosclerosis. Their continuous recruitment from the bone marrow and accumulation in the arterial wall, giving rise to macrophages and DCs, is one of the earliest events in the disease process and is proportional to the extent of atherosclerosis[89]. Monocytosis is caused by hypercholesterolemia in mice[90] and has been found in humans to correlate with cardiovascular disease[91]. This is initiated by chemokines which are produced by activated ECs and SMCs, but also by activated macrophages and DCs within lesions[92]. Recruitment of monocytes from the bone marrow occurs in a CCL2- and CCL7-dependent manner[93,94], both being ligands for CCR2. The increase of circulating monocytes is a result of increased HSPCs in the bone marrow, which give rise to common myeloid progenitors (CMPs), which again generate macrophage DC progenitors (MDPs)[95]. Moreover, recently an extramedullary pool of splenic HSPCs has been proposed[77,96] that can be mobilized and give rise to monocytes under inflammatory conditions.

The population of circulating murine monocytes ($CD11b^+CD115^+F4/80^{int-lo}Ly\text{-}6G^-$) consists of two main subsets differentiated by their expression of Ly-6C. $Ly\text{-}6C^{hi}$ monocytes share properties with human classical $CD14^+CD16^-$ monocytes based on gene expression profiles. They are pro-inflammatory and preferentially recruited to inflamed tissues, while $Ly\text{-}6C^{low}$ monocytes share several properties with human non-classical $CD14^{dim}CD16^+$ monocytes and are considered to patrol the vasculature and home to non-inflamed tissues[97,98]. $Ly\text{-}6C^{hi}$ monocytes are predominant during hypercholesterolemia and give rise to macrophages in the lesion[90]. They express high

levels of CCR2, CCR5, CCR1, and low levels of CX_3CR1, CD62L, and rely on CCL2/CCR2 for egression from the bone marrow[99]. Ly-$6C^{low}$ monocytes on the other hand express high levels of CX_3CR1 and low levels of CCR2[100]. Ly-$6C^{hi}$ monocytes have been found to be able to convert to Ly-$6C^{low}$ monocytes showing plasticity at the monocyte level[97]. Chemokine deficiencies as well as monocyte deficiencies of chemokines receptors reduce lesional monocyte infiltration and lesion sizes in murine models of atherosclerosis.

The importance of the CCL2-CCR2 axis has been extensively investigated. Both deficiencies of CCL2[101] and its receptor CCR2[102] have been shown to strongly inhibit lesion formation in murine models of atherosclerosis, as a result of reduced leukocyte recruitment to lesions. However, we have shown that CCR2 does not affect lesion progression as reconstitution of $ApoE^{-/-}$ mice with CCR2-deficient bone marrow did not affect lesion progression, macrophage content or lesion stability[103]. This indicates that either CCL2/CCR2-mediated recruitment plays a less profound role in advanced lesions, in line with a recent study suggesting that lesion progression is largely independent of monocyte recruitment[104], or that reduced recruitment of protective regulatory T cells (Tregs) via CCR2 compensates for the potential beneficial effect of reduced monocyte recruitment. This dual role of CCR2 has previously also been observed in collagen-induced arthritis[105].

Nonetheless, CCR2 and CCL2 are promising targets for cardiovascular disease therapy and interference of the CCL2-CCR2 axis has been explored. In mice, targeting liposomes containing siRNA against CCR2 to monocytes has been shown to prevent their recruitment and accumulation in atherosclerotic lesions[106]. Additionally, gene therapy by transfection of an N-terminal deletion mutant of the human CCL2 gene into the skeletal muscle significantly inhibited lesion formation in $ApoE^{-/-}$ mice[107]. Interestingly, while CCR2 deficiency does not affect lesion progression, blockade of CCL2 by this N-terminal deletion mutant of the CCL2 gene was found to limit lesion progression[108]. In humans CCL2[109,110] serum levels and CCR2 on monocytes[110] have been shown to be associated with atherosclerosis. In a phase II clinical an anti-CCR2 monoclonal antibody potently reduced CRP serum levels, a risk factor for cardiovascular disease, for up to three months in cardiovascular risk patients and was well tolerated[111].

CX_3CL1 (also known as fractalkine) has been shown to be crucially involved in the recruitment of monocytes to atherosclerotic lesions in $ApoE^{-/-}$ mice[112]. Both deficiency of CX_3CL1[113] and its receptor CX_3CR1[114,115] reduces atherosclerosis in $ApoE^{-/-}$ mice. Interestingly, also in humans single nucleotide polymorphisms (SNPs) of CX_3CR1 have been associated with cardiovascular disease[116–119]. CX_3CR1 signaling plays an additional role, besides CCR2, in the recruitment of monocytes to lesions. $CX_3CR1^{-/-}CCR2^{-/-}ApoE^{-/-}$ mice have significantly less atherosclerotic lesions than single deficiencies of CX_3CR1 and CCR2[120]. It was found that retention of monocytes is at least in part mediated by their loss of CCR2 and gain of CX_3CR1, which results from exposure to oxidized lipids in the vessel wall[121].

Additionally, CCL5 has been associated with an unstable atherosclerotic lesion phenotype in carotid endarterectomy patients[122]. It is thought that CCL5/CCR5

interactions are needed for firm adhesion of rolling monocytes[123]. However, while some studies indeed indicate that CCR5 expression is pro-atherogenic[124–126], others show that CCR5 deficiency does not affect atherosclerosis[127]. We have shown that an HIV entry inhibitor (TAK-779), a CCR5 antagonist, shows a significant reduction of atherosclerosis[128], further suggesting CCR5 as a potential therapeutic target. However, it should be noted that CCR5 is highly expressed by pro-atherogenic T helper (Th)1 cells and is involved in their recruitment to lesions. To what extent reduction of macrophage recruitment by CCR5 contributes to effects of lesion development still needs to be addressed. A clue might be provided in a study by Combadière *et al.* where $CCL2^{-/-}CX3CR1^{-/-}ApoE^{-/-}$ mice were treated with a CCR5 antagonist. These mice showed a significant additional reduction of circulating ($Ly\text{-}6C^{hi}$) monocytes compared to non-treated mice, indicating that all three chemokine receptors are involved in monocyte recruitment[129]. However, again it cannot be excluded that the additional decrease in lesion size of CCR5 antagonist-treated $CCL2^{-/-}CX3CR1^{-/-}ApoE^{-/-}$ mice is not due to an additional reduction in Th1 recruitment.

In addition to the three main chemokines involved in recruitment of monocytes, also CXCL8 (IL-8; in mice there is no true homologue) and CXCL1 (GRO-α; in mice KC is accepted as the closest homologue), which are best known as neutrophil chemoattractants[130], play a role. These related chemokines signal via CXCR1 and CXCR2. They have been shown to result in firm adhesion of monocytes to the vasculature[131] and their accumulation in murine atherosclerotic lesions[132,133]. Additionally $CXCR2^{-/-}LDLr^{-/-}$ mice and $CXCR1^{-/-}LDLr^{-/-}$ show reduced atherosclerotic lesion sizes with reduced amounts of lesional macrophages[132]. However, as these chemokines also play a crucial role in neutrophil recruitment to lesions, future studies will have to assess monocyte-specific effects on atherosclerosis.

Interestingly, some studies have suggested that monocytes can emigrate from atherosclerotic lesions during lesion regression, but not during lesion progression[134] and CCR7 has been implicated in this process[135].

2.3 Macrophages

Macrophages are found at all stages of atherosclerotic lesion development and outnumber any other cell type in the lesion[136,137]. The majority of lesional macrophages is derived from circulating monocytes[26]. The important role of macrophages during atherosclerosis is exemplified by $ApoE^{-/-}$ mice deficient in M-CSF, which show reduced macrophage numbers with altered functions and a dramatic 86% reduction in atherosclerosis[138]. However, it should be noted that these mice also already have reduced monocytes. The same accounts for studies that have used clodronate liposomes. These liposomes deliver clodronate to macrophages, resulting in their apoptotis. This treatment has been shown to reduce neointimal hyperplasia, but again circulating monocytes are also significantly reduced by this treatment[139]. CD11b-diphtheria toxin receptor (DTR) mice, which display a depletion of macrophages and monocytes, show a similar profound reduction of initial lesion development[140]. However, no effect on lesion progression is observed, indicating a less pronounced role of monocytes and

macrophages in established lesions[140]. The role of macrophages was more specifically assessed by systemic administrations of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which only resulted in apoptosis of macrophages but not of other cells. This significantly reduced progression of advanced atherosclerotic lesions[141].

Macrophages play a crucial role in clearing debris, such as modified lipids, in the arterial wall. OxLDL can be efficiently cleared by macrophages as they recognize it via scavenger receptors (SRs), including SR-A1[142] and CD36[143], which together mediate up to 90% of oxLDL uptake in vitro[144]. Eventually macrophages undergo apoptosis and contribute to necrotic core formation and further lesion progression. Therefore, LDL is accepted as a key factor in the pathogenesis of atherosclerosis and often referred to as the "bad cholesterol". Once oxLDL is taken up by macrophages, it is delivered to lysosomes, where cholesterol esters are hydrolyzed to free cholesterol and fatty acids. Cholesterol can then either be effluxed or be re-esterified by acyl-CoA cholesterol ester transferase (ACAT) and stored in cytosolic lipid droplets, if an acceptor is lacking[145]. Interestingly, recent studies have also indicated that native LDL particles[146] and cholesterol crystals[147] can be taken up by macrophages and contribute to foam cell formation. Cholesterol crystals are additionally potent activators of the inflammasome resulting in strong IL-1β responses[148].

The accumulation of lipid droplets in macrophage results in a shutdown of endogenous cholesterol biosynthesis and LDLr expression by inhibition of the sterol regulatory element-binding protein (SREBP) pathway[149]. However, this does not restore cholesterol homeostasis in the macrophage as cholesterol is continuously taken up by SRs. Excessive cholesterol accumulation results in the activation of liver X receptor (LXR) signaling. LXRα and LXRβ induce specific target genes upon binding of oxysterols (oxygenated derivatives of cholesterol). While LXRβ is widely expressed, LXRα is mostly expressed in the liver and the intestine, but gets significantly induced in macrophages upon lipid accumulation. LXRs and retinoid X receptors (RXRs) form heterodimers, which induce three classes of target genes: (1) ATP-binding cassette (ABC) transporter genes, (2) ApoE in macrophages, both promoting cholesterol efflux, and (3) fatty acid synthesis genes, promoting re-esterification of cholesterol. ABC transporters are crucially involved in cholesterol efflux from the macrophages and strongly induced by LXR and RXR signaling. The most studied cholesterol transporters are ABCA1 and ABCG1[26]. Cholesterol efflux from macrophages can occur passively or actively via high-density lipoprotein (HDL) or apolipoprotein A-I (ApoA-I) cholesterol acceptors. ApoA-I is the major apolipoprotein of HDL and is produced in the liver or intestine. ApoA-I can accept cholesterol esters from ABCA1, thereby forming nascent HDL, which is subsequently converted to mature HDL by esterification of free cholesterol by lecithin cholesterol acyltransferase (LCAT). Mature HDL can in turn accept free cholesterol from ABCG1 and SR-BI[28]. HDL then transports the cholesterol esters back to the liver, where they are either excreted into the bile or reused for lipoprotein assembly. For this beneficial role in maintaining cholesterol homeostasis, HDL is also referred to as the "good cholesterol". Several epidemiological studies have shown an inverse correlation between plasma HDL and cardiovascular diseases[150]. We

and others have demonstrated the important role of cholesterol efflux in macrophages by reconstituting LDLr$^{-/-}$ mice with ABCA1$^{-/-}$ or ABCA1$^{-/-}$ABCG1$^{-/-}$ bone marrow. These mice show increased foam cell formation and atherosclerosis[151,152]. In contrast, results of ABCG1 deficiency are less clear, possibly due to a compensatory upregulation of ABCA1 or a less pronounced role of ABCG1. LDLr$^{-/-}$ mice transplanted with ABCG1$^{-/-}$ bone marrow either showed no effect on atherosclerotic lesions[151,153], only moderately increased lesion formation[154], or even decreased lesion development[155,156] and progression[157]. Reduced atherosclerosis was attributed to increased macrophage apoptosis upon ABCG1 deficiency[156,157], since apoptotic cell clearance induces anti-inflammatory responses[158,159]. However, because apoptotic cell clearance in advanced human and murine lesions is impaired[160-162] it is likely that other processes, such as a previously observed compensatory ABCA1 and ApoE upregulation[155], also contributed. Interestingly, it has recently been suggested that desmosterol, a cholesterol-precursor present in oxLDL, can induce an anti-inflammatory macrophage phenotype through activation of LXR[163]. VLDL has also been associated with cardiovascular disease risk and VLDL levels are often increased in metabolic syndrome. VLDL is mostly processed in adipose tissue and muscle by vascular endothelial cell-anchored lipoprotein lipase (LPL)[164]. The remaining cholesterol-rich remnants are readily cleared by the liver or result in the formation of LDL. VLDL can also enter lesions and can be taken up by macrophages, which can generate cholesterol ester-rich remnants via LPL. Therefore, while LPL has beneficial effects in the periphery[165], its expression in lesions seems pro-atherogenic by production of cholesterol ester-rich lipoproteins, which in turn can be oxidized and taken up by macrophages[166].

In addition to their role in local lipid metabolism in the arterial wall, macrophages are involved in modulating immune responses. They express several pattern recognition receptors (PRRs) by which they can sense PAMPs or DAMPs in the lesions. This results in their activation and production of pro-inflammatory cytokines. TLR4 in particular has been implicated to induce pro-inflammatory cytokine production in lesional macrophages[167,168]. Moreover, they can present antigens derived from the recognized PAMPs/DAMPs on their major histocompatibility complex (MHC) I and MHCII molecules and interact with memory /effector CD8$^+$ T cells and CD4$^+$ T cells, respectively[25]. Additionally, foam cells were found to express CD1d[169], which allows them to interact with NKT cells[170].

Macrophages are influenced by their microenvironment: inflammatory mediators and microbial products can modulate macrophage phenotype. Naïve macrophages (M0) are unpolarized macrophages generated by M-CSF, which can then be polarized based on the cues from their environment. However, it has to be acknowledged that macrophage phenotypes are only a snap-shot of a current situation and are a simplification of a continuum of different functions that macrophages can adopt. In fact, macrophages have been shown to be able to re-polarize under certain micro-environmental circumstances[171–174]. This, in addition to the fact that many subsets express similar markers, makes it difficult to assess the specific role of each macrophage subset in atherosclerosis (Figure 2). Moreover, the similarity of macrophages and DCs, especially upon hypercholesterolemia, has to be noted[175].

Nonetheless, current studies suggest that macrophages in early lesions are of an M2 phenotype and switch to an M1 phenotype as lesions progress[172]. Moreover, M1 macrophages were found to localize to rupture-prone regions, while M2 macrophages are located in the adventitia[3]. Induction of an M2 phenotype by Schistosoma mansoni eggs was found to reduce atherosclerotic lesion development[3], further suggesting an anti-atherogenic role of M2 and a pro-atherogenic role of M1 macrophages.

2.3.1 Classically Activated Macrophages (M1)

Classically activated macrophages were originally derived by treatment with IFN-γ and TNF-α, but were also induced by TLR agonists (e.g. LPS) and IFN-γ. They produce pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, IL-8, IL-12p70, and MMP's and possess increased anti-microbial activity[31,176]. They are thus the "classical" macrophage found upon infection. Failure to downregulate M1 activation eventually results in tissue damage. In atherosclerotic lesions, a wide availability of IFN-γ and TNF-α enables polarization of M0 macrophages to an M1 phenotype. M1 macrophages have been described to be present in both human[177] and murine lesions[171]. They have been shown to be explicitly capable of taking up oxLDL and secreting MMPs, while showing poor capacity to clear apoptotic cells. M1-derived pro-inflammatory mediators may trigger EC and SMC activation, induce Th1 and Th17 generation, may promote formation of a necrotic core, thereby further exacerbating the disease process[145,178].

2.3.2 Alternatively Activated Macrophages (M2)

IL-4 polarizes macrophages to a so-called "alternative" state characterized e.g. by upregulation of the mannose receptor (CD206)[179]. These macrophages show reduced pro-inflammatory cytokine production, but enhanced anti-inflammatory cytokine production such as IL-1 receptor antagonist and IL-10. Moreover they have an enhanced endocytic clearance capacity for mannosylated ligands[179] and apoptotic debris[180]. This, in addition to their ability to recruit fibroblasts and to remodel extracellular matrix, makes them potent in promoting wound healing[180]. More recently this phenotype has been subdivided into M2a macrophages (induced by IL-4 and IL-13), M2b macrophages (induced by immune complexes with IL-1β or LPS) and M2c macrophages (induced by glucocorticoids, TGF-β, or IL-10)[31,178]. Next to M1 macrophages, also M2 macrophages are found in both human[177,181] and murine lesions[175]. Due to the production of anti-inflammatory mediators, M2 macrophages are thought to be anti-atherogenic. Furthermore, as M2 macrophages phagocytose apoptotic debris efficiently[181], they might be able to help resolve early atherosclerosis. However, it has to be noted that M2 macrophages produce high levels of MMPs due to their matrix remodeling capacity, which in advanced lesions might result in destabilization and plaque rupture.

2.3.3 Mox, Mha and M4 Macrophages

As mentioned, the microenvironment is crucial in determining the macrophage phenotype. Therefore, it has been investigated whether the atherosclerotic

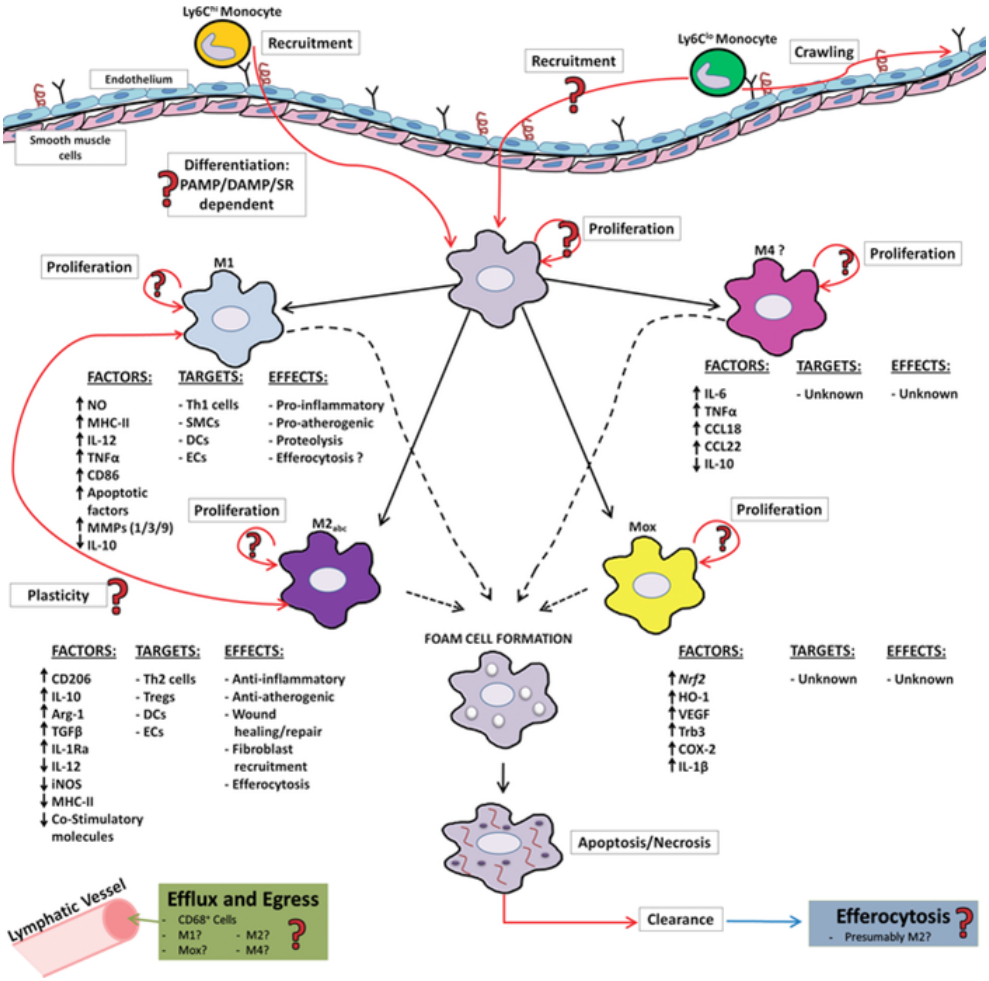


Figure 2. Different lesional macrophage phenotypes. Recruited monocytes give rise to lesional macrophages. As a result of environmental factors monocytes differentiate to macrophages, which can polarize to various subsets. Factors that expressed by these subsets, their target cells and assumed effects are shown. '?' indicates recently proposed mechanisms that still need to be further elucidate. It has e.g. been proposed that lesional macrophages proliferate locally, that monocytes and macrophages can efflux in lesion regression and that possibly M2 macrophages are crucially involved in apoptotic cell clearance. Reproduced with permission from John Wiley and Sons. Taghavie-Moghadam P, Butcher MJ, Galkina EV. The dynamic lives of macrophage and dendritic cell subsets in atherosclerosis. *Ann N Y Acad Sci.* 2014;1319:19-37.

environment can result in specific macrophage subsets. Indeed, oxidized phospholipids present in the arterial wall or oxidized red blood cells, associated with hemorrhages in lesions, can influence macrophage phenotype. Both Mox macrophages (induced by oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine, OxPAPC[171]) and Mha macrophages (resulting from intraplaque haemorrhage[182]) show increased expression of heme oxygenase-1 (HO-1), vascular endothelial growth factor (VEGF) and IL-10. It is likely that these macrophages function to protect other cells from

oxidative stress by their production of anti-oxidizing enzymes and help to suppress other immune cells by their production of anti-inflammatory mediators. Interestingly Mox macrophages were shown to have a significantly different gene expression than M1 and M2 macrophages and only showed approximately 26% and 12% similar genes expressed, respectively. This suggests that oxLDL does induce a distinct gene signature in macrophages. The exact contribution of these macrophages to the atherosclerotic disease process however still remains elusive. CXCL4, deposited by platelets onto the inflamed endothelium, plays a role in recruiting monocytes to the atherosclerotic lesion[183]. The deficiency of CXCL4 has been found to result in reduced atherosclerotic lesions[184], suggesting a pro-atherogenic effect of this chemokine. Recently, it was shown that CXCL4 can directly affect the macrophage phenotype by inducing a robust expression of CD86, ABCG1, CCL18, CCL22, while inhibiting expression of CD36, HO-1 and CD163 expression[185,186]. M4 macrophages show reduced cholesterol uptake and increased efflux, culminating in decreased foam cell formation. However, whether this has any implications for atherosclerosis has yet to be established[186]. Mha and M4 macrophage phenotypes are specifically defined by factors in the microenvironment that induce distinct functions. However, if and to what extend they also show characteristics of M1 or M2 macrophages, and could thus be classified as specialized M1/M2 macrophages remains to be established.

2.3.4 Regulatory Macrophages (Mregs) and Tumor-associated Macrophages (TAMs)

Several studies have described anti-inflammatory macrophages, which have been termed Mreg or TAM, dependent on the disease/mouse model. Mregs have been shown to be induced by the presence of immune complexes and TLR ligands (similar to M2b macrophages), prostaglandins, glucocorticoids, apoptotic cells or IL-10[31]. Moreover, the presence of IFN-γ in the final stages of macrophage differentiation seems to induce a regulatory phenotype in murine as well as human macrophages[187,188]. These Mregs show T cell suppressive effects via production of iNOS and are capable of prolonging allograft survival in a murine heart transplantation model[188]. The administration of human Mregs shows beneficial effects in renal transplantation patients, which was determined to be due to their production of indoleamine-2,3-dioxygenase (IDO), and they are currently being investigated within The ONE Study, a multinational clinical trial of immunomodulatory cell therapy in renal transplantation[189,190]. TAMs are M2-like macrophages, which as the name suggests are found in the tumor microenvironment. They support tumor formation by promoting angiogenesis (via VEGF production) and metastasis (via MMP production). TAMs also produce IL-10 and TGF-β[191,192], as well as large amounts of chemokines, such as CCL22, which results in the recruitment of Th2 cells, atheroprotective Tregs, and monocytes. Moreover, CCL2 and CCL18 production results in the recruitment of naïve T cells, which undergo anergy in the tumor micro-environment[191]. Whether Mregs and TAMs represent a distinct macrophage subtype or whether they are related to other macrophage subsets remains to be elucidated by gene expression analysis. The identification of specific regulatory elements, such as

unique transcription factors or miRNAs, could potentially help.

As macrophages are increasingly found to show high functional diversity and plasticity, there is an increased need for experimental guidelines to establish specific phenotypes, and a nomenclature for macrophages. Murray *et al.* have recently proposed a nomenclature to reduce confusions in the macrophage field. They recommend to define macrophages by origin/source, activators, and a consensus collection of markers. For activated macrophages, they propose to indicate the activator in parentheses, e.g. M(LPS+IFN-γ) and M(IL-4). However, for the in vivo situation they suggest to state the closest relative along the spectrum of in vitro activated macrophages and to additionally provide a combination of markers[193].

2.4 Dendritic Cells

DCs are the most potent antigen-presenting cells (APCs) and can activate naïve and memory T cells[194]. They are present in all lymphoid and most non-lymphoid tissues, particularly at sites in close contact with the environment[195], thereby enabling a quick recognition of possible invading pathogens or other danger signals. In general four types of DCs exist: conventional DCs (cDCs), which can be of a myeloid or lymphoid origin, plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs), and Langerhans cells (DCs of the skin).

cDCs can be further subdivided into migratory inflammatory DCs, which sample the periphery and migrate to lymph nodes, and lymphoid tissue-resident DCs. Lymphoid tissue-resident DCs, which are recognized as $CD8\alpha^{+}CD4^{-}CD11b^{-}CD11c^{+}$ DCs, $CD8\alpha^{-}CD4^{+}CD11b^{+}CD11c^{+}$ DCs, or $CD8\alpha^{-}CD4^{-}CD11c^{+}$ DCs, which can either be CD11b positive or negative[196,197]. CD8α-expressing DCs are highly capable of cross-presenting and priming $CD8^{+}$ T cells[198]. They act to maintain tolerance in steady state, but upon activation produce vast amounts of IL-12 and IFN-γ[198]. $CD4^{+}CD11b^{+}CD11c^{+}$ DCs and $CD4^{-}CD11b^{-}CD11c^{+}$ DCs efficiently activate $CD4^{+}$ T cell responses[199]. Migratory cDCs are commonly divided into $CD103^{+}CD11c^{+}$ DCs and $CD11b^{+}CD11c^{+}$ DCs. $CD103^{+}CD11c^{+}$ DCs are capable of cross-presentation and can induce Tregs or Th2 responses[200]. $CD11b^{+}CD11c^{+}$ DCs are found in most peripheral tissues and mainly promote Th1 responses[161].

Certain DCs in peripheral tissues, e.g. Langerhans cells in the skin and intestinal lamina propria DCs, can be derived from monocytes. Additionally, under inflammatory conditions, monocytes can differentiate into DCs. For example monocytes can differentiate into Tip-DCs (TNF-α/iNOS-producing DCs) which show a strong anti-microbial defense[197]. MoDCs, which are of myeloid origin, can adapt multiple different phenotypes depending on cues from the microenvironment. MoDCs are in general recognized by a high expression of CD11c and MHCII molecules, and can express other markers, such as CD11b, F4/80, CD103, DEC-205. Most of these markers, including CD11c and MHCII, are however also expressed by macrophages and monocytes, especially under inflammatory and hyperlipidemic conditions, making it extremely difficult to identify moDCs. Recently other markers, such as Flt3, c-kit, and CD26 have been suggested to be exclusively expressed by DCs and not

macrophages[161]. Additionally, the zinc finger transcription factor Zbtb46 was recently identified only in cDCs and not pDCs or monocytes[201,202]. pDCs in contrast to other DC subsets express low levels of CD11c and MHCII and undergo terminal differentiation in the bone marrow, while other DC subsets do so in the periphery. They are poor APCs in steady state, but respond with strong type I interferon responses to pathogen recognition and then become fully capable of antigen presentation[203].

2.4.1 DC presence in healthy and diseased aorta

In the vasculature, DCs are found to localize mostly in areas of the aorta susceptible to atherosclerosis[204,205] and their numbers increase during lesion development and progression[205,206]. Upon atherosclerotic lesion formation, large numbers of DCs are found within atherosclerotic lesions. These were initially identified by ultrastructural features, such as a well-developed smooth endoplasmic reticulum and several dendrites[207], while later expression of CD11c served to identify DCs[208–210]. However, it has to be noted that monocytes and macrophages/foam cells also express CD11c and could account for some "DCs" found in these later studies[209,211]. Nonetheless, Choi *et al.* isolated $CD11c^+$ cells from aortas and found them to be as effective as splenic DCs to induce T cell responses[208], providing evidence that these were indeed DCs. Moreover, the majority of lesional DCs were found to lack CD11b expression, indicating that they are distinct from monocytes and macrophages[210]. The precise contribution of DCs versus macrophage in lesions however will only be completely understood when either exclusive markers or a panel of markers for these cells are identified.

In atherosclerotic lesions, DCs form stable contacts with T cells, mostly in the shoulder and rupture-prone regions[212], indicating their crucial role in ongoing immune responses. Indeed, DCs present in murine aortas can activate T cells[208,213,214], suggesting that T cell activation can in fact directly occur within the atherosclerotic lesion. In line with this, DCs in advanced lesions were found to have a mature phenotype[215]. Interestingly, unstable lesions show a higher number of DCs[215], suggesting that DCs contribute to lesion instability by activating T cells. Nonetheless, it still remains unclear whether T cell activation by DCs occurs mainly in the lesion itself or in secondary lymphoid tissues, while activation of naïve T cells will mostly occur in lymphoid tissues[161].

To establish the contribution of DCs to atherosclerosis, the CD11c-DTR $LDLr^{-/-}$ mouse model was used to deplete DCs and a 55% decrease of lesion development was found[209,216]. Conversely, increased DC numbers due to an expanded lifespan of DCs (CD11c-specific expression of anti-apoptotic hBcl-2) resulted in increased numbers of Th1 and Th17 cells, and increased Th1-driven IgG2c autoantibodies, while interestingly lesion size was not affected[216]. These studies indicate a pro-atherogenic role for DCs in atherosclerosis. However, it has to be noted that monocytes and macrophages also express CD11c under hypercholesterolemic conditions, making it difficult to determine whether the effects are due to a depletion and expanded lifespan of DCs only. Recently, Choi *et al.* established that at least two phenotypes of DCs exist in the intima of murine aortas: *bona fide* classical Flt3-Flt3L signaling-

dependent CD103$^+$Langerin$^+$CD11b$^-$F4/80$^-$CD8$^-$CD205$^-$CX$_3$CR1$^-$33D1$^-$ DCs and M-CSF-dependent CD14$^+$CD11b$^+$F4/80$^+$DC-SIGN$^+$TLR4$^+$ monocyte-derived DCs[217]. While both cells expand during atherosclerosis, the Flt3-Flt3L signaling-dependent DCs are most likely tolerogenic and protect from atherosclerosis by inducing Tregs, while M-CSF-dependent monocyte-derived DCs exacerbate atherosclerosis[217,218]. CCL17-expressing DCs in atherosclerotic lesions were found to limit the induction of Tregs, while at the same time recruiting and activating CD4$^+$ T cells, rendering them pro-atherogenic[213]. However, whether they are a unique subset or directly related to M-CSF-derived DCs remains to be established. Additionally, pDCs have been found to cluster with cDCs in shoulder regions of the lesions[219]. Their production of IFN-α enhances TRAIL expression by CD4$^+$ T cells (enabling an efficient killing of SMCs)[220] and induces the production of pro-inflammatory cytokines in other APCs[219]. As pDCs specifically express TLR7 and TLR9, they are equipped to recognize nucleotides or DNA from cellular debris in lesions[203]. These studies suggest that different types of DCs that control T cell homeostasis exist in atherosclerotic lesions and that while some are resident DCs, others are derived from recruited monocytes.

The accumulation of moDCs in atherosclerotic lesions has been shown to critically depend on CX$_3$CR1[221], indicating a possible role for Ly-6C^{low} monocytes in lesional DC accumulation. Indeed, high cholesterol levels were found to increase CD11c expression on Ly-6C^{low} monocytes, which was found to be crucial for monocyte adherence to endothelium (via VCAM-1 and E-Selectin)[222]. Hypercholesterolemia also results in an increased recruitment of pro-inflammatory Ly-6C^{hi} monocytes, which can also give rise to DCs when exposed to granulocyte-macrophage colony-stimulating factor (GM-CSF), a growth factor inducing DC development[223]. However, it has to be noted that increased GM-CSF-dependent DC proliferation in the lesions also persists, and even increases over time, when monocyte recruitment is inhibited[210]. While it will need to be clarified to what amount moDCs contribute to lesional DCs, it is clear that GM-CSF is specifically needed to maintain lesional DCs, as GM-CSF deficiency results in a significant reduction of DCs in lesions without any effects on other monocyte-derived cells[224]. A future challenge will be to decipher the relationship of various DC phenotypes, as well as their origin, in atherosclerotic lesions (Figure 3).

Furthermore, studies have demonstrated that under hypercholesterolemic conditions, DCs are impaired in their migration towards draining lymph nodes, as a result of inhibitory signals from platelet-activating factor (PAF) or oxLDL that acts as a PAF mimetic[134,225]. This appears to be due to imparied CCR7 upregulation, as in the aorta transplantation model for regression (transplantation of atherosclerotic aorta into wild type mice) emigration of DCs was strongly dependent on CCR7[135], which was also shown to be involved in DC emigration from the skin[226]. Additionally statins were shown to promote lesion regression via activation of CCR7-dependent emigration of cells from lesions[227]. However reconstitution of ApoE expression in ApoE$^{-/-}$ mice, which induces regression in these mice, did not show an involvement of CCR7[228]. Therefore, it still remains to be established if CCR7 is the main chemokine receptor involved, and if other factors besides high cholesterol levels determine DC emigration. Nonetheless,

despite their reduced capacity to emigrate from lesions, DCs still retain their capacity to activate T cells[214]. Monocyte recruitment, local DC proliferation, and reduced DC emigration result in increased lesional DC content over time and eventually DCs can comprise up to 20% of lesion volume[224].

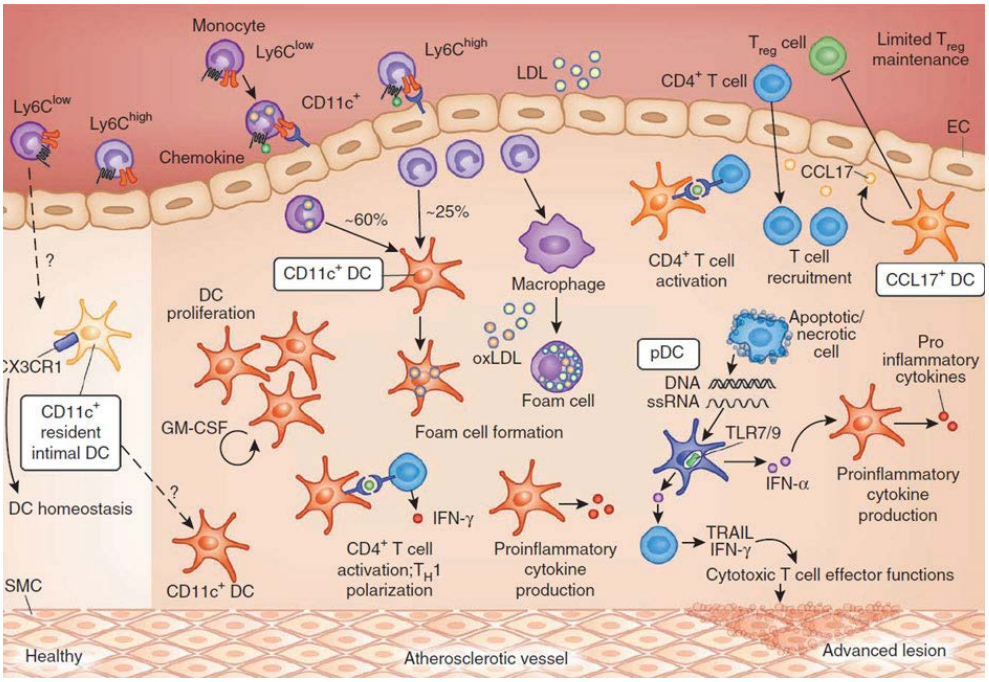


Figure 3. DCs in atherosclerotic lesions. Resident intimal dendritic cell numbers increase in a GM-CSF-dependent manner during lesion development and progression. Circulating monocytes can differentiate to moDCs in lesions. Due to a lack of specific markers, distinction of DCs and macrophages, which can express CD11c upon lipid-loading, is difficult. DCs also engulf lipids in lesions and become foam cell-like cells. DCs produce pro-inflammatory cytokines, activate T cells and induce a Th1 phenotype. CCL17+ DCs can limit Treg expansion and increase T cell recruitment. pDCs can also promote inflammation in lesions. Reproduced with permission from Nature Publishing Group & Palgrave Macmillan. Weber C and Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med.* 2011;17(11):1410-22.

2.4.2 DC function in steady state

Under steady state conditions DCs migrate at a low rate to the draining lymph node without undergoing activation. In the draining lymph node they present self-antigens, derived from apoptotic bodies and/or cellular debris arising from normal cell turnover, to T cells. The T cells subsequently become anergic or apoptotic. It is believed that this represents an important physiological process designed to induce peripheral tolerance and contribute to limiting autoimmunity[229]. This self-tolerance needs to be tightly regulated. However, under certain circumstances this proves problematic as foreign antigens are very similar to self-antigens, creating a considerable challenge for the immune system. This is for example the case with endogenous heat shock proteins (HSPs), which resembles HSP expressed by pathogens such as *Chlamydia pneumonia* and *Helicobacter pylori*[230]. Therefore, under these conditions, DCs may

fail to distinguish between self and foreign antigens and induce immune responses against self-antigens, as observed in the context of atherosclerosis.

As mentioned, DCs together with macrophages are crucially involved in the clearance of apoptotic cells. This process, also termed efferocytosis, is enabled by recognition of molecules exposed on the cell membrane of apoptotic cells. These so-called 'eat-me-signals' consist of modified membrane molecules, such as altered carbohydrates and oxidized molecules that resemble oxLDL[231], and newly exposed molecules on the plasma membrane of dying cells, such as phosphatidylserine (PS)[232]. Modified membrane molecules enable a tethering of APCs to the apoptotic cells, while it was shown that recognition of PS then results in the engulfment of the cells and downstream production of anti-inflammatory cytokines, such as IL-10[158,159].

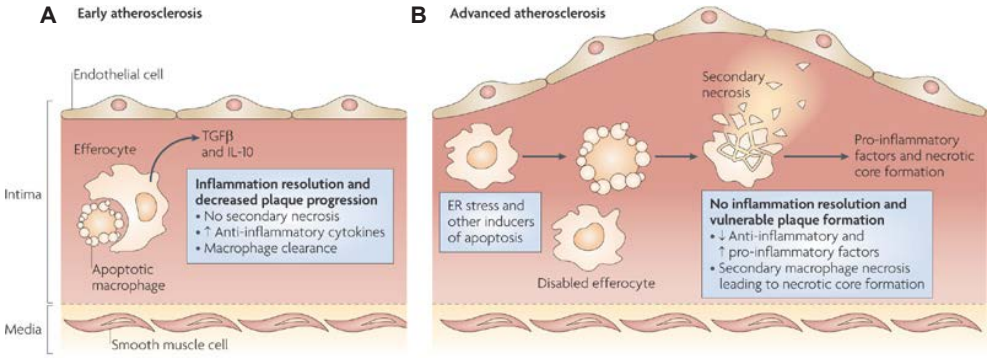


Figure 4. Efferocytosis in early and advanced atherosclerotic lesions. A. Efficient efferocytosis in early atherosclerosis results in a rapid clearance of apoptotic macrophages and concomitant release of anti-inflammatory mediators, reducing lesion progression. **B.** Efferocytosis in advanced lesion does not function properly, resulting in increased necrotic material. Inflammation resolution fails, a necrotic core builds, and lesions progress. Reproduced with permission from Nature Publishing Group & Palgrave Macmillan. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol.* 2010 Jan;10(1):36-46.

Efferocytosis has been shown to be essential to maintain homeostasis and when disrupted it can result in the onset of inflammation and autoimmunity, including atherosclerosis. In atherosclerosis, apoptotic foam cells should help to resolve inflammation as efferocytosis results in anti-inflammatory responses (Figure 4A). However, as the lesion progresses increasing amounts of oxLDL and apoptotic cells will accumulate, possibly exceeding the clearance capabilities of APCs present in lesions. As DCs in atherosclerotic lesions are exposed to maturation signals and mature DCs lose their efferocytosis capabilities[161] this might be a reason for defective efferocytosis in atherosclerosis. Furthermore, the presence of oxLDL in the environment of apoptotic cells could be the main reason that apoptosis cannot help to resolve inflammation for four main reasons: 1) OxLDL and apoptotic cells are recognized by some of the same receptors and might therefore compete for uptake by APCs. 2) OxLDL contains a high amount of lysophosphatidylcholine, which is a chemoattractant that usually recruits DCs to apoptotic cells, and could therefore interfere with DC recruitment to apoptotic cells. 3) DCs that have taken up high amounts of oxLDL cannot emigrate from lesions to modulate T cell responses in draining lymph nodes[134,233]. 4) If uptake of apoptotic

cells takes place in an inflammatory environment shaped by oxLDL, e.g. by activated endothelium and other activated leukocytes, this may possibly override the inhibitory signals of apoptotic cells. Additionally, if apoptotic cells are not cleared, they will undergo secondary necrosis which will promote inflammation (Figure 4B). Indeed it has been suggested that in atherosclerosis post-apoptotic secondary necrosis is due to inefficient or defective efferocytosis[160,234]. In fact, human lesions contain significant amounts of apoptotic cells that are not engulfed by adjacent phagocytes[160], which is striking when comparing to tissues with high cell turnover such as the thymus, where apoptotic cells are rarely detected[235].

2.4.3 DC maturation and immune responses in atherosclerosis

Immature DCs readily detect PAMPs of microbes or DAMPs released by damaged cells via PRRs, including TLRs and cell surface C-type lectin receptors (CLRs)[236]. TLRs dimerize and undergo conformational changes that enable them to bind Toll/IL-1R (TIR)-domain-containing adaptor molecules. There are four adaptor molecules, of which myeloid differentiation primary response gene (MyD) 88 and TIR-domain-containing adapter protein inducing IFN-β (TRIF) are responsible for the activation of distinct pathways, resulting in the maturation of DCs[236]. All TLR signaling results in the activation of the transcription factor nuclear factor-κB (NF-κB), which induces the expression of pro-inflammatory mediators[237]. For NF-κB activation, inhibitory κB (IκB) proteins need to be degraded by the proteasome. It should be noted that in the context of atherosclerosis, modified LDL can trigger TLR2 and TLR4 signaling through CD36 and result in activation of the inflammasome[238,239]. In line, TLR2 and TLR4 deficient LDLr$^{-/-}$ mice have been found to have reduced atherosclerosis[240,241]. In addition to these activation signals through PRRs, immature DCs can also be activated through pathogen-induced non-specific tissue responses, for example pro-inflammatory cytokine production by ECs[242]. Furthermore, ECs and T lymphocytes can also contribute to DC maturation by direct cell-to-cell contact and CD40-CD40L-interaction, respectively[195]. The exact maturation signals for DCs in atherosclerotic lesions remain unknown, but likely inflammatory cytokines and cellular debris, such as RNA and DNA from necrotic cells, activate DCs.

The maturation process can be described as DCs evolving from immature, antigen-capturing cells, over an initial upregulation of antigen-sampling, to mature, only antigen-presenting, T cell priming cells[229]. The maturation process is determined by the maturation signal itself and results in extensive plasticity in DCs[243]. Morphological changes such as the formation of veils and dendrites, which could augment DC-T cell contacts, and an increased cellular motility can be observed upon maturation[244]. DCs will migrate to secondary lymphoid organs, where they encounter T cells and induce their clonal proliferation and expansion[34]. DCs can present lipid antigens on CD1d molecules to NKT cells, providing the immune system with a mechanism for detecting lipid antigens.

DC-induced T cell activation in draining lymph nodes is crucially dependent on three signals of the DCs: 1) antigen presentation, 2) co-stimulatory molecule

expression, and 3) cytokine production by DCs (Figure 5). One of the most well-established and confirmed antigens in atherosclerosis is oxLDL. Interestingly, oxLDL results in the differentiation of monocytes to mature DCs that secrete IL-12, but does not result in the maturation of DCs[245]. Other antigens such as β-2 glycoprotein I (β2GPI), HSP60 and HSP65 have been implicated as pro-atherogenic. OxLDL and HSP60 remain the most prominent antigens in humans and experimental models[246]. Native LDL can also provide antigens for atherosclerosis development and it remains interesting that the immune response in atherosclerosis is directed towards components of native LDL[247], suggesting that fully oxidized LDL might not induce an immune response. MHCI molecules acquire peptides from intracellular antigens, which are generated by proteasomal processing, in the endoplasmatic reticulum; while MHCII molecules acquire peptides from extracellular antigens in the endosome. Some extracellular antigens can also escape into the cytosol and be degraded by the proteasome and then be presented on MHCI molecules by cross-presentation[248]. The vital importance of MHCII-mediated antigen presentation to $CD4^+$ T cells in atherosclerosis was shown in mice deficient in the invariant chain CD74, mediating antigen loading on MHCII, which show decreased atherosclerosis. This study assessed both initial and advanced lesions and showed that while deficiency of antigen presentation already reduced early lesions, it much more dramatically halted lesion progression. Advanced lesions showed significant reductions in activated T cells[249], further substantiating that antigen presentation and ensuing adaptive T cell responses critically promote atherosclerotic lesion progression. Moreover, a specific subset of MHCII molecules (IAb, expressed in atherosclerosis-susceptible C57BL/6 mice) was found to be essential for inducing Th1 responses[250]. The identification of involved MHCII molecules will enable a more directed search for atherosclerosis-specific antigenic epitopes by binding prediction models, which has already resulted in the discovery of two peptide fragments[251].

We and others have shown that co-stimulatory molecules are crucially involved in the activation of T cells and development of atherosclerosis. Deficiencies of CD40L[253], both CD80 and CD86[252], as well as the blockade of CD40L[254], OX40L[255,256], CD30L[257], among others, have been shown to dramatically reduce lesion development. However, it should be noted that co-stimulation is also crucial for the development and maintenance of Tregs, as $CD28^{-/-}$ and $CD80^{-/-}CD86^{-/-}$ mice show a significant reduction in Tregs[258–260]. In line with the protective role of Tregs, $LDLr^{-/-}$ mice reconstituted with $CD28^{-/-}$ bone marrow or $CD80^{-/-}CD86^{-/-}$ bone marrow showed increased atherosclerotic lesion development[258]. The difference in the two studies assessing the effect of deficiency of CD80 and CD86 could be due to the experimental setup (bone marrow transplantation versus $CD80^{-/-}CD86^{-/-}$ mice backcrossed to $LDLr^{-/-}$ mice), but are more likely due to a difference in expression of the co-stimulatory molecules CD28 (stimulating function) versus cytotoxic T-lymphocyte-associated protein-4 (CTLA-4; inhibiting function) on T cells, which both interact with these co-stimulatory molecules. In addition to co-stimulatory molecules the absence of "co-inhibitory" molecules, such as programmed death-ligand 1 (PD-L1) and PD-L2, has been shown to aggravate atherosclerosis[261,262]. This illustrates that simple inhibition of all co-stimulation is not

a feasible approach to reduce atherosclerosis, but that specific "fine-tuning" of co-stimulatory signals is needed.

Antigen recognition as well as co-stimulation both influence T helper lineage commitment (Figure 5), but cytokine production by DCs most potently shapes T cell responses[263]. Indeed, we and others have shown that IL-12 and IL-18 play a crucial role in atherosclerosis development. IL-12[264] and IL-18 deficiencies[265] as well as vaccination against IL-12[266] reduces atherosclerosis, while IL-12[267] and IL-18[268] injections increase atherosclerosis burden. Additionally, DCs can express a variety of chemokines, e.g. CCL17, which can attract T cells to the lesions[213].

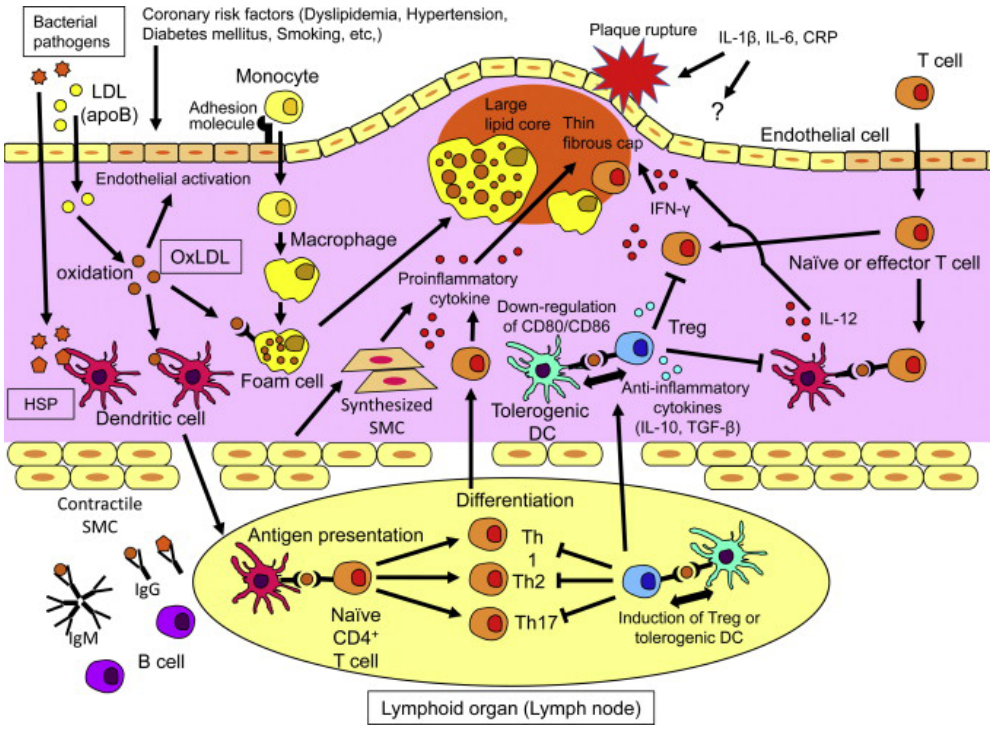


Figure 5. Immune responses in atherogenesis. OxLDL and coronary risk factors activate the endothelium and induce adhesion molecule expression. Monocytes migrate into the subendothelial space using adhesion molecules, differentiate into macrophages, take up oxLDL, and become foam cells. The protein components of the oxLDL particle are processed and presented as antigens to T cells by macrophages and DCs. Other self and foreign antigens may also trigger similar immune reactions. DCs initially migrate to draining lymph nodes and induce naïve T cells differentiation into effector T cells (Th1, Th2, and Th17). For this three signals of the DCs are needed, namely antigen presentation, co-stimulatory molecule expression, and cytokine production by DCs. Tregs suppress effector T cell activation, the differentiation of naïve T cell into effector T cells. Tolerogenic DCs, characterized e.g. by downregulated expressions of CD80/CD86, maintain the tolerance to self-antigens by inducing Tregs or by inhibiting effector T cells. In lesions macrophages and DCs release cytokines and chemokines, and stimulate the migration of smooth muscle cell (SMC) and other inflammatory reactions. Proatherogenic cytokines including IFN-γ secreted by Th1, and IL-12 secreted by DCs and macrophages are associated with destabilizing of the plaque and induce plaque rupture. apo B, apolipoprotein B; CRP, C-reactive protein; HSP, heat shock protein; IFN, interferon; Ig, immunoglobulin. Reproduced with permission from Elsevier. Yamashita T, Sasaki N, Kasahara K, Hirata KI. Anti-inflammatory and immune-modulatory therapies for preventing atherosclerotic cardiovascular disease. *J Cardiol.* 2015 Mar 2.

2.4.4 DC role in lipid metabolism in atherosclerosis

Besides their function as APCs, DCs also engulf lipids (via micropinocytosis, endocytosis, scavenger receptors, and efferocytosis) and form foam cells, contributing to atherosclerotic lesion development[161,209,269]. As they share phenotypic and functional properties with macrophages, which attain a DC-like phenotype upon foam cell formation[211], it has been difficult to dissect the role of DCs and macrophages in lipid uptake in atherosclerosis and the contribution of DCs to foam cell formation in atherosclerotic lesions. As discussed, the uptake of oxLDL results in DC maturation, enhanced antigen presentation to T cells and increased T cell proliferation[245,270]. On the other hand, it was found that uptake of oxidized phospholipids by DCs can impair maturation upon CD40 and TLR2, TLR3 and TLR4 ligation[271]; possibly providing a control for excessive DC activation[271]. Therefore, it will be interesting to determine which components of oxLDL are responsible for both pro-inflammatory as well as anti-inflammatory responses and whether these responses correlate with macrophage function. As mentioned, in macrophages desmosterol was shown to induce an anti-inflammatory response[163]. Additionally, oxysterols, which are found at high concentrations within oxLDL, have been found to activate LXR and similar to macrophages it has been found that LXR can induce an anti-inflammatory phenotype in DCs[272]. As LXR can modulate DCs, it will also be interesting to determine if cholesterol efflux pathways exist in DCs.

Interestingly, a correlation between circulating cholesterol levels and DCs numbers has been found. DC-hBcl2 mice, which express the anti-apoptotic Bcl-2 under the CD11c promoter, have highly increased numbers of DCs as well as significantly reduced plasma cholesterol levels[216]. Conversely, reduced levels of DCs in CD11c-DTR ApoE$^{-/-}$ mice results in enhanced systemic cholesterol levels[216], while lesional lipid accumulation was decreased[209]. The mechanisms behind this effect of DCs on cholesterol metabolism have yet to be identified.

2.4.5 DCs as a therapy for atherosclerosis

DC therapy to induce immune responses has been extensively investigated and used in the field of cancer, where in 2014 alone 289 clinical trials were registered and in 2010 a DC-based vaccine for prostate cancer was approved by the United States Food and Drug Administration (FDA)[273]. Different approaches have been investigated, e.g. the administration of ex vivo modulated DCs, DC-targeted administration of cancer antigens, administration of peptides with adjuvants or blockade of certain receptors on DCs to block signaling pathways (Figure 6).

The crucial role of DCs in enhancing atherosclerosis was shown by adoptive transfer of malondialdehyde-modified LDL-pulsed DCs which aggravated atherosclerosis[274]. This indicated that adoptive transfer of DCs could modulate immune responses in atherosclerosis. Indeed, a study by our laboratory showed that adoptive transfer of oxLDL-pulsed mature DCs could reduce atherosclerosis in an antigen-specific manner, likely due to induction of oxLDL-specific antibodies[275]. However, as atherosclerosis is an inflammatory disease, immunosuppression rather than induction

of immune responses appears a more intuitive and promising approach.

In the past two decades, the recognition of the crucial role of DCs in maintaining steady state homeostasis has resulted in a great interest for DC therapies for autoimmune diseases. Initial studies showed that when antigens were targeted to immature lymphoid DCs in vivo this would result in the generation of Tregs or T cell anergy[276–279], proving the potential of such immature antigen-presenting DCs.

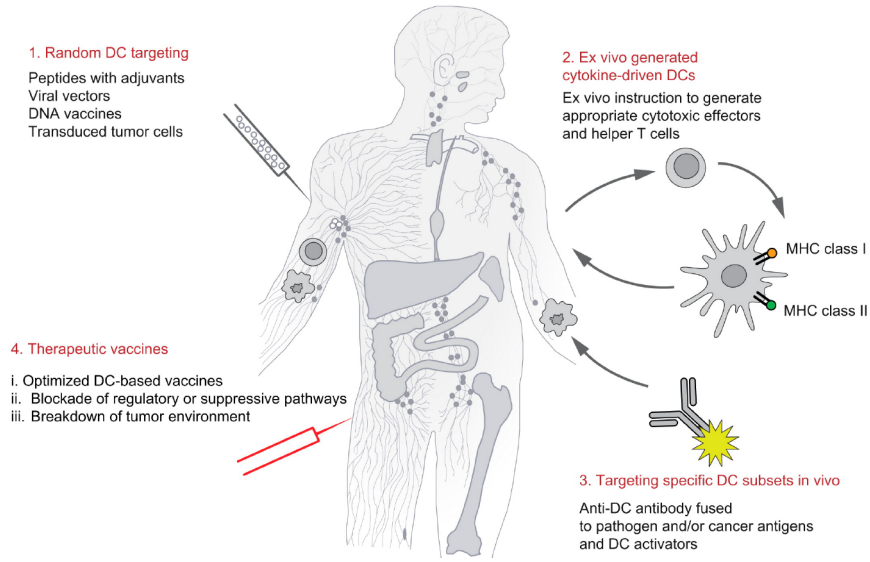


Figure 6. Different approaches for DC-based vaccines in cancer that can be potentially translated to the field of atherosclerosis. 1. Vaccines can target DCs randomly by e.g. by administration of antigens that DCs will take up. As no specific targeting of DCs is involved, these may cause unwanted side-effects. **2.** Ex vivo generated DCs can be instructed to specifically modulate T cell responses. **3.** Anti-DC antibodies linked to antigens can specifically target these to DCs. By combining with an activator (or inhibitor) immune responses to these antigens can be modulated. **4.** Combination of DC therapy with approaches to modulate the microenvironment or blocking (induction) of Tregs. Reproduced with permission from Elsevier. Palucka K, Banchereau J, Mellman I. Designing vaccines based on biology of human dendritic cell subsets. *Immunity.* 2010 Oct 29;33(4):464-78

The simple administration of immature DCs, however, harbors the danger of the cells maturing, especially in inflammatory environments such as atherosclerosis. Additionally, it proved difficult to induce antigen presentation and migratory capacity in immature DCs to ensure interaction with T cells upon injection, without inducing maturation. Several studies in the past years have therefore explored ways to induce so-called tolerogenic DCs that would not further mature once they encounter a pro-inflammatory stimulus, would present the antigen in question, migrate to secondary lymphoid tissues and induce tolerance.

To generate immature tolerogenic DCs, the majority of studies have assessed the feasibility of biological factors (e.g. IL-10, 1α,25-dihydroxyvitamin D3, TGF-β, M-CSF, or vasoactive intestinal peptide) or pharmacological drugs (e.g. dexamethasone, aspirin or rapamycin)[199,280]. However, also pathogen-derived molecules, e.g. of *C. albicans*, or tumor-derived molecules, e.g. mucins, have been shown to induce

immature tolerogenic DCs[280]. Some studies have also used the approach of genetically modifying DCs, e.g. by knocking out suppressor of cytokine signaling 3 (SOCS3) or RelB[280]. These DCs all show low co-stimulatory molecules, increased anti-inflammatory cytokine production, while pro-inflammatory cytokines are reduced, and can induce anergy in T cells and/or Tregs.

Despite inhibiting maturation of DCs, one can imagine that partial maturation of DCs would also prevent them from maturing further in vivo. Several studies have addressed this possibility by showing that a semi-mature DC phenotype can be achieved, by e.g. TNF-α[281], adrenomedullin[282], and disruption of E-Cadherin clusters[283]. The latter mediate mainly cell-cell adhesions and likely disruption of these by mechanical disruption ensures that DCs migrating to secondary lymphoid tissues under steady state induce tolerance. These semi-mature tolerogenic DCs have a marked upregulation of co-stimulatory molecules, chemokines receptors, and antigen-presenting molecules. They also do not produce pro-inflammatory cytokines, but anti-inflammatory cytokines such as IL-10. Moreover, DCs, which are exposed to maturation stimuli such as LPS for a prolonged time in vitro lose their capacity to induce Th1 responses. Instead they produce IL-10 and induce non-polarized memory T cells or Th2 responses[284]. Interestingly, many of the reagents found to induce a tolerogenic phenotype are also produced under physiological conditions in tissues. They could provide a mechanism whereby spontaneously maturing DCs are prevented from inducing immune responses to self in vivo. Up till now, however, it remains unclear which exact signals are needed to promote and maintain the tolerogenic phenotype of DCs.

Different types of tolerogenic DCs have been shown in the past years to decrease ongoing immune responses in various autoimmune diseases[281,285–287]. Indeed, Hermansson et al. have shown that tolerogenic DCs (ApoB100-pulsed) can also protect against atherosclerosis[288]. These initial experiments of DC therapies provide some promising results. However, several questions remain to be answered to improve a potential DC therapy: Should an antigen be used with the treatment and if so, which antigen should be used? Should an adjuvant be used? What is the optimal DC phenotype for vaccination? Future experiments will have to establish the best approach for atherosclerosis and will have to show its feasibility for clinical translation.

2.5 T cells

Both $CD4^+$ and $CD8^+$ T cells have been found in human atherosclerotic lesions, with the majority being $CD4^+$ T cells[289]. T cell infiltration already occurs in early stages of lesion development, in a similar manner as monocyte recruitment[290]. It was found that T cells present in lesions are in an activated state[291], with more than 60% of $CD4^+$ T cells being memory T cells[292]. Recently, increased memory $CD4^+$ T cells and reduced naïve T cells were associated with coronary artery calcification and carotid artery intimal media thickness in subclinical atherosclerosis[293], indicating a pro-atherogenic role of these cells. The presence of such a high number of memory T cells suggests an antigen-specific response as these cells have previously encountered their cognate

antigen. Indeed, a fraction of $CD4^+$ T cells isolated from human atherosclerotic lesions were found to be oxLDL-specific[294].

The overall role of T cells was established in murine models where T cell depletion by anti-CD3 treatment resulted in a reduction of initial lesion development[295], a reduction of lesion progression[295], as well as increased lesion regression[296]. Additionally, nude mice (which are deficient in T cells) were found to have an almost complete (90%) reduction in lesion size compared to their heterozygote littermates[297]. Similarly, $ApoE^{-/-}$ mice crossed to Severe combined immunodeficiency (SCID) mice (deficient in B and T cells)[298], $LDLr^{-/-}$ mice crossed with $Rag1^{-/-}$ mice (no mature B and T cells)[299,300], and $ApoE^{-/-}$ mice crossed with $Rag2^{-/-}$ mice show significant inhibition of atherosclerotic lesion development[301].

2.5.1 Cytotoxic T cells

$CD8^+$ T cells (cytotoxic T cells) kill their targets by release of cytotoxins, such as granzymes and perforins, and expression of FasL. They have also been found to produce large amounts of IFN-γ upon activation, such as in atherosclerosis[302]. Studies have shown a correlation between $CD8^+$ T cells and coronary heart disease[303,304] and up to 50% of lymphocytes found in human lesions are $CD8^+$ T cells[305]. In animal models there is contradictory evidence for the role of $CD8^+$ T cells. $CD8^+$ T cells were found to be the first responders to high fat diet, upregulating their IFN-γ expression weeks before $CD4^+$ T cells were activated[306]. In line with this, antibody-mediated $CD8^+$ T cell depletion in $ApoE^{-/-}$ mice improved atherosclerosis[307], while adoptive transfer of $CD8^+$ T cells into Rag-$2^{-/-}$ApoE-/- mice aggravated atherosclerosis[307]. In contrast, $CD8^{-/-}$ mice backcrossed to $ApoE^{-/-}$ mice showed no significant effects on lesion size[69]. Other studies indicate an atheroprotective effect of $CD8^+$ T cells, as MHCI deficient C57BL/6 mice show increased atherosclerosis[308] and ApoB-100 peptide immunization mediates protective effects in $ApoE^{-/-}$ mice via $CD8^+$ T cells[309]. The reason for these observed differences could be due to the heterogeneity of $CD8^+$ T cells. $CD8^+$ T cells can be further subdivided into type 1 (Tc1) and type 2 (Tc2), with Tc1 being predominant in human lesions[310]. A recent study found that adoptive transfer of the CD25-positive fraction of $CD8^+$ cells, possibly including regulatory $CD8^+$ T cells[311], reduced atherosclerotic lesion development in $ApoE^{-/-}$ mice[312]. This indicates that indeed different subsets of $CD8^+$ T cells likely have distinct functions in atherosclerosis. It is therefore of significant interest to further dissect the role of different $CD8^+$ T cell subsets in atherosclerosis.

2.5.2 Helper T cells

The crucial role of $CD4^+$ T cells (T helper cells) in atherosclerosis has been established in several mouse models. $CD4^+$ T cell depletion was shown to reduce lesions by 70% in C57BL/6 mice[297]. Adoptive transfer of $CD4^+$ T cells into SCID mice dramatically increased lesions by 1.6-fold[298]. Moreover, MHCII transgenic mice (expressing other MHCII molecules than the IAb of athero-susceptible C57Bl/6 mice) were found to have a dramatically reduced atherosclerotic lesion development[250], further confirming the crucial role of $CD4^+$ T cells.

nterestingly, recent evidence suggests that the lipid rich environment in atherosclerotic lesions can directly modulate T cell function. Lysophosphatidylcholine, one of the main phospholipid components of oxLDL, can enhance the expression of CD40, CXCR4, and IFN-γ in human $CD4^+$ T cells, in the absence of APCs[313]. Moreover, increased membrane cholesterol was found to activate T cells, which were more prone to differentiate towards Th1 cells[314]. It will be interesting to establish how hypercholesterolemia affects T cell metabolism, and thereby T cell activation and proliferation. T cell deficiencies were also associated with a significant reduction of total cholesterol levels[300], predominantly due to decreased VLDL, indicating that T cells might not only be affected by cholesterol levels, but can also directly affect these themselves.

Different T helper cell subsets can be induced by cytokines that DCs produce. The most studied in atherosclerosis are Th1 cells, Th2 cells, Th17 cells and Tregs and will be discussed in more detail. However, recently discovered T cell subsets, such as Th5, Th9, Th22 cells might also play a role in atherosclerosis.

2.5.2.1 Th1 cells

The most predominant $CD4^+$ T cell subset in human[291,315] and early murine[316] atherosclerotic lesions are Th1 cells. Th1 cells produce a plethora of pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, IL-2 and IL-12) and express the transcription factor T-bet (T-box expressed in T cells). IFN-γ, IL-12p70 and IL-18 promote T-bet expression in $CD4^+$ T cells[263,317,318]. T-bet induces Th1 cytokine production and inhibits Th2 cytokine expression[263,317,318]. IFN-γ was shown to promote vascular inflammation by enhancing macrophage lipid uptake, activating APCs and ECs, with subsequent recruitment of inflammatory cells, while reducing collagen production by SMCs[319].

Several studies have assessed the role of Th1 cells in atherosclerosis. Inhibition of Th1 differentiation[320] as well as deficiency of T-bet[321] have been shown to significantly reduce atherosclerotic lesion development. In line with the role of Th1 immune responses, a deficiency of IFN-γ results in a 60% reduction of atherosclerosis[302], while IFN-γ injections result in a significant 2-fold increase of atherosclerotic lesions[322]. Additionally, as mentioned previously, IL-12 and IL-18, cytokines involved in Th1 generation, are also crucially involved in atherosclerosis[264–268]. These studies indicate that atherosclerosis is strongly driven by Th1 cells and the production of pro-inflammatory cytokines associated with their presence. Nonetheless, it will be interesting to establish to what extent the recently described IFN-γ producing $T\text{-}bet^+$ innate lymphoid cells contribute to atherosclerosis[323].

2.5.2.2 Th2 cells

Th2 cell numbers in atherosclerotic lesions is low, but gradually increases with disease progression[316]. When comparing atherosclerosis development in $LDLr^{-/-}$ mice on a C57BL/6 (Th1 dominated) versus BALB/c (Th2 dominated) background, it was found that atherosclerosis development was significantly increased in Th1 biased mice, indicating that Th2 cells may be atheroprotective or at least do not contribute to the

same extent as Th1 cells to atherosclerosis development[324]. Indeed, in humans it was found that Th2 cells were associated with a reduced risk of MI[325]. However, in mice the role of Th2 cells has not been perfectly elucidated. Our laboratory showed that Th2 cells might actually promote atherosclerosis progression, as interference of the OX40-OX40L pathway was associated with a reduced amount of Th2 cells and reduced atherosclerotic lesion progression[256]. Most studies have assessed the role of Th2 cells by studying the cytokines that induce Th2 cells (IL-4, IL-33) and the cytokines that Th2 cells produce themselves (IL-4, IL-5, IL-10 and IL-13). IL-4 induces GATA-binding protein 3 (GATA-3) which increases IL-4 expression of the T cells and inhibits IFN-γ[326].

IL-4 administration or deficiency was found to have no effect on early atherosclerosis in ApoE$^{-/-}$ mice[327]. While IL-4 deficiency in LDLr$^{-/-}$ mice was also found to have no effect on early lesions in the aortic arch[327], the same group showed later on that transplantation of bone marrow from IL-4$^{-/-}$ mice into LDLr$^{-/-}$ mice resulted in reduced lesions in the aortic arch and the thoracic aorta, while aortic root lesions were not affected[328]. This could be due to differences induced by the bone marrow transplantation, as aortic root lesions develop faster and thoracic aorta lesions develop slower after γ-irradiation[329]. This already indicates that the role of Th2 cells seems to critically depend on the site of lesion development, the stage of the lesions and the animal model used.

Two other cytokines that Th2 cells produce are IL-5 and IL-13. IL-5 has been shown to be anti-atherogenic by promoting the development of B-1 cells that produce protective IgM antibodies, resulting in reduced atherosclerosis[61]. Recently anti-IL-5 autoantibodies have been shown to be associated with human atherosclerosis, as antibody titers were much higher in patients with coronary and peripheral artery disease than in controls, and additionally correlated with well-known risk factors, such as plasma LDL-cholesterol[330]. It was found that IL-33 can specifically induce IL-5 production in CD4^{+} T cells[331,332] and that administration of IL-33 reduces atherosclerosis development in ApoE$^{-/-}$ mice[332]. In line with this, anti-OX40L treatment, which results in IL-5 producing T cells (via IL-33) and increased IgM levels results in reduced atherosclerotic lesion progression[256]. IL-13 has also been shown to reduce atherosclerotic lesion development by skewing macrophages towards an M2 phenotype[333].

The different effects of IL-4, IL-5 and IL-13 on atherosclerosis indicate the difficulty of dissecting the role of Th2 cells in atherosclerosis. This is further complicated by the fact that the studied cytokines are not solely produced by Th2 cells and also affect other cell types. Moreover, innate lymphoid cells have also been found to express Gata-3[334]. Overall, there is a necessity for specific markers and mouse models to establish the role of Th2 cells in atherosclerosis in more detail. The general consensus at the moment is however that Th2 cells are anti-atherogenic in early lesion development and pro-atherogenic in later stages of atherosclerosis.

2.5.2.3 Th17 cells

Th17 cells are found in both human[335] and murine[336] lesions. In mice, TGF-β and IL-6 results in the activation of signal transducer and activator of transcription (STAT)3 and subsequently retinoic acid-related orphan receptor γT (RORγT) and RORα, resulting in Th17 induction[326]. IL-21 is required for Th17 proliferation, while IL-23 is necessary for their maintenance[326]. In humans, IL-1β, IL-6 and IL-23 result in the induction of Th17 cells[326.] Moreover, also the combination of IL-21 and TGF-β was found to induce Th17 cells in humans[337]. Th17 cells produce IL-17A/F, IL-21 and IL-22. The effect of Th17 cells in atherosclerosis remains controversial. T cells isolated from human atherosclerotic coronary arteries were found to produce IFN-γ as well as IL-17 upon stimulation[338]. Moreover, Th17 cells were found to be increased in unstable angina patients[339] and increased IL-17 mRNA levels in unstable lesions were found[335]. While both these findings indicate a pro-atherogenic role for Th17 cells, circulating IL-17A levels showed no association with carotid intima-media thickness in humans[340]. As with Th2 cells, studies in mice have investigated the role of cytokines that are produced by Th17 cells. Blocking IL-17A in ApoE$^{-/-}$ mice was found to significantly reduce atherosclerosis[336]. In line with this, both IL-17A$^{-/-}$ApoE$^{-/-}$ mice and IL-17RA$^{-/-}$ApoE$^{-/-}$ mice were found to have reduced atherosclerosis[341]. However, other groups found that IL-17A deficiency in ApoE$^{-/-}$ mice had either no effect[340] or resulted in significantly more atherosclerosis[342]. Similar contradictory data exist for LDLr$^{-/-}$ mice. We have shown that transplantation of IL-17R$^{-/-}$ bone marrow into LDLr$^{-/-}$ mice resulted in significantly reduced atherosclerosis[343], indicating a pro-atherogenic role for Th17 cells. However, SOCS3$^{-/-}$LDLr$^{-/-}$ mice, which have increased STAT3-mediated expression of IL-17A in T cells, were found to have a significant 50% reduction in atherosclerosis[344]. Additionally, the same study showed that an IL-17A blocking antibody increased atherosclerosis[344], indicating a protective role for Th17 cells. Future studies will have to resolve this issue; again T cell specific knockout models should prove crucial to rule out the effect of other immune cells, such as innate lymphoid cells, NKT cells, NK cells and γδ T cells.

2.5.2.4 Regulatory T cells

Regulatory T cells (Tregs) are, as the name suggests, regulators of immune responses and their main function is thought to be the inhibition of self-reactive T cells in the periphery. Tregs that derive from the thymus are referred to as natural Tregs (FoxP3^{+}CD25^{hi}) and they produce IL-10 and TGF-β. The constitutive high expression of CD25 (the α-subunit of the IL-2 receptor) enables them to respond to physiologically low IL-2 levels. IL-2 is crucial for Treg generation and maintenance[345], and induces FoxP3, which controls key molecules expressed in Tregs, such as CD25, CTLA4, GITR and ICOS[346]. Genetic defects in FoxP3 are the cause of a severe autoimmune disease in humans, the X-linked autoimmune syndrome (IPEX)[347]. Scurfy mice and FoxP3-null mice, both lacking Tregs, as well as neonatal thymectomy were all found to result in spontaneous autoimmune manifestations[348,349]. Helios has recently been suggested to be a specific marker of natural Tregs[350]. However, at least in humans, it was found that natural Tregs do not all express Helios[351] and in mice Helios has been also found to be

expressed in Th2 cells[352]. Additionally neuropilin 1 has been suggested as a marker for natural Tregs in mice[353]. However, in humans neuropilin 1 was found to be less specific as it could also be detected on other T cells in secondary lymphoid organs and its expression could be induced in vitro upon activation of T cells[354]. Tregs can also be induced from naïve T cells, by ligation of the T cell receptor (TCR), CD28 ligation and/or TGF-β and IL-2, IL-10 or retinoic acid[326,355] and these are thus referred to as inducible Tregs (iTregs). These are further subdivided into Tr1 (mainly IL-10-producing and induced by IL-10) and Th3 cells (mainly TGF-β-producing and induced by TGF-β). iTregs also express CD25, but do not need FoxP3 expression to be functional[326]. So far, no exclusive markers for these cells exist making it difficult to dissect their specific roles in immune responses and atherosclerosis. Currently, the distinction between natural Tregs and iTregs can only be derived from the methylation profile of the FoxP3 promoter[356].

In addition to the distinction between natural Tregs and iTregs, Tregs can be divided into central Tregs (patrolling characteristics), effector Treg populations (enhanced function due to recent antigen encounter, present in lymphoid organs) and polarized tissue-resident Tregs (present in non-lymphoid organs)[355]. Whether these Treg populations derive from natural Tregs or iTregs is still unclear. Central Tregs can specialize into effector Tregs and specific tissue-resident Tregs. It appears that TCR ligation and IL-2 are needed for this differentiation and that the specific tissue microenvironment is pivotal as well[355]. Recent studies indicate that transcription factors specific for the differentiation of $CD4^+$ T cells result in distinct capacities in Tregs to inhibit these specific T helper subunits, thereby matching specific Tregs with their specific Th target cells. For example in response to IFN-γ, Tregs upregulate T-bet, which in turn induces the upregulation of CXCR3 enabling the migration of Tregs to sites of Th1-dominated inflammation[357]. Moreover, it was found that T-bet is essential in maintaining Treg function and homeostasis at the site of inflammation[357]. Interestingly, these $FoxP3^+IFN\text{-}\gamma^+$ Tregs were also found in atherosclerotic lesions of $ApoE^{-/-}$ mice[358]. STAT3 and RORγt have also been found to play a role in limiting Th17 responses and were shown to enhance the expression of IL-10 and IL-35[359,360]. Gata-3 however was found to be more generally needed for Treg function[361,362].

Tregs can suppress numerous immune cells, such as (naïve, effector, and memory) T cells, B cells, monocytes, macrophages, DCs, NK cells, and NKT cells. Treg production of IL-10 and TGF-β plays a crucial role in inhibiting immune responses, most likely through the inhibition of APC function and direct inhibition of T cell proliferation[346]. Their production of TGF-β can e.g. induce apoptosis in activated T cells[363]. Treg suppression requires cellular interactions and results in reduced proliferation of target T cells[346]. Addition of IL-2 can overcome Treg suppression, suggesting that limiting IL-2 is crucial for the T cell suppression[364]. As IL-2 is crucial for Treg maintenance and function, the current understanding is that Tregs sequester IL-2 produced by target T cells[355]. Another molecule that has been shown to be involved in the suppression of target T cells is CTLA-4. CTLA-4 can bind CD80 and CD86 on either APCs or target T cells and induce a suppressive signal in these cells or prevent binding of CD28 and activation via this molecule[365]. Tregs can also exert direct cytotoxicity by their

expression of granzymes, which can kill activated $CD4^+$ and $CD8^+$ T cells, monocytes, and B cells[366,367]. Tregs were also found to suppress CCL2 production, resulting in reduced recruitment of Ly-$6C^{hi}$ monocytes[368]. Tregs are capable of suppressing immune responses via multiple mechanisms and which mechanism is predominant likely depends on the microenvironment and the presence of antigens and APCs.

In atherosclerotic lesions, Tregs constitute about 1-5% of all T cells[326]. Interestingly, this is much lower than in other inflammatory diseases[326]. Indeed, low levels of Tregs are associated with increased risk for MI[369] and coronary syndromes[370]. During murine atherosclerosis it was found that Treg numbers significantly decrease with lesion progression[371,372]. Moreover, oxLDL was found to negatively affect the suppressive capacity of Tregs[371,372]. All of this indicates that the lack of Treg responses might be crucially involved in the progression of the disease and that restoration of these would beneficially affect atherosclerosis.

The beneficial role of Tregs in atherosclerosis has been extensively studied and is well-established (Figure 7). As mice with a deficiency of IL-2 or its receptor (CD25), or mice reconstituted with bone marrow from IL-2 or CD25-deficient mice die early from severe autoimmune diseases the effect of Tregs in atherosclerosis has been assessed by other models[258]. The co-stimulatory molecules CD80 and CD86 have been shown to be crucial for the development of Tregs. $LDLr^{-/-}$ mice reconstituted with $CD80^{-/-}CD86^{-/-}$ bone marrow showed a significant reduction of Tregs and increased atherosclerotic lesions[258]. Also specific depletion of CD25-positive cells increased

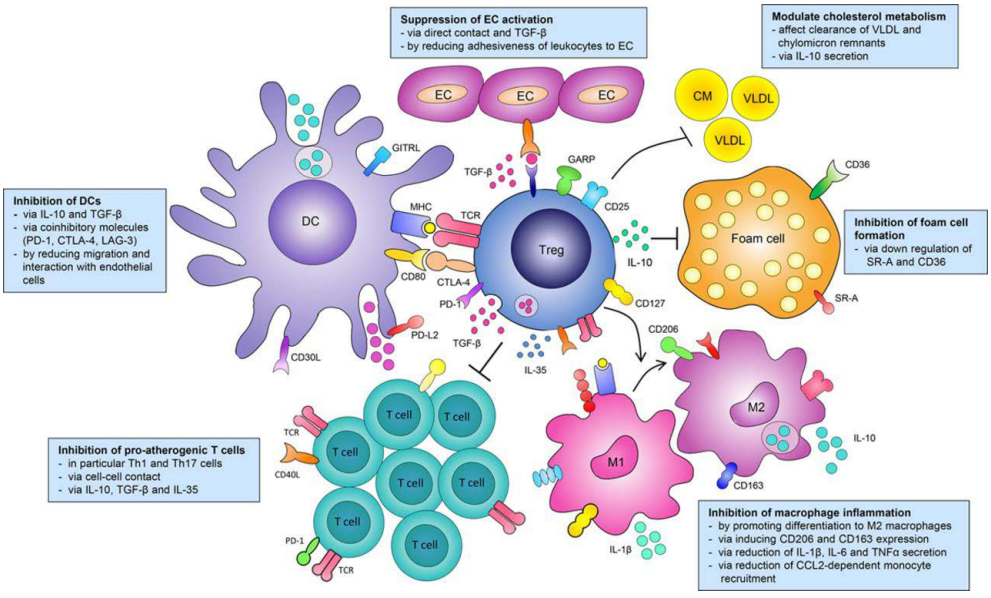


Figure 7. Regulatory T cells in atherosclerosis. Regulatory T cells can modulate several processes involved in the development of atherosclerosis. Tregs can inhibit proatherogenic T cells, DC activation and migration, macrophage inflammation, foam cell formation, endothelial cell (EC) activation and can affect cholesterol metabolism. Reproduced with permission from the Wolters Kluwer Health. Foks AC, Lichtman AH, Kuiper J. Treating atherosclerosis with regulatory T cells. *Arterioscler Thromb Vasc Biol.* 2015 Feb;35(2):280-7.

atherosclerotic lesion development in ApoE$^{-/-}$ mice[258]. Reconstitution of LDLr$^{-/-}$ mice with bone marrow from depletion of regulatory T cell (DEREG) mice (expressing DTR under the control of FoxP3) and depletion of Tregs by diphtheria toxin injection were found to significantly increase atherosclerotic lesions[373,374]. Interestingly also increased VLDL and plasma cholesterol levels were found in these mice indicating a link between Tregs and cholesterol homeostasis[374]. Our laboratory has shown that vaccination against FoxP3, resulting in the depletion of Tregs, significantly increases atherosclerotic lesion development[375]. The opposite approach to increase Tregs by adoptive transfer also dramatically decreased atherosclerosis[258,371,376]. Moreover, treatment of LDLr$^{-/-}$ mice with an IL-2/anti-IL-2 antibody complex, which induces Treg expansion, dramatically reduced lesion development[377]. It has recently been shown that the presence of CD103^{+} DCs[217] and MyD88^{+} DCs[368] is needed within aortas to maintain functional Tregs and that loss of these DCs results in increased atherosclerosis. All of these studies clearly demonstrate a protective role of Tregs. Additionally, a surfeit of studies that used approaches to induce/expand Tregs have all been shown to be able to reduce atherosclerosis, such as oral tolerance induction[378,379], anti-CD3 antibody treatment[380], and tolerogenic DC therapy[288]. Interestingly, statins were also shown to increase Tregs[373]. Overall, Treg expansion has been shown to be a feasible goal of any immune therapy to induce tolerance.

3. Outline of this Thesis

Recent years have clearly shown the crucial role of inflammatory immune responses in atherosclerotic lesion development. However, although our knowledge of how various immune cells contribute to the disease process has increased substantially, little progress has been made in finding an optimal and easily translatable immune cell-based therapy for atherosclerosis. In this book, we use several approaches to induce tolerance by targeting various immune cells, of which some resulted in effects on lipid metabolism (Figure 8).

In **chapter 2**, we treated LDLr$^{-/-}$ mice with oxLDL-induced apoptotic DCs and show that this is a novel therapy for both initial and advanced atherosclerosis. This treatment results in induction of tolerogenic DCs, enhanced Treg numbers and reduced inflammatory monocyte responses. Earlier studies have shown that inhibition of efferocytosis increases atherosclerosis, which demonstrates the crucial role of apoptotic cell clearance. As DCs within atherosclerotic lesions are impaired in their emigration from these lesions, this may contribute to the substantial lack of tolerance induction towards cleared antigens from apoptotic cells in atherosclerotic lesions. We show that our *intravenous* administration of oxLDL-loaded apoptotic DCs circumvents this by inducing tolerance in splenic DCs and consequently Tregs. Our study not only provides interesting aspects for research in the field of cardiovascular research (a potent easily translatable therapy for atherosclerosis and evidence for the vital importance of enhancing apoptotic cell clearance and DC emigration from atherosclerotic lesions), but also provides an interesting new concept that apoptotic cells may affect monocyte responses directly. The latter may be of crucial interest for

other (autoimmune) diseases that are at least partly mediated by monocytes, such as rheumatoid arthritis.

In **chapter 3**, we determined whether enhanced β-catenin signaling in DCs, shown to induce a tolerogenic phenotype, can reduce atherosclerosis. We generated CD11c-βcat^{EX3}/LDLr$^{-/-}$ bone marrow chimeras to assess the effect of overall stabilization of β-catenin in DCs and also adoptively transferred DCs from CD11c-βcat^{EX3} mice into LDLr$^{-/-}$ mice. CD11c-βcat^{EX3} mice have a CD11c-promoter-driven Cre recombinase and the exon 3 of the β-catenin gene floxed, which results in excision of the sequence encoding the ubiquitination site needed for degradation of β-catenin. We show that these DCs result in the induction of Tregs and reduce overall inflammation in mice, indicating that strategies to enhance β-catenin signaling in DCs are feasible to reduce atherosclerotic lesion development.

In **chapter 4**, we investigated the potential of an adoptive transfer of MSCs into LDLr$^{-/-}$ mice. These cells have been shown to have profound immunomodulatory capacities. As they can suppress functions of many immune cells involved in atherosclerosis, we assessed whether they were capable of inhibiting inflammatory responses in atherosclerosis. Indeed, we found reduced inflammation but interestingly also a significant reduction of VLDL synthesis and total serum cholesterol levels. Our study clearly demonstrates the potential of MSCs for the treatment of atherosclerosis.

In **chapter 5**, we show that TLR2-mediated signaling is not all bad in atherosclerosis. We use the bacterial cell wall of *S. aureus* to induce TLR2/PI3K-mediated IL-10 responses in peritoneal macrophages and shift the macrophage phenotype towards an M2b phenotype. These cells produce strong IL-10 responses and inhibit inflammation, resulting in reduced atherosclerosis. In this study we demonstrate that specific targeting of macrophages with components of the bacterial cell wall could prove a beneficial strategy to treat atherosclerosis.

In **chapter 6**, we administered an IL-2/anti-IL-2 complex to expand Tregs in LDLr$^{-/-}$ mice. The almost 10-fold increase of Tregs potently suppressed T cell responses and resulted in reduced initial atherosclerotic lesion development. Furthermore, in a regression model, our treatment resulted in significantly enhanced lesions stability.

In **chapter 7**, we determine whether proteasomal inhibition by Bortezomib, an FDA-approved proteasome inhibitor for multiple myeloma, can reduce immune responses in atherosclerosis. Indeed, we observed reduced inflammation in these mice. Interestingly, we also observed significant effects on cholesterol metabolism, with a dramatic decrease in total cholesterol levels. This resulted from a dramatic reduction of hepatic VLDL production and increased hepatic lipoprotein clearance. Bortezomib is already FDA-approved, which should make the translation of our findings to the clinic more straightforward.

Overall we provide strategies to decrease inflammatory monocyte responses, enhance anti-inflammatory cytokine production by macrophages, increase the presence of tolerogenic DCs and increase the amount of Tregs. Moreover, we show that adoptive transfer of tolerogenic DCs and MSCs may also prove beneficial in treating atherosclerosis. Interestingly, while our strategies aimed to modulate immune responses in atherosclerosis, we observe that MSC therapy and proteasomal inhibition

dramatically affect VLDL metabolism. This as well as other conclusions that can be drawn from our studies for future immune therapies will be discussed in **Chapter 8**.

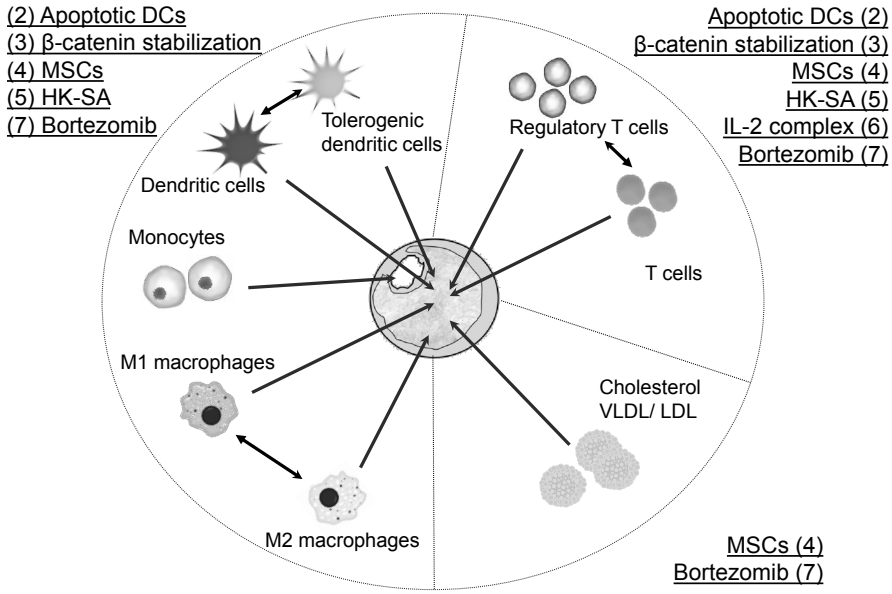


Figure 8. Different experimental approaches used in this thesis affect different disease processes. We used several treatment options to modulate innate immunity in LDLr$^{-/-}$ mice, resulting in tolerogenic DCs (apoptotic DCs, MSCs, β-catenin stabilization), M2 macrophages (HK-SA), and reduced circulating monocytes (apoptotic DCs, MSCs, HK-SA, Bortezomib). Furthermore we reduced T cell responses and induced Tregs (apoptotic DCs, MSCs, β-catenin stabilization, HK-SA, Bortezomib, IL-2/ anti-IL-2 complex). Some of our treatments also affected cholesterol metabolism (MSCs, Bortezomib).

References

1. Go, A. S. et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* 129, e28–e292 (2014).
2. Conen, D. Alcohol consumption and incident cardiovascular disease: not just one unifying hypothesis. *Eur. Heart J.* (2015). doi:10.1093/eurheartj/ehv021
3. Legein, B., Temmerman, L., Biessen, E. A. L. & Lutgens, E. Inflammation and immune system interactions in atherosclerosis. *Cell. Mol. Life Sci.* 70, 3847–69 (2013).
4. Nicholas, M., Townsend, N., Scarborough, P. & Rayner, M. Cardiovascular disease in Europe 2014: epidemiological update. *Eur. Heart J.* (2014). doi:10.1093/eurheartj/ehu489
5. Go, A. S. et al. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation* 127, e6–e245 (2013).
6. Smolina, K., Wright, F. L., Rayner, M. & Goldacre, M. J. Determinants of the decline in mortality from acute myocardial infarction in England between 2002 and 2010: linked national database study. *BMJ* 344, d8059 (2012).
7. Finucane, M. M. et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9·1 million participants. *Lancet* 377, 557–67 (2011).
8. Danaei, G. et al. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *Lancet* 378, 31–40 (2011).
9. Finegold, J. A., Asaria, P. & Francis, D. P. Mortality from ischaemic heart disease by country, region, and age: statistics from World Health Organisation and United Nations. *Int. J. Cardiol.* 168, 934–45 (2013).

10. Leal, J., Luengo-Fernández, R., Gray, A., Petersen, S. & Rayner, M. Economic burden of cardiovascular diseases in the enlarged European Union. *Eur. Heart* J. 27, 1610–9 (2006).
11. Keele, K. D. Leonardo da Vinci's views on arteriosclerosis. *Med. Hist.* 17, 304–8 (1973).
12. Konstantinov, I. E., Mejevoi, N. & Anichkov, N. M. Nikolai N. Anichkov and his theory of atherosclerosis. *Tex. Heart Inst.* J. 33, 417–23 (2006).
13. Mayerl, C. *et al.* Atherosclerosis research from past to present--on the track of two pathologists with opposing views, Carl von Rokitansky and Rudolf Virchow. *Virchows Arch.* 449, 96–103 (2006).
14. Hodgson, J. A Treatise on the Diseases of Arteries and Veins, Containing the Pathology and Treatment of Aneurysms and Wounded Arteries. *London: Thomas Underwood* (1811).
15. Hope, J. Von den Krankheiten des Herzens und der grossen Gefässe. Berlin: Enslin (1833).
16. Marchand, F. Über Arteriosklerose. Verhandlungen des Kongresses für Innere Medizin. 21. Kongress, Leipzig 23–59 (1904).
17. Stary, H. C. Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. *Am. J. Clin. Nutr.* 72, 1297S–1306S (2000).
18. Gonçalves, I. et al. Short communication: Dating components of human atherosclerotic plaques. *Circ. Res.* 106, 1174–7 (2010).
19. Zhou, J., Li, Y.-S. & Chien, S. Shear stress-initiated signaling and its regulation of endothelial function. *Arterioscler. Thromb. Vasc. Biol.* 34, 2191–8 (2014).
20. Skålén, K. et al. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature* 417, 750–4 (2002).
21. Li, A. C. & Glass, C. K. The macrophage foam cell as a target for therapeutic intervention. *Nat. Med.* 8, 1235–42 (2002).
22. Hansson, G. K. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 352, 1685–95 (2005).
23. Soehnlein, O. *et al.* Distinct functions of chemokine receptor axes in the atherogenic mobilization and recruitment of classical monocytes. *EMBO Mol. Med.* 5, 471–81 (2013).
24. Tacke, F. et al. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* 117, 185–94 (2007).
25. Lichtman, A. H., Binder, C. J., Tsimikas, S. & Witztum, J. L. Adaptive immunity in atherogenesis: new insights and therapeutic approaches. *J. Clin. Invest.* 123, 27–36 (2013).
26. Moore, K. J., Sheedy, F. J. & Fisher, E. A. Macrophages in atherosclerosis: a dynamic balance. *Nat. Rev. Immunol.* 13, 709–21 (2013).
27. De Villiers, W. J., Fraser, I. P., Hughes, D. A., Doyle, A. G. & Gordon, S. Macrophage-colony-stimulating factor selectively enhances macrophage scavenger receptor expression and function. *J. Exp. Med.* 180, 705–9 (1994).
28. Tall, A. R., Yvan-Charvet, L., Terasaka, N., Pagler, T. & Wang, N. HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. Cell Metab. 7, 365–75 (2008).
29. Ross, R. Atherosclerosis--an inflammatory disease. *N. Engl. J. Med.* 340, 115–26 (1999).
30. Bobryshev, Y. V. Dendritic cells and their role in atherogenesis. *Lab. Invest.* 90, 970–84 (2010).
31. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–69 (2008).
32. Eriksson, E. E. Intravital microscopy on atherosclerosis in apolipoprotein e-deficient mice establishes microvessels as major entry pathways for leukocytes to advanced lesions. *Circulation* 124, 2129–38 (2011).
33. Campbell, K. A. et al. Lymphocytes and the adventitial immune response in atherosclerosis. *Circ. Res.* 110, 889–900 (2012).
34. Nilsson, J. & Hansson, G. K. Autoimmunity in atherosclerosis: a protective response losing control? *J. Intern. Med.* 263, 464–78 (2008).
35. Allahverdian, S., Pannu, P. S. & Francis, G. A. Contribution of monocyte-derived macrophages and smooth muscle cells to arterial foam cell formation. *Cardiovasc. Res.* 95, 165–72 (2012).
36. Shah, P. K. Molecular mechanisms of plaque instability. *Curr. Opin. Lipidol.* 18, 492–9 (2007).
37. Newby, A. C. et al. Vulnerable atherosclerotic plaque metalloproteinases and foam cell phenotypes. *Thromb. Haemost.* 101, 1006–11 (2009).
38. Arbustini, E. et al. Comparison of coronary lesions obtained by directional coronary atherectomy in unstable angina, stable angina, and restenosis after either atherectomy or angioplasty. *Am. J. Cardiol.* 75, 675–82 (1995).
39. De Feyter, P. J. et al. Ischemia-related lesion characteristics in patients with stable or unstable angina. A study with intracoronary angioscopy and ultrasound. *Circulation* 92, 1408–13 (1995).
40. Moreno, P. R. et al. Macrophages, smooth muscle cells, and tissue factor in unstable angina. Implications for cell-mediated thrombogenicity in acute coronary syndromes. *Circulation* 94, 3090–7 (1996).
41. Schoenhagen, P. et al. Extent and direction of arterial remodeling in stable versus unstable

coronary syndromes : an intravascular ultrasound study. *Circulation* 101, 598–603 (2000).
42. Van Lammeren, G. W. et al. Time-dependent changes in atherosclerotic plaque composition in patients undergoing carotid surgery. *Circulation* 129, 2269–76 (2014).
43. Wang, X., Wolf, M. P., Keel, R. B., Lehner, R. & Hunziker, P. R. Polydimethylsiloxane embedded mouse aorta ex vivo perfusion model: proof-of-concept study focusing on atherosclerosis. *J. Biomed. Opt.* 17, 076006 (2012).
44. Silver, M. J., Sedar, A. W., Nissenbaum, M., Ingerman, C. & Smith, J. B. A model system for studying initial events in atherosclerosis. *Artery* 8, 80–4 (1980).
45. Monaco, C. et al. Toll-like receptor-2 mediates inflammation and matrix degradation in human atherosclerosis. *Circulation* 120, 2462–9 (2009).
46. Kakisis, J. D., Liapis, C. D., Breuer, C. & Sumpio, B. E. Artificial blood vessel: the Holy Grail of peripheral vascular surgery. *J. Vasc. Surg.* 41, 349–54 (2005).
47. Hoch, E., Tovar, G. E. M. & Borchers, K. Bioprinting of artificial blood vessels: current approaches towards a demanding goal. *Eur. J. Cardiothorac. Surg.* (2014). doi:10.1093/ejcts/ezu242
48. Stemerman, M. B. & Ross, R. Experimental arteriosclerosis. I. Fibrous plaque formation in primates, an electron microscope study. *J. Exp. Med*. 136, 769–89 (1972).
49. Luginbühl, H., Pauli, B. & Ratcliffe, H. L. Atherosclerosis in swine and swine as a model for the study of atherosclerosis. *Adv. Cardiol.* 13, 119–26 (1974).
50. Shore, B. & Shore, V. Rabbits as a model for the study of hyperlipoproteinemia and atherosclerosis. *Adv. Exp. Med. Biol.* 67, 123–41 (1976).
51. Koletsky, S. Obese spontaneously hypertensive rats--a model for study of atherosclerosis. *Exp. Mol. Pathol.* 19, 53–60 (1973).
52. Paigen, B., Ishida, B. Y., Verstuyft, J., Winters, R. B. & Albee, D. Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. *Arteriosclerosis* 10, 316–23
53. Véniant, M. M., Withycombe, S. & Young, S. G. Lipoprotein size and atherosclerosis susceptibility in Apoe(-/-) and Ldlr(-/-) mice. *Arterioscler. Thromb. Vasc. Biol.* 21, 1567–70 (2001).
54. Zhang, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258, 468–71 (1992).
55. Plump, A. S. et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71, 343–53 (1992).
56. Pendse, A. A., Arbones-Mainar, J. M., Johnson, L. A., Altenburg, M. K. & Maeda, N. Apolipoprotein E knock-out and knock-in mice: atherosclerosis, metabolic syndrome, and beyond. *J. Lipid Res.* 50 Suppl, S178–82 (2009).
57. Murphy, A. J. et al. ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. *J. Clin. Invest.* 121, 4138–49 (2011).
58. Ishibashi, S. et al. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* 92, 883–93 (1993).
59. Poston, R. N. & Davies, D. F. Immunity and inflammation in the pathogenesis of atherosclerosis. A review. Atherosclerosis 19, 353–67
60. Libby, P. Inflammation in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 32, 2045–51 (2012).
61. Tsiantoulas, D., Diehl, C. J., Witztum, J. L. & Binder, C. J. B cells and humoral immunity in atherosclerosis. *Circ. Res.* 114, 1743–56 (2014).
62. Bot, I., Shi, G.-P. & Kovanen, P. T. Mast Cells as Effectors in Atherosclerosis. Arterioscler. Thromb. Vasc. Biol. (2014). doi:10.1161/ATVBAHA.114.303570
63. Doring, Y., Drechsler, M., Soehnlein, O. & Weber, C. Neutrophils in Atherosclerosis: From Mice to Man. *Arterioscler. Thromb. Vasc. Biol.* (2014). doi:10.1161/ATVBAHA.114.303564
64. Niccoli, G. & Cosentino, N. Eosinophils: a new player in coronary atherosclerotic disease. *Hypertens. Res. 3*5, 269–71 (2012).
65. Bondarenko, S., Catapano, A. L. & Norata, G. D. The CD1d-natural killer T cell axis in atherosclerosis. *J. Innate Immun.* 6, 3–12 (2014).
66. Selathurai, A. et al. Natural killer (NK) cells augment atherosclerosis by cytotoxic-dependent mechanisms. *Cardiovasc. Res.* 102, 128–37 (2014).
67. Cheng, H.-Y., Wu, R. & Hedrick, C. C. Gammadelta (γδ) T lymphocytes do not impact the development of early atherosclerosis. *Atherosclerosis* 234, 265–9 (2014).
68. Vu, D. M. et al. γδT Cells Are Prevalent in the Proximal Aorta and Drive Nascent Atherosclerotic Lesion Progression and Neutrophilia in Hypercholesterolemic Mice. *PLoS One* 9, e109416 (2014).
69. Elhage, R. *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.* 165, 2013–8 (2004).
70. Gabrilovich, D. I. & Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9, 162–74 (2009).
71. He, S., Nakada, D. & Morrison, S. J. Mechanisms of stem cell self-renewal. *Annu. Rev. Cell Dev. Biol.* 25, 377–406 (2009).

72. Bolli, R. *et al.* Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. *Lancet* 378, 1847–57 (2011).
73. Makkar, R. R. *et al.* Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *Lancet* 379, 895–904 (2012).
74. Yvan-Charvet, L. *et al.* ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation. *Science* 328, 1689–93 (2010).
75. Heidt, T. *et al.* Chronic variable stress activates hematopoietic stem cells. *Nat. Med.* 20, 754–8 (2014).
76. Leuschner, F. et al. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *J. Exp. Med.* 209, 123–37 (2012).
77. Dutta, P. *et al.* Myocardial infarction accelerates atherosclerosis. *Nature* 487, 325–9 (2012).
78. Bernardo, M. E. & Fibbe, W. E. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 13, 392–402 (2013).
79. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–7 (1999).
80. Uccelli, A., Moretta, L. & Pistoia, V. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 8, 726–36 (2008).
81. Wen, Z., Zheng, S., Zhou, C., Wang, J. & Wang, T. Repair mechanisms of bone marrow mesenchymal stem cells in myocardial infarction. *J. Cell. Mol. Med.* 15, 1032–43 (2011).
82. Sivanathan, K. N., Gronthos, S., Rojas-Canales, D., Thierry, B. & Coates, P. T. Interferon-gamma modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. *Stem Cell Rev.* 10, 351–75 (2014).
83. Bartholomew, A. *et al.* Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp. Hematol.* 30, 42–8 (2002).
84. Reinders, M. E. J. *et al.* Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. S*tem Cells Transl. Med.* 2, 107–11 (2013).
85. Zappia, E. *et al.* Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106, 1755–61 (2005).
86. Augello, A., Tasso, R., Negrini, S. M., Cancedda, R. & Pennesi, G. Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum. 5*6, 1175–86 (2007).
87. Fan, H. *et al.* Pre-treatment with IL-1β enhances the efficacy of MSC transplantation in DSS-induced colitis. *Cell. Mol. Immunol.* 9, 473–81 (2012).
88. Le Blanc, K. *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 371, 1579–86 (2008).
89. Swirski, F. K. *et al.* Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease. *Proc. Natl. Acad. Sci. U. S. A.* 103, 10340–5 (2006).
90. Swirski, F. K. *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J. Clin. Invest.* 117, 195–205 (2007).
91. Yang, J., Zhang, L., Yu, C., Yang, X.-F. & Wang, H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark. Res.* 2, 1 (2014).
92. Ley, K., Miller, Y. I. & Hedrick, C. C. Monocyte and macrophage dynamics during atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 31, 1506–16 (2011).
93. Serbina, N. V & Pamer, E. G. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 7, 311–7 (2006).
94. Tsou, C.-L. *et al.* Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest.* 117, 902–9 (2007).
95. Fogg, D. K. et al. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311, 83–7 (2006).
96. Robbins, C. S. *et al.* Extramedullary hematopoiesis generates Ly-6C(high) monocytes that infiltrate atherosclerotic lesions. *Circulation* 125, 364–74 (2012).
97. Weber, C., Zernecke, A. & Libby, P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat. Rev. Immunol.* 8, 802–15 (2008).
98. Geissmann, F., Jung, S. & Littman, D. R. Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties. *Immunity* 19, 71–82 (2003).
99. Li, J. & Ley, K. Lymphocyte Migration into Atherosclerotic Plaque. *Arterioscler. Thromb. Vasc. Biol.* (2014).
100. Geissmann, F. *et al.* Development of monocytes, macrophages, and dendritic cells. *Science* 327, 656–61 (2010).
101. Gosling, J. *et al.* MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress

human apolipoprotein B. J. *Clin. Invest.* 103, 773–8 (1999).
102. Boring, L., Gosling, J., Cleary, M. & Charo, I. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 394, 894–897 (1998).
103. Guo, J. *et al.* Repopulation of apolipoprotein E knockout mice with CCR2-deficient bone marrow progenitor cells does not inhibit ongoing atherosclerotic lesion development. *Arterioscler. Thromb. Vasc. Biol.* 25, 1014–9 (2005).
104. Robbins, C. S. *et al.* Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat. Med.* 19, 1166–72 (2013).
105. Brühl, H. *et al.* Dual role of CCR2 during initiation and progression of collagen-induced arthritis: evidence for regulatory activity of CCR2+ T cells. *J. Immunol.* 172, 890–8 (2004).
106. Leuschner, F. *et al.* Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat. Biotechnol.* 29, 1005–10 (2011).
107. Ni, W. *et al.* New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 103, 2096–101 (2001).
108. Inoue, S. *et al.* Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 106, 2700–6 (2002).
109. De Lemos, J. A. *et al.* Serial measurement of monocyte chemoattractant protein-1 after acute coronary syndromes: results from the A to Z trial. J. Am. Coll. Cardiol. 50, 2117–24 (2007).
110. Van Wijk, D. F. et al. Chemokine ligand 2 genetic variants, serum monocyte chemoattractant protein-1 levels, and the risk of coronary artery disease. Arterioscler. Thromb. Vasc. Biol. 30, 1460–6 (2010).
111. Gilbert, J. et al. Effect of CC chemokine receptor 2 CCR2 blockade on serum C-reactive protein in individuals at atherosclerotic risk and with a single nucleotide polymorphism of the monocyte chemoattractant protein-1 promoter region. *Am. J. Cardiol.* 107, 906–11 (2011).
112. Schulz, C. et al. Chemokine fractalkine mediates leukocyte recruitment to inflammatory endothelial cells in flowing whole blood: a critical role for P-selectin expressed on activated platelets. *Circulation* 116, 764–73 (2007).
113. Teupser, D. *et al.* Major reduction of atherosclerosis in fractalkine (CX3CL1)-deficient mice is at the brachiocephalic artery, not the aortic root. *Proc. Natl. Acad. Sci. U. S. A.* 101, 17795–800 (2004).
114. Combadière, C. *et al.* Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. *Circulation* 107, 1009–16 (2003).
115. Lesnik, P., Haskell, C. A. & Charo, I. F. Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis. *J. Clin. Invest.* 111, 333–40 (2003).
116. Moatti, D. et al. Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. *Blood* 97, 1925–8 (2001).
117. McDermott, D. H. *et al.* Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. *J. Clin. Invest.* 111, 1241–50 (2003).
118. Lavergne, E. et al. Adverse associations between CX3CR1 polymorphisms and risk of cardiovascular or cerebrovascular disease. *Arterioscler. Thromb. Vasc. Biol.* 25, 847–53 (2005).
119. Niessner, A. et al. Opposite effects of CX3CR1 receptor polymorphisms V249I and T280M on the development of acute coronary syndrome. A possible implication of fractalkine in inflammatory activation. *Thromb. Haemost.* 93, 949–54 (2005).
120. Saederup, N., Chan, L., Lira, S. A. & Charo, I. F. Fractalkine deficiency markedly reduces macrophage accumulation and atherosclerotic lesion formation in CCR2-/- mice: evidence for independent chemokine functions in atherogenesis. *Circulation* 117, 1642–8 (2008).
121. Barlic, J., Zhang, Y., Foley, J. F. & Murphy, P. M. Oxidized lipid-driven chemokine receptor switch, CCR2 to CX3CR1, mediates adhesion of human macrophages to coronary artery smooth muscle cells through a peroxisome proliferator-activated receptor gamma-dependent pathway. *Circulation* 114, 807–19 (2006).
122. Herder, C. *et al.* RANTES/CCL5 and risk for coronary events: results from the MONICA/KORA Augsburg case-cohort, Athero-Express and CARDIoGRAM studies. *PLoS One* 6, e25734 (2011).
123. Weber, C., Schober, A. & Zernecke, A. Chemokines: key regulators of mononuclear cell recruitment in atherosclerotic vascular disease. Arterioscler. Thromb. Vasc. Biol. 24, 1997–2008 (2004).
124. Weber, C. *et al.* Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-like/CD45RO(+) T cells. Blood 97, 1144–6 (2001).
125. Braunersreuther, V. *et al.* Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arterioscler. Thromb. Vasc. Biol.* 27, 373–9 (2007).
126. Potteaux, S. *et al.* Role of bone marrow-derived CC-chemokine receptor 5 in the development of atherosclerosis of low-density lipoprotein receptor knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 26, 1858–63 (2006).

127. Kuziel, W. A. et al. CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice. *Atherosclerosis* 167, 25–32 (2003).
128. Van Wanrooij, E. J. A. *et al.* HIV entry inhibitor TAK-779 attenuates atherogenesis in low-density lipoprotein receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 25, 2642–7 (2005).
129. Combadière, C. *et al.* Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation* 117, 1649–57 (2008).
130. Soehnlein, O. Multiple roles for neutrophils in atherosclerosis. *Circ. Res.* 110, 875–88 (2012).
131. Gerszten, R. E. *et al.* MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* 398, 718–23 (1999).
132. Boisvert, W. A., Santiago, R., Curtiss, L. K. & Terkeltaub, R. A. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J. Clin. Invest.* 101, 353–63 (1998).
133. Huo, Y. et al. The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J. Clin. Invest.* 108, 1307–14 (2001).
134. Llodrá, J. *et al.* Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc. Natl. Acad. Sci. U. S. A.* 101, 11779–84 (2004).
135. Trogan, E. *et al.* Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 103, 3781–6 (2006).
136. Watanabe, T., Hirata, M., Yoshikawa, Y., Nagafuchi, Y. & Toyoshima, H. Role of macrophages in atherosclerosis. Sequential observations of cholesterol-induced rabbit aortic lesion by the immunoperoxidase technique using monoclonal antimacrophage antibody. *Lab. Invest.* 53, 80–90 (1985).
137. Gown, A. M., Tsukada, T. & Ross, R. Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am. J. Pathol.* 125, 191–207 (1986).
138. Smith, J. D. *et al.* Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc. Natl. Acad. Sci. U. S. A.* 92, 8264–8 (1995).
139. Danenberg, H. D. *et al.* Macrophage depletion by clodronate-containing liposomes reduces neointimal formation after balloon injury in rats and rabbits. *Circulation* 106, 599–605 (2002).
140. Stoneman, V. *et al.* Monocyte/macrophage suppression in CD11b diphtheria toxin receptor transgenic mice differentially affects atherogenesis and established plaques. *Circ. Res.* 100, 884–93 (2007).
141. Secchiero, P. *et al.* Systemic tumor necrosis factor-related apoptosis-inducing ligand delivery shows antiatherosclerotic activity in apolipoprotein E-null diabetic mice. *Circulation* 114, 1522–30 (2006).
142. Suzuki, H. *et al.* A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* 386, 292–6 (1997).
143. Endemann, G. *et al.* CD36 is a receptor for oxidized low density lipoprotein. J. Biol. Chem. 268, 11811–6 (1993).
144. Kunjathoor, V. *et al*. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J. Biol. Chem.* 277, 49982–8 (2002).
145. Moore, K. J. & Tabas, I. Macrophages in the pathogenesis of atherosclerosis. *Cell* 145, 341–55 (2011).
146. Kruth, H. S. Receptor-independent fluid-phase pinocytosis mechanisms for induction of foam cell formation with native low-density lipoprotein particles. *Curr. Opin. Lipidol.* 22, 386–93 (2011).
147. Freigang, S. *et al.* Nrf2 is essential for cholesterol crystal-induced inflammasome activation and exacerbation of atherosclerosis. *Eur. J. Immunol.* 41, 2040–51 (2011).
148. Duewell, P. *et al.* NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464, 1357–61 (2010).
149. Brown, M. S. & Goldstein, J. L. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. U. S. A.* 96, 11041–8 (1999).
150. Boden, W. E. High-density lipoprotein cholesterol as an independent risk factor in cardiovascular disease: assessing the data from Framingham to the Veterans Affairs High--Density Lipoprotein Intervention Trial. *Am. J. Cardiol.* 86, 19L–22L (2000).
151. Yvan-Charvet, L. *et al.* Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. *J. Clin. Invest.* 117, 3900–8 (2007).
152. Out, R. *et al.* Combined deletion of macrophage ABCA1 and ABCG1 leads to massive lipid accumulation in tissue macrophages and distinct atherosclerosis at relatively low plasma cholesterol levels. *Arterioscler. Thromb. Vasc. Biol.* 28, 258–64 (2008).
153. Lammers, B. *et al.* Independent protective roles for macrophage Abcg1 and Apoe in the atherosclerotic lesion development. *Atherosclerosis* 205, 420–6 (2009).

154. Out, R. *et al.* Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 26, 2295–300 (2006).
155. Ranalletta, M. *et al.* Decreased atherosclerosis in low-density lipoprotein receptor knockout mice transplanted with Abcg1-/- bone marrow. *Arterioscler. Thromb. Vasc. Biol.* 26, 2308–15 (2006).
156. Baldán, A. *et al.* Impaired development of atherosclerosis in hyperlipidemic Ldlr-/- and ApoE-/- mice transplanted with Abcg1-/- bone marrow. *Arterioscler. Thromb. Vasc. Biol.* 26, 2301–7 (2006).
157. Meurs, I. *et al.* The effect of ABCG1 deficiency on atherosclerotic lesion development in LDL receptor knockout mice depends on the stage of atherogenesis. *Atherosclerosis* 221, 41–7 (2012).
158. Albert, M. L. Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nat. Rev. Immunol.* 4, 223–31 (2004).
159. Hoffmann, P. R. *et al.* Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J. Cell Biol.* 155, 649–59 (2001).
160. Schrijvers, D. M., De Meyer, G. R. Y., Kockx, M. M., Herman, A. G. & Martinet, W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 25, 1256–61 (2005).
161. Subramanian, M. & Tabas, I. Dendritic cells in atherosclerosis. *Semin. Immunopathol.* 36, 93–102 (2014).
162. Thorp, E. & Tabas, I. Mechanisms and consequences of efferocytosis in advanced atherosclerosis. *J. Leukoc. Biol.* 86, 1089–95 (2009).
163. Spann, N. J. *et al.* Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell* 151, 138–52 (2012).
164. Goldberg, I. J. & Merkel, M. Lipoprotein lipase: physiology, biochemistry, and molecular biology. *Front. Biosci.* 6, D388–405 (2001).
165. Yagyu, H. *et al.* Overexpressed lipoprotein lipase protects against atherosclerosis in apolipoprotein E knockout mice. *J. Lipid Res.* 40, 1677–85 (1999).
166. Babaev, V. R. *et al.* Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J. Clin. Invest.* 103, 1697–705 (1999).
167. Miller, Y. I. *et al.* Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. *J. Biol. Chem.* 278, 1561–8 (2003).
168. Xu, X. H. et al. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 104, 3103–8 (2001).
169. Melián, A., Geng, Y. J., Sukhova, G. K., Libby, P. & Porcelli, S. A. CD1 expression in human atherosclerosis. A potential mechanism for T cell activation by foam cells. *Am. J. Pathol.* 155, 775–86 (1999).
170. Braun, N. A., Covarrubias, R. & Major, A. S. Natural killer T cells and atherosclerosis: form and function meet pathogenesis. *J. Innate Immun.* 2, 316–24 (2010).
171. Kadl, A. *et al.* Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circ. Res.* 107, 737–46 (2010).
172. Khallou-Laschet, J. et al. Macrophage plasticity in experimental atherosclerosis. *PLoS One* 5, e8852 (2010).
173. Mylonas, K. J., Nair, M. G., Prieto-Lafuente, L., Paape, D. & Allen, J. E. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *J. Immunol.* 182, 3084–94 (2009).
174. Stout, R. D. *et al.* Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J. Immunol.* 175, 342–9 (2005).
175. Butcher, M. J. & Galkina, E. V. Phenotypic and functional heterogeneity of macrophages and dendritic cell subsets in the healthy and atherosclerosis-prone aorta. *Front. Physiol.* 3, 44 (2012).
176. Murray, P. J. & Wynn, T. A. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* 11, 723–37 (2011).
177. Bouhlel, M. A. *et al.* PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab.* 6, 137–43 (2007).
178. Wolfs, I. M. J., Donners, M. M. P. C. & de Winther, M. P. J. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. *Thromb. Haemost.* 106, 763–71 (2011).
179. Stein, M., Keshav, S., Harris, N. & Gordon, S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176, 287–92 (1992).
180. Gordon, S. & Martinez, F. O. Alternative activation of macrophages: mechanism and functions. *Immunity* 32, 593–604 (2010).
181. Chinetti-Gbaguidi, G. *et al.* Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARγ and LXRα

pathways. *Circ. Res.* 108, 985–95 (2011).
182. Boyle, J. J. *et al.* Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. *Am. J. Pathol.* 174, 1097–108 (2009).
183. Ley, K., Laudanna, C., Cybulsky, M. I. & Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7, 678–89 (2007).
184. Sachais, B. S. *et al.* Elimination of platelet factor 4 (PF4) from platelets reduces atherosclerosis in C57Bl/6 and apoE-/- mice. *Thromb. Haemost.* 98, 1108–13 (2007).
185. Gleissner, C. A. *et al.* CXCL4 downregulates the atheroprotective hemoglobin receptor CD163 in human macrophages. *Circ. Res.* 106, 203–11 (2010).
186. Gleissner, C. A., Shaked, I., Little, K. M. & Ley, K. CXC chemokine ligand 4 induces a unique transcriptome in monocyte-derived macrophages. *J. Immunol.* 184, 4810–8 (2010).
187. Hutchinson, J. A., Riquelme, P., Geissler, E. K. & Fändrich, F. Human regulatory macrophages. *Methods Mol. Biol.* 677, 181–92 (2011).
188. Riquelme, P. *et al.* IFN-γ-induced iNOS expression in mouse regulatory macrophages prolongs allograft survival in fully immunocompetent recipients. *Mol. Ther.* 21, 409–22 (2013).
189. Geissler, E. K. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplant. Res.* 1, 11 (2012).
190. Hutchinson, J. A. *et al.* Cutting Edge: Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. *J. Immunol.* 187, 2072–8 (2011).
191. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23, 549–55 (2002).
192. Liu, Y. & Cao, X. The origin and function of tumor-associated macrophages. *Cell. Mol. Immunol. (*2014). doi:10.1038/cmi.2014.83
193. Murray, P. J. et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* 41, 14–20 (2014).
194. Steinman, R. M. Dendritic cells: understanding immunogenicity. *Eur. J. Immunol.* 37 Suppl 1, S53–60 (2007).
195. Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C. & Amigorena, S. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* 20, 621–67 (2002).
196. Lewis, K. L. & Reizis, B. Dendritic cells: arbiters of immunity and immunological tolerance. *Cold Spring Harb. Perspect. Biol.* 4, a007401 (2012).
197. Turley, S. J., Fletcher, A. L. & Elpek, K. G. The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs. *Nat. Rev. Immunol.* 10, 813–25 (2010).
198. Den Haan, J. M. M., Lehar, S. M. & Bevan, M. J. Cd8+ but Not Cd8- Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo. *J. Exp. Med.* 192, 1685–1696 (2000).
199. Chistiakov, D. A., Sobenin, I. A., Orekhov, A. N. & Bobryshev, Y. V. Dendritic cells in atherosclerotic inflammation: the complexity of functions and the peculiarities of pathophysiological effects. *Front. Physiol.* 5, 196 (2014).
200. Del Rio, M.-L., Bernhardt, G., Rodriguez-Barbosa, J.-I. & Förster, R. Development and functional specialization of CD103+ dendritic cells. *Immunol. Rev.* 234, 268–81 (2010).
201. Satpathy, A. T. *et al.* Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *J. Exp. Med.* 209, 1135–52 (2012).
202. Meredith, M. M. *et al.* Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J. Exp. Med.* 209, 1153–65 (2012).
203. Biessen, E. A. L. & Christ, A. Plasmacytoid Dendritic Cells in Atherosclerosis: Knocking at T-Cell's Door. *Circulation* 130, 1340–2 (2014).
204. Millonig, G. *et al.* Network of vascular-associated dendritic cells in intima of healthy young individuals. *Arterioscler. Thromb. Vasc. Biol.* 21, 503–8 (2001).
205. Jongstra-Bilen, J. *et al.* Low-grade chronic inflammation in regions of the normal mouse arterial intima predisposed to atherosclerosis. *J. Exp. Med.* 203, 2073–83 (2006).
206. Galkina, E. *et al.* Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J. Exp. Med.* 203, 1273–82 (2006).
207. Bobryshev, Y. V & Lord, R. S. Ultrastructural recognition of cells with dendritic cell morphology in human aortic intima. Contacting interactions of Vascular Dendritic Cells in athero-resistant and athero-prone areas of the normal aorta. *Arch. Histol. Cytol.* 58, 307–22 (1995).
208. Choi, J.-H. *et al.* Identification of antigen-presenting dendritic cells in mouse aorta and cardiac valves. *J. Exp. Med.* 206, 497–505 (2009).
209. Paulson, K. E. *et al.* Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. *Circ. Res.* 106, 383–90 (2010).
210. Zhu, S.-N., Chen, M., Jongstra-Bilen, J. & Cybulsky, M. I. GM-CSF regulates intimal cell proliferation

in nascent atherosclerotic lesions. *J. Exp. Med.* 206, 2141–9 (2009).
211. Cho, H. J. *et al.* Induction of dendritic cell-like phenotype in macrophages during foam cell formation. *Physiol. Genomics* 29, 149–60 (2007).
212. Bobryshev, Y. V & Lord, R. S. Mapping of vascular dendritic cells in atherosclerotic arteries suggests their involvement in local immune-inflammatory reactions. *Cardiovasc. Res.* 37, 799–810 (1998).
213. Weber, C. *et al.* CCL17-expressing dendritic cells drive atherosclerosis by restraining regulatory T cell homeostasis in mice. *J. Clin. Invest.* 121, 2898–910 (2011).
214. Packard, R. R. S. *et al.* CD11c(+) dendritic cells maintain antigen processing, presentation capabilities, and CD4(+) T-cell priming efficacy under hypercholesterolemic conditions associated with atherosclerosis. *Circ. Res.* 103, 965–73 (2008).
215. Yilmaz, A. *et al.* Emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques. *Atherosclerosis* 176, 101–10 (2004).
216. Gautier, E. L. *et al.* Conventional dendritic cells at the crossroads between immunity and cholesterol homeostasis in atherosclerosis. *Circulation* 119, 2367–75 (2009).
217. Choi, J.-H. *et al.* Flt3 signaling-dependent dendritic cells protect against atherosclerosis. *Immunity* 35, 819–31 (2011).
218. Jaensson, E. *et al.* Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J. Exp. Med.* 205, 2139–49 (2008).
219. Niessner, A. *et al.* Synergistic proinflammatory effects of the antiviral cytokine interferon-alpha and Toll-like receptor 4 ligands in the atherosclerotic plaque. *Circulation* 116, 2043–52 (2007).
220. Niessner, A. *et al.* Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha. *Circulation* 114, 2482–9 (2006).
221. Liu, P. *et al.* CX3CR1 deficiency impairs dendritic cell accumulation in arterial intima and reduces atherosclerotic burden. *Arterioscler. Thromb. Vasc. Biol.* 28, 243–50 (2008).
222. Wu, H. *et al.* Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 119, 2708–17 (2009).
223. Yona, S. & Jung, S. Monocytes: subsets, origins, fates and functions. *Curr. Opin. Hematol.* 17, 53–9 (2010).
224. Shaposhnik, Z., Wang, X., Weinstein, M., Bennett, B. J. & Lusis, A. J. Granulocyte macrophage colony-stimulating factor regulates dendritic cell content of atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* 27, 621–7 (2007).
225. Angeli, V. *et al.* Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. Immunity 21, 561–74 (2004).
226. Ohl, L. *et al.* CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* 21, 279–88 (2004).
227. Feig, J. E. *et al.* Statins promote the regression of atherosclerosis via activation of the CCR7-dependent emigration pathway in macrophages. *PLoS One* 6, e28534 (2011).
228. Potteaux, S. *et al.* Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of Apoe-/- mice during disease regression. J. Clin. Invest. 121, 2025–36 (2011).
229. Reis e Sousa, C. Dendritic cells in a mature age. *Nat. Rev. Immunol.* 6, 476–83 (2006).
230. Lamb, D. J., El-Sankary, W. & Ferns, G. A. A. Molecular mimicry in atherosclerosis: a role for heat shock proteins in immunisation. *Atherosclerosis* 167, 177–85 (2003).
231. Chang, M. K. *et al.* Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6353–8 (1999).
232. Fadok, V. A. *et al.* Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148, 2207–16 (1992).
233. Pagler, T. A. *et al.* Deletion of ABCA1 and ABCG1 impairs macrophage migration because of increased Rac1 signaling. *Circ. Res.* 108, 194–200 (2011).
234. Tabas, I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler. Thromb. Vasc. Biol.* 25, 2255–64 (2005).
235. Surh, C. D. & Sprent, J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* 372, 100–3 (1994).
236. Reis e Sousa, C. Toll-like receptors and dendritic cells: for whom the bug tolls. *Semin. Immunol.* 16, 27–34 (2004).
237. Kawai, T. & Akira, S. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol. Med.* 13, 460–9 (2007).
238. Seimon, T. A. *et al.* Atherogenic lipids and lipoproteins trigger CD36-TLR2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress. *Cell Metab.* 12, 467–82 (2010).

239. Sheedy, F. J. *et al.* CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat. Immunol.* 14, 812–20 (2013).
240. Curtiss, L. K. & Tobias, P. S. Emerging role of Toll-like receptors in atherosclerosis. *J. Lipid Res.* 50 Suppl, S340–5 (2009).
241. Cole, J. E., Kassiteridi, C. & Monaco, C. Toll-like receptors in atherosclerosis: a 'Pandora's box' of advances and controversies. *Trends Pharmacol.* Sci. 34, 629–36 (2013).
242. Blanco, P., Palucka, A. K., Pascual, V. & Banchereau, J. Dendritic cells and cytokines in human inflammatory and autoimmune diseases. *Cytokine Growth Factor Rev.* 19, 41–52 (2008).
243. Huang, Q. *et al.* The plasticity of dendritic cell responses to pathogens and their components. *Science* 294, 870–5 (2001).
244. Winzler, C. *et al.* Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med.* 185, 317–28 (1997).
245. Perrin-Cocon, L. et al. Oxidized low-density lipoprotein promotes mature dendritic cell transition from differentiating monocyte. *J. Immunol.* 167, 3785–91 (2001).
246. Hansson, G. K. & Libby, P. The immune response in atherosclerosis: a double-edged sword. *Nat. Rev. Immunol.* 6, 508–19 (2006).
247. Hermansson, A. *et al.* Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis. *J. Exp. Med.* 207, 1081–93 (2010).
248. Kasturi, S. P. & Pulendran, B. Cross-presentation: avoiding trafficking chaos? Nat. Immunol. 9, 461–3 (2008).
249. Sun, J. *et al.* Deficiency of antigen-presenting cell invariant chain reduces atherosclerosis in mice. *Circulation* 122, 808–20 (2010).
250. Huber, S. A., Sakkinen, P., David, C., Newell, M. K. & Tracy, R. P. T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia. *Circulation* 103, 2610–6 (2001).
251. Tse, K. *et al.* Atheroprotective Vaccination with MHC-II Restricted Peptides from ApoB-100. *Front. Immunol.* 4, 493 (2013).
252. Buono, C. *et al.* B7-1/B7-2 costimulation regulates plaque antigen-specific T-cell responses and atherogenesis in low-density lipoprotein receptor-deficient mice. *Circulation* 109, 2009–15 (2004).
253. Lutgens, E. *et al.* Requirement for CD154 in the progression of atherosclerosis. *Nat. Med.* 5, 1313–6 (1999).
254. Mach, F., Schönbeck, U., Sukhova, G. K., Atkinson, E. & Libby, P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature* 394, 200–3 (1998).
255. Van Wanrooij, E. J. A. *et al.* Interruption of the Tnfrsf4/Tnfsf4 (OX40/OX40L) pathway attenuates atherogenesis in low-density lipoprotein receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 27, 204–10 (2007).
256. Foks, A. C. *et al.* Interruption of the OX40-OX40 ligand pathway in LDL receptor-deficient mice causes regression of atherosclerosis. *J. Immunol.* 191, 4573–80 (2013).
257. Foks, A. C. *et al.* Interference of the CD30-CD30L pathway reduces atherosclerosis development. *Arterioscler. Thromb. Vasc. Biol.* 32, 2862–8 (2012).
258. Ait-Oufella, H. *et al.* Natural regulatory T cells control the development of atherosclerosis in mice. *Nat. Med.* 12, 178–80 (2006).
259. Tang, Q. *et al.* Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J. Immunol.* 171, 3348–52 (2003).
260. Salomon, B. *et al.* B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12, 431–40 (2000).
261. Gotsman, I. *et al.* Proatherogenic immune responses are regulated by the PD-1/PD-L pathway in mice. J. Clin. Invest. 117, 2974–82 (2007).
262. Lichtman, A. H. T cell costimulatory and coinhibitory pathways in vascular inflammatory diseases. *Front. Physiol.* 3, 18 (2012).
263. Glimcher, L. H. & Murphy, K. M. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* 14, 1693–1711 (2000).
264. Davenport, P. & Tipping, P. G. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. *Am. J. Pathol.* 163, 1117–25 (2003).
265. Elhage, R. *et al.* Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc. Res.* 59, 234–40 (2003).
266. Hauer, A. D. *et al.* Blockade of interleukin-12 function by protein vaccination attenuates atherosclerosis. *Circulation* 112, 1054–62 (2005).
267. Lee, T. S., Yen, H. C., Pan, C. C. & Chau, L. Y. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. A*rterioscler. Thromb. Vasc. Biol.* 19, 734–42 (1999).
268. Whitman, S. C., Ravisankar, P. & Daugherty, A. Interleukin-18 enhances atherosclerosis in

apolipoprotein E(-/-) mice through release of interferon-gamma. *Circ. Res.* 90, E34–8 (2002).
269. Bobryshev, Y. V & Watanabe, T. Subset of Vascular Dendritic Cells Transforming into Foam Cells in Human Atherosclerotic Lesions. *Cardiovasc. Pathol.* 6, 321–331 (1997).
270. Alderman, C. J. J. *et al*. Effects of oxidised low density lipoprotein on dendritic cells: a possible immunoregulatory component of the atherogenic micro-environment? *Cardiovasc. Res.* 55, 806–19 (2002).
271. Blüml, S. *et al.* Oxidized Phospholipids Negatively Regulate Dendritic Cell Maturation Induced by TLRs and CD40. *J. Immunol.* 175, 501–508 (2005).
272. Geyeregger, R. *et al.* Liver X receptors regulate dendritic cell phenotype and function through blocked induction of the actin-bundling protein fascin. *Blood* 109, 4288–95 (2007).
273. Ahmed, M. S. & Bae, Y.-S. Dendritic cell-based therapeutic cancer vaccines: past, present and future. *Clin. Exp. Vaccine Res.* 3, 113–6 (2014).
274. Hjerpe, C., Johansson, D., Hermansson, A., Hansson, G. K. & Zhou, X. Dendritic cells pulsed with malondialdehyde modified low density lipoprotein aggravate atherosclerosis in Apoe(-/-) mice. *Atherosclerosis* 209, 436–41 (2010).
275. Habets, K. L. L. *et al.* Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc. Res.* 85, 622–30 (2010).
276. Probst, H. C., Lagnel, J., Kollias, G. & van den Broek, M. Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8+ T cell tolerance. *Immunity* 18, 713–20 (2003).
277. Bonifaz, L. *et al.* Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral CD8+ T Cell Tolerance. *J. Exp. Med.* 196, 1627–1638 (2002).
278. Hawiger, D. *et al.* Dendritic Cells Induce Peripheral T Cell Unresponsiveness under Steady State Conditions in Vivo. J. Exp. Med. 194, 769–780 (2001).
279. Liu, K. et al. Immune Tolerance After Delivery of Dying Cells to Dendritic Cells In Situ. *J. Exp. Med.* 196, 1091–1097 (2002).
280. Maldonado, R. A. & von Andrian, U. H. How tolerogenic dendritic cells induce regulatory T cells. *Adv. Immunol.* 108, 111–65 (2010).
281. Menges, M. *et al.* Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med.* 195, 15–21 (2002).
282. Rullé, S. *et al.* Adrenomedullin, a neuropeptide with immunoregulatory properties induces semi-mature tolerogenic dendritic cells. *Immunology* 136, 252–64 (2012).
283. Jiang, A. *et al.* Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27, 610–24 (2007).
284. Langenkamp, A. *et al.* T cell priming by dendritic cells: thresholds for proliferation, differentiation and death and intraclonal functional diversification. *Eur. J. Immunol.* 32, 2046–54 (2002).
285. Hackstein, H. *et al.* Aspirin inhibits in vitro maturation and in vivo immunostimulatory function of murine myeloid dendritic cells. *J. Immunol.* 166, 7053–62 (2001).
286. Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J. & Enk, A. H. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* 159, 4772–80 (1997).
287. Sato, K., Yamashita, N., Baba, M. & Matsuyama, T. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood* 101, 3581–9 (2003).
288. Hermansson, A. *et al. I*mmunotherapy with tolerogenic apolipoprotein B-100-loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice. *Circulation* 123, 1083–91 (2011).
289. Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G. K. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6, 131–8 (1986).
290. Galkina, E. & Ley, K. Leukocyte influx in atherosclerosis. *Curr. Drug Targets* 8, 1239–48 (2007).
291. Hansson, G. K., Holm, J. & Jonasson, L. Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am. J. Pathol.* 135, 169–75 (1989).
292. Stemme, S., Holm, J. & Hansson, G. K. T lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and the integrin VLA-1. *Arterioscler. Thromb.* 12, 206–11 (1992).
293. Olson, N. C. *et al.* Decreased naive and increased memory CD4(+) T cells are associated with subclinical atherosclerosis: the multi-ethnic study of atherosclerosis. *PLoS One* 8, e71498 (2013).
294. Stemme, S. *et al.* T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* 92, 3893–3897 (1995).
295. Steffens, S. *et al.* Short-term treatment with anti-CD3 antibody reduces the development and progression of atherosclerosis in mice. *Circulation* 114, 1977–84 (2006).
296. Kita, T. *et al.* Regression of atherosclerosis with anti-CD3 antibody via augmenting a regulatory T-cell response in mice. *Cardiovasc. Res.* 102, 107–17 (2014).
297. Emeson, E. E., Shen, M. L., Bell, C. G. & Qureshi, A. Inhibition of atherosclerosis in CD4 T-cell-ablated and nude (nu/nu) C57BL/6 hyperlipidemic mice. *Am. J. Pathol.* 149, 675–85 (1996).

298. Zhou, X., Nicoletti, A., Elhage, R. & Hansson, G. K. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation* 102, 2919–22 (2000).
299. Song, L., Leung, C. & Schindler, C. Lymphocytes are important in early atherosclerosis. *J. Clin. Invest.* 108, 251–9 (2001).
300. Reardon, C. A., Blachowicz, L., Lukens, J., Nissenbaum, M. & Getz, G. S. Genetic background selectively influences innominate artery atherosclerosis: immune system deficiency as a probe. *Arterioscler. Thromb. Vasc. Biol.* 23, 1449–54 (2003).
301. Dansky, H. M., Charlton, S. A., Harper, M. M. & Smith, J. D. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc. Natl. Acad. Sci. U. S. A.* 94, 4642–6 (1997).
302. Gupta, S. *et al.* IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J. Clin. Invest.* 99, 2752–61 (1997).
303. Bergström, I., Backteman, K., Lundberg, A., Ernerudh, J. & Jonasson, L. Persistent accumulation of interferon-γ-producing CD8+CD56+ T cells in blood from patients with coronary artery disease. *Atherosclerosis* 224, 515–20 (2012).
304. Kolbus, D. *et al.* Association between CD8+ T-cell subsets and cardiovascular disease. *J. Intern. Med.* 274, 41–51 (2013).
305. Gewaltig, J., Kummer, M., Koella, C., Cathomas, G. & Biedermann, B. C. Requirements for CD8 T-cell migration into the human arterial wall. *Hum. Pathol.* 39, 1756–62 (2008).
306. Kolbus, D. *et al.* CD8+ T cell activation predominate early immune responses to hypercholesterolemia in Apoe−(/)− mice. *BMC Immunol.* 11, 58 (2010).
307. Kyaw, T. *et al.* Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. *Circulation* 127, 1028–39 (2013).
308. Fyfe, A. I., Qiao, J. H. & Lusis, A. J. Immune-deficient mice develop typical atherosclerotic fatty streaks when fed an atherogenic diet. *J. Clin. Invest.* 94, 2516–20 (1994).
309. Chyu, K.-Y. *et al.* CD8+ T cells mediate the athero-protective effect of immunization with an ApoB-100 peptide. *PLoS One* 7, e30780 (2012).
310. Benagiano, M. *et al.* T helper type 1 lymphocytes drive inflammation in human atherosclerotic lesions. *Proc. Natl. Acad. Sci. U. S. A.* 100, 6658–63 (2003).
311. Mahic, M. *et al.* Generation of highly suppressive adaptive CD8(+)CD25(+)FOXP3(+) regulatory T cells by continuous antigen stimulation. *Eur. J. Immunol.* 38, 640–6 (2008).
312. Zhou, J. *et al.* CD8(+)CD25(+) T cells reduce atherosclerosis in apoE(-/-) mice. Biochem. *Biophys. Res. Commun.* 443, 864–70 (2014).
313. Han, K. H. *et al.* Lysophosphatidylcholine up-regulates CXCR4 chemokine receptor expression in human CD4 T cells. *J. Leukoc. Biol.* 76, 195–202 (2004).
314. Surls, J. *et al.* Increased membrane cholesterol in lymphocytes diverts T-cells toward an inflammatory response. *PLoS One* 7, e38733 (2012).
315. Frostegård, J. *et al.* Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 145, 33–43 (1999).
316. Zhou, X., Paulsson, G., Stemme, S. & Hansson, G. K. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J. Clin. Invest.* 101, 1717–25 (1998).
317. Bachmann, M., Dragoi, C., Poleganov, M. A., Pfeilschifter, J. & Mühl, H. Interleukin-18 directly activates T-bet expression and function via p38 mitogen-activated protein kinase and nuclear factor-kappaB in acute myeloid leukemia-derived predendritic KG-1 cells. *Mol. Cancer Ther.* 6, 723–31 (2007).
318. Afkarian, M. *et al.* T-bet is a STAT1-induced regulator of IL-12R expression in naïve CD4+ T cells. *Nat. Immunol.* 3, 549–57 (2002).
319. Voloshyna, I., Littlefield, M. J. & Reiss, A. B. Atherosclerosis and interferon-γ: new insights and therapeutic targets. *Trends Cardiovasc. Med.* 24, 45–51 (2014).
320. Laurat, E. *et al.* In Vivo Downregulation of T Helper Cell 1 Immune Responses Reduces Atherogenesis in Apolipoprotein E-Knockout Mice. *Circulation* 104, 197–202 (2001).
321. Buono, C. *et al.* T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proc. Natl. Acad. Sci. U. S. A.* 102, 1596–601 (2005).
322. Whitman, S. C., Ravisankar, P., Elam, H. & Daugherty, A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice. *Am. J. Pathol.* 157, 1819–24 (2000).
323. Leavy, O. Innate-like lymphocytes: Will the real ILC1 please stand up? *Nat. Rev. Immunol.* 13, 67 (2013).
324. Schulte, S., Sukhova, G. K. & Libby, P. Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis. *Am. J. Pathol.* 172, 1500–8 (2008).
325. Engelbertsen, D. *et al.* T-helper 2 immunity is associated with reduced risk of myocardial infarction

and stroke. *Arterioscler. Thromb. Vasc. Biol.* 33, 637–44 (2013).
326. Tse, K., Tse, H., Sidney, J., Sette, A. & Ley, K. T cells in atherosclerosis. *Int. Immunol.* 25, 615–22 (2013).
327. King, V. L., Cassis, L. A. & Daugherty, A. Interleukin-4 does not influence development of hypercholesterolemia or angiotensin II-induced atherosclerotic lesions in mice. *Am. J. Pathol.* 171, 2040–7 (2007).
328. King, V. L. Interleukin-4 Deficiency Decreases Atherosclerotic Lesion Formation in a Site-Specific Manner in Female LDL Receptor-/- Mice. *Arterioscler. Thromb. Vasc. Biol.* 22, 456–461 (2002).
329. Schiller, N. K., Kubo, N., Boisvert, W. A. & Curtiss, L. K. Effect of gamma-irradiation and bone marrow transplantation on atherosclerosis in LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 21, 1674–80 (2001).
330. Ishigami, T. *et al.* Anti-interleukin-5 and multiple autoantibodies are associated with human atherosclerotic diseases and serum interleukin-5 levels. *FASEB J.* 27, 3437–45 (2013).
331. Kurowska-Stolarska, M. *et al.* IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J. Immunol.* 181, 4780–90 (2008).
332. Miller, A. M. *et al.* IL-33 reduces the development of atherosclerosis. *J. Exp. Med.* 205, 339–46 (2008).
333. Cardilo-Reis, L. *et al.* Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype. *EMBO Mol. Med.* 4, 1072–86 (2012).
334. Serafini, N. *et al*. Gata3 drives development of RORγt+ group 3 innate lymphoid cells. *J. Exp. Med.* 211, 199–208 (2014).
335. Erbel, C. *et al.* Expression of IL-17A in human atherosclerotic lesions is associated with increased inflammation and plaque vulnerability. *Basic Res. Cardiol.* 106, 125–34 (2011).
336. Smith, E. *et al.* Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 121, 1746–55 (2010).
337. Yang, L. *et al.* IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* 454, 350–2 (2008).
338. Eid, R. E. *et al.* Interleukin-17 and Interferon- Are Produced Concomitantly by Human Coronary Artery-Infiltrating T Cells and Act Synergistically on Vascular Smooth Muscle Cells. *Circulation* 119, 1424–1432 (2009).
339. Cheng, X. *et al.* The Th17/Treg imbalance in patients with acute coronary syndrome. *Clin. Immunol.* 127, 89–97 (2008).
340. Madhur, M. S. *et al.* Role of interleukin 17 in inflammation, atherosclerosis, and vascular function in apolipoprotein e-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 31, 1565–72 (2011).
341. Butcher, M. J., Gjurich, B. N., Phillips, T. & Galkina, E. V. The IL-17A/IL-17RA axis plays a proatherogenic role via the regulation of aortic myeloid cell recruitment. *Circ. Res*. 110, 675–87 (2012).
342. Danzaki, K. *et al*. Interleukin-17A deficiency accelerates unstable atherosclerotic plaque formation in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 32, 273–80 (2012).
343. Van Es, T. *et al*. Attenuated atherosclerosis upon IL-17R signaling disruption in LDLr deficient mice. *Biochem. Biophys. Res. Commun.* 388, 261–5 (2009).
344. Taleb, S. *et al.* Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *J. Exp. Med.* 206, 2067–77 (2009).
345. Papiernik, M., de Moraes, M. L., Pontoux, C., Vasseur, F. & Pénit, C. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. *Int. Immunol.* 10, 371–8 (1998).
346. Maloy, K. J. & Powrie, F. Regulatory T cells in the control of immune pathology. *Nat. Immunol.* 2, 816–22 (2001).
347. Sakaguchi, S. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J. Clin. Invest.* 112, 1310–2 (2003).
348. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 4, 330–6 (2003).
349. Sakaguchi, S. Animal models of autoimmunity and their relevance to human diseases. *Curr. Opin. Immunol.* 12, 684–90 (2000).
350. Thornton, A. M. *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J. Immunol.* 184, 3433–41 (2010).
351. Himmel, M. E., MacDonald, K. G., Garcia, R. V, Steiner, T. S. & Levings, M. K. Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. *J. Immunol.* 190, 2001–8 (2013).
352. Serre, K. *et al.* Helios is associated with CD4 T cells differentiating to T helper 2 and follicular helper T cells in vivo independently of Foxp3 expression. *PLoS One* 6, e20731 (2011).
353. Bruder, D. *et al.* Neuropilin-1: a surface marker of regulatory T cells. *Eur. J. Immunol.* 34, 623–30

(2004).
354. Milpied, P. *et al.* Neuropilin-1 is not a marker of human Foxp3+ Treg. *Eur. J. Immunol.* 39, 1466–71 (2009).
355. Liston, A. & Gray, D. H. D. Homeostatic control of regulatory T cell diversity. *Nat. Rev. Immunol.* 14, 154–65 (2014).
356. Lal, G. *et al.* Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J. Immunol.* 182, 259–73 (2009).
357. Koch, M. A. *et al.* The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat. Immunol.* 10, 595–602 (2009).
358. Butcher, M., McGary, C. & Galkina, E. The pro-atherosclerotic milieu promotes the conversion of murine T regulatory cells to a spectrum of Foxp3+IFNγ+ T cell phenotypes. (IRC8P.481). *J. Immunol.* 192, 190.9 (2014).
359. Voo, K. S. *et al.* Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc. Natl. Acad. Sci. U. S. A.* 106, 4793–8 (2009).
360. Chaudhry, A. *et al.* CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* 326, 986–91 (2009).
361. Wang, Y., Su, M. A. & Wan, Y. Y. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity* 35, 337–48 (2011).
362. Wohlfert, E. A. *et al.* GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. *J. Clin. Invest.* 121, 4503–15 (2011).
363. Chung, E. J. *et al.* Transforming growth factor-beta induces apoptosis in activated murine T cells through the activation of caspase 1-like protease. *Cell. Immunol.* 204, 46–54 (2000).
364. Takahashi, T. *et al.* Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 10, 1969–80 (1998).
365. Paust, S., Lu, L., McCarty, N. & Cantor, H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc. Natl. Acad. Sci. U. S. A.* 101, 10398–403 (2004).
366. Grossman, W. J. *et al.* Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21, 589–601 (2004).
367. Gondek, D. C., Lu, L.-F., Quezada, S. A., Sakaguchi, S. & Noelle, R. J. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J. Immunol.* 174, 1783–6 (2005).
368. Subramanian, M., Thorp, E., Hansson, G. K. & Tabas, I. Treg-mediated suppression of atherosclerosis requires MYD88 signaling in DCs. *J. Clin. Invest.* 123, 179–88 (2013).
369. Wigren, M. *et al*. Low levels of circulating CD4+FoxP3+ T cells are associated with an increased risk for development of myocardial infarction but not for stroke. *Arterioscler. Thromb. Vasc. Biol.* 32, 2000–4 (2012).
370. Mor, A., Luboshits, G., Planer, D., Keren, G. & George, J. Altered status of CD4(+)CD25(+) regulatory T cells in patients with acute coronary syndromes. *Eur. Heart J.* 27, 2530–7 (2006).
371. Mor, A. *et al.* Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 27, 893–900 (2007).
372. Maganto-García, E., Tarrio, M. L., Grabie, N., Bu, D. & Lichtman, A. H. Dynamic changes in regulatory T cells are linked to levels of diet-induced hypercholesterolemia. *Circulation* 124, 185–95 (2011).
373. Meng, X. *et al.* Statins induce the accumulation of regulatory T cells in atherosclerotic plaque. *Mol. Med.* 18, 598–605 (2012).
374. Klingenberg, R. *et al.* Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis. J. Clin. Invest. 123, 1323–34 (2013).
375. Van Es, T. *et al.* Vaccination against Foxp3(+) regulatory T cells aggravates atherosclerosis. *Atherosclerosis* 209, 74–80 (2010).
376. Mallat, Z. *et al.* Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 108, 1232–7 (2003).
377. Foks, A. C. *et al.* Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis* 218, 53–60 (2011).
378. Harats, D., Yacov, N., Gilburd, B., Shoenfeld, Y. & George, J. Oral tolerance with heat shock protein 65 attenuates Mycobacterium tuberculosis-induced and high-fat-diet-driven atherosclerotic lesions. *J. Am. Coll. Cardiol.* 40, 1333–8 (2002).
379. Van Puijvelde, G. H. M. *et al.* Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis. *Circulation* 114, 1968–76 (2006).
380. Ochi, H. *et al.* Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4+ CD25- LAP+ T cells. *Nat. Med.* 12, 627–35 (2006).

Vanessa Frodermann[1]
Gijs H.M. van Puijvelde[1]
Laura Wierts[1]
H. Maxime Lagraauw[1]
Amanda C. Foks[1,2]
Peter J. van Santbrink[1]
Ilze Bot[1]
Johan Kuiper[1]
Saskia C.A. de Jager[1,3]

2

[1] Division of Biopharmaceutics, LACDR, Leiden University, Leiden, The Netherlands
[2] Present address: Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, USA
[3] Present address: Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

OxLDL-Induced Apoptotic Dendritic Cells as a Novel Therapy for Atherosclerosis

Adapted with permission from
J Immunol. 2015 Mar 1;194(5):2208-18.

Abstract

Objective Modulation of immune responses may form a powerful approach to treat atherosclerosis. It has been shown that clearance of apoptotic cells results in tolerance induction to cleared antigens by DCs; however, this seems impaired in atherosclerosis as antigen-specific tolerance is lacking. This could partially result from a reduced emigration of DCs from atherosclerotic lesions due to the high cholesterol environment. Nonetheless, local induction of anti-inflammatory responses by apoptotic cell clearance seems to dampen atherosclerosis, as inhibition of apoptotic cell clearance worsens atherosclerosis. In this study we assessed whether intravenous administration of oxLDL-induced apoptotic ($apop^{ox}$-DCs), and as a control unpulsed apoptotic DCs ($apop^{ctrl}$-DCs), could modulate atherosclerosis by inducing tolerance.

Methods and Results Adoptive transfer of $apop^{ox}$-DCs into $LDLr^{-/-}$ mice either before or during Western-type diet resulted in increased numbers of $CD103^{+}$ tolerogenic splenic DCs, with a concomitant increase in regulatory T cells. Interestingly, both types of apoptotic DCs induced an immediate 40% decrease in Ly-$6C^{hi}$ monocyte numbers and a 50% decrease in circulating CCL2 levels, but only $apop^{ox}$-DC treatment resulted in long-term effects on monocytes and CCL2 levels. While initial lesion development was reduced by 40% in both treatment groups, only $apop^{ox}$-DC treatment prevented lesion progression by 28%. Interestingly these lesions displayed enhanced stability, determined by a robust 45% increase in collagen content.

Conclusion Our findings clearly show that apoptotic DC treatment significantly decreases lesion development, but only $apop^{ox}$-DCs can positively modulate lesion progression and stability. These findings may translate into a safe treatment of patients with established cardiovascular diseases using patient-derived oxLDL-induced apoptotic DCs.

Introduction

Immune responses are, besides dyslipidemia, driving forces behind atherosclerotic disease development and progression[1]. Dendritic cells (DCs) have been shown to play a crucial role in atherosclerosis. They engulf autoantigens, such as oxidized low-density lipoprotein (oxLDL), in the vessel wall and induce differentiation and clonal expansion of antigen-specific T cells in secondary lymphoid organs. Subsequently, T cells migrate to the lesions and exacerbate local inflammatory responses. In more advanced atherosclerotic lesions, however, the cholesterol-rich environment may prevent DC migration[2]. DCs can then also activate T cells locally, which correlates with rupture-prone regions of the lesions[3].

DCs play a critical role in maintaining homeostasis and self-tolerance. They are crucially involved in apoptotic cell clearance, termed efferocytosis, which does not result in maturation of the phagocytes[4]. While it was assumed that clearance of potentially harmful apoptotic cell content is essential to avoid induction of (auto) immune responses, it was soon discovered that apoptotic cells can actively suppress inflammatory responses. The production of pro-inflammatory cytokines, e.g. TNF-α and IL-6, in response to LPS is decreased in phagocytes upon efferocytosis, while the production of e.g. IL-10, prostaglandin E2, and TGF-β is increased[5]. Furthermore, DCs produce nitrite in response to apoptotic cell uptake, which can inhibit T cell responses[6]. Both early and late apoptotic cells[7] of different origin, e.g. apoptotic peripheral blood lymphocytes[8], neutrophils[9], T cells[10] and DCs[11], can have anti-inflammatory effects. In vivo, apoptotic cells have been shown to downregulate the immunostimulatory capacity of both splenic marginal zone macrophages and DCs[12,13].

By clearing apoptotic cells, DCs can present (auto)antigens derived from ingested apoptotic cells on MHC class I/II molecules, whereas macrophages fail to do so[14,15]. DCs can induce anergy in both $CD4^+$ T cells[16] and $CD8^+$ T cells[13,15], while potentiating Treg responses[11,17], thereby inducing tolerance.

Antigen-presenting cells recognize apoptotic cells due to newly exposed or modified membrane molecules on their cell surface. Disruption of this recognition results in the onset of inflammation and autoimmunity. Mice lacking receptors (such as TIM receptors and TAM receptors, e.g. Mertk) or bridging molecules (e.g. milk fat globule-epidermal growth factor 8)) involved in the recognition of apoptotic cells show a similar phenotype: inefficient apoptotic cell clearance resulting in enhanced activation of $CD4^+$ T cells and/ or B cells, elevated levels of autoantibodies and spontaneous systemic autoimmunity[18]. $LDLr^{-/-}$ mice reconstituted with bone marrow lacking Mertk or milk fat globule-epidermal growth factor 8, $ApoE^{-/-}$ mice with a defective Mertk, and C1qa-deficient $LDLr^{-/-}$ all develop more atherosclerosis[19–22]. Moreover, we have recently shown that blockade of TIM-3 has the same effect[23].

As apoptotic cell clearance is crucial for maintaining tolerance, apoptotic cell administration may be used for anti-inflammatory therapies. Indeed, apoptotic cells can protect mice against LPS-induced shock[24] and myelin cross-linked apoptotic cells can reduce antigen-specific responses in experimental autoimmune encephalomyelitis[25]. In the present study we determined whether apoptotic DCs could be used to treat

atherosclerosis. We induced apoptosis in DCs by oxLDL (apop^{ox}-DCs), which possibly also delivers an atherosclerosis-specific antigen in the context of regulatory signals to viable DCs, or by proteasomal inhibition (apop^{ctrl}-DC). Both apoptotic DCs were injected *intravenously* to enable their uptake by DCs to modulate T cell responses in developing and established atherosclerosis. We show here for the first time that apoptotic DC-treatment is highly effective in reducing initial atherosclerosis. Interestingly, the presence of oxLDL in apoptotic DCs has additional beneficial effects on lesion progression and stability.

Material and Methods

Animals

C57BL/6 and LDLr$^{-/-}$ mice were originally obtained from the Jacksons Laboratory, kept under standard laboratory conditions, and administered food and water *ad libitum*. All animal work was approved by the Ethics Committee for Animal Experiments of Leiden University and conforms to Dutch government guidelines.

DC cultures and stimulations

Bone marrow cells were isolated from the tibias and femurs of C57BL/6 mice. The cells were cultured for ten days at 37°C and 5% CO_2 in 10 cm petri dishes (Greiner Bio-One) in IMDM supplemented with 10% FCS, 100 U/mL penicillin/streptomycin (all obtained from PAA), 2mM glutamax (Thermo Fisher Scientific) and 20 µm β-mercaptoethanol (Sigma Aldrich) in the presence of granulocyte-macrophage colony-stimulating factor. DC purity was assessed by CD11c expression (flow cytometry) and routinely found to be above 85%. Apoptosis for in vivo treatments was induced by 0.1 µM epoxomicin (apop^{ctrl}-DCs) or 50 µg/mL oxLDL (apop^{ox}-DCs). LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave *et al.*[26]. The isolated LDL was dialyzed against 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[27].

For in vitro DC stimulations, 5 x 10^5 DCs per well were plated in 24-well plates with indicated ratios of apop^{ox}-DCs or apop^{ctrl}-DCs for 24 hrs. After extensive washing with PBS, DCs were stimulated with 100 ng/mL LPS to determine cytokine responses.

Co-cultures

Single cell suspensions of spleens from LDLr$^{-/-}$ mice were obtained by using a 70 µm cell strainer (Falcon). Red blood cells were lysed with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). CD4$^+$ T cells (>95% purity) were isolated from splenocytes by using the BD IMagTM mouse CD4 T lymphocyte enrichment set according to manufacturer's protocol (BD Biosciences). CD11c$^+$ cells (>85% purity) were isolated from splenocytes by using CD11c MicroBeads according to manufacturer's protocol (Miltenyi Biotec).

For DC-T cell co-cultures 0.1 x 10^5 DCs were cultured with 1 x 10^5 T cells per well, and for splenocyte proliferation 2 x 10^5 splenocytes per well were cultured

in quintuplicates in 96-well round-bottom plates (Greiner Bio-One) in the presence or absence of αCD3, or αCD3 and αCD28 (2 μg/mL, eBioscience) for 72 hours in complete RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2mM L-glutamine (all obtained from PAA) and 20 μm β-mercaptoethanol (Sigma Aldrich). Proliferation was measured by Ki-67 expression. Relative Ki-67 expression was measured by flow cytometry and is expressed as mean Ki-67% with stimulation divided by mean Ki-67% without stimulation. T cell subsets in the culture were determined by flow cytometry.

Atherosclerosis

Atherosclerosis was induced in 10-12 weeks old male $LDLr^{-/-}$ mice by feeding a Western-type diet (WTD) (0.25% cholesterol and 15% cocoa butter; Special Diet Services). For the initial atherosclerosis study, mice were treated with 3 *i.v.* injections of PBS, 1.5 x 10^6 $apop^{ox}$-DCs or 1.5 x 10^6 $apop^{ctrl}$-DCs every other day prior to nine weeks WTD. For the progression study, a baseline group was sacrificed after ten weeks WTD, while the rest received 3 *i.v.* injections of PBS, 1.5 x 10^6 $apop^{ox}$-DCs or 1.5 x 10^6 $apop^{ctrl}$-DCs every other day and were put another nine weeks on WTD.

Flow Cytometry

At sacrifice, blood, spleen, bone marrow, and aorta were harvested. Single cell suspensions were obtained as described above. For aortic single cell suspensions, aortas were isolated and adventitial fat was removed. The aorta was then dissected and digested in a 20mM HEPES/ PBS buffer containing 125 U/ml Collagenase type XI, 60 U/ml hyaluronidase type 1-s, 60 U/ml DNase I, and 450 U/ml Collagenase type I at 37°C for 1 hour. Subsequently, 3 x 10^5 cells per sample were stained with the appropriate antibodies. To determine aortic Tregs, we pre-gated on $CD3^+$ cells within $CD45^+$ cells. The following antibodies were used: CCR2-purified (Abcam), anti-Rabbit-IgG-FITC (BD Biosciences), CD3-PerCP (BD Biosciences), CD4-PerCP (BD Biosciences), CD8-PerCP (BD Biosciences), CD11b-FITC, CD11c-APC, CD25-FITC, CD44-FITC, CD45- Pacific Blue, CD62L-Pacific Blue, CD103-FITC, FoxP3-APC, Gata-3-PE, Ki67-FITC, Ly-6C-PerCP, MHCII-Pacific Blue, RORyt-PE, and T-bet-Alexa Fluor 647. All antibodies were purchased from eBioscience, unless stated otherwise. For intracellular staining, cells were fixed and permeabilized according to the manufacturer's protocol (eBioscience). Flow cytometry analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (BD Biosciences).

Histological analysis

To determine plaque size, 10 μm cryosections of the aortic root were stained with Oil-Red-O and haematoxylin (Sigma Aldrich). Corresponding sections were stained for collagen fibers using the Masson's Trichrome staining (Sigma Aldrich) or immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b, 1:1000, Serotec Ltd.). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100; Sigma Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as

enzyme substrates. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, ThermoScientific). BrightVision Anti-rabbit-HRP was used as secondary antibody (Immunologic). The section with the largest lesion and four flanking sections were analyzed for lesion size and collagen content, two flanking sections were analyzed for macrophage and T cell content. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2-positive area by the total lesion surface area.

Real-time PCR

mRNA was isolated from the aortic arch using the guanidium isothiocyanate method and reverse transcribed (RevertAid Moloney murine leukemia virus reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast real-time PCR system (Applied Biosystems) using SYBR Green technology. The expression was determined relative to the average expression of three household genes: acidic ribosomal phosphoprotein PO (36B4), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). For used primer pairs refer to Table 1.

Gene	Forward	Reverse
36B4	CTGAGTACACCTTCCCACTTACTGA	CGACTCTTCCTTTGCTTCAGCTTT
CCL2	CTGAAGCCAGCTCTCTCTTCCTC	GGTGAATGAGTAGCAGCAGGTGA
CD68	TGCCTGACAAGGGACACTTCGGG	GCGGGTGATGCAGAAGGCGATG
IL-6	AGACAAAGCCAGAGTCCTTCAGAGA	GGAGAGCATTGGAAATTGGGGTAGG
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG

Table 1. Primer Pairs used for qPCR analysis. The relative expression of genes was determined relative to the average expression of the three household genes: ribosomal protein 36B4, hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27).

Cytokine and nitrite analysis

IL-10, TNF-α, IL-6 (BD Biosciences), and CCL2 (eBioscience) were determined by ELISA, according to manufacturer's protocol. Nitrite production was assessed by mixing cell culture supernatant with an equal amount of Griess reagent (Sigma Aldrich) and measuring absorbance at 540 nm. Culture medium served as background control.

Serum cholesterol levels

Serum concentrations of total cholesterol were determined by enzymatic colorimetric assays (Roche Diagnostics). Absorbance was read at 490 nm. Precipath (standardized serum; Roche Diagnostics) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 µl of serum of each mouse using a Superose 6 column (3.2 x 300 mm, Smart-System; Pharmacia). Total cholesterol content of the effluent was determined as described above.

Statistical analysis

Values are expressed as mean±SEM. Data of three groups were analyzed with one-way ANOVA and data of two or more groups with more than one variable were analyzed by two-way ANOVA, both followed by Bonferroni post-testing. Statistical analysis was performed using Prism (GraphPad). Probability values of $P<0.05$ were considered significant.

Results

OxLDL-induced apoptotic DCs induce tolerogenic DCs and modulate T cell responses in vitro

To induce DC apoptosis and simultaneously load DCs with an atherosclerosis-relevant antigen, we incubated bone marrow-derived DCs with increasing concentrations of oxLDL for 24 and 48 hours. After 48 hours, 50 µg/mL oxLDL was sufficient to induce late apoptosis in 97% of the DCs. We therefore chose this condition to generate oxLDL-induced apoptotic DCs (apop^{ox}-DCs) for further experiments. As a control, we generated apoptotic DCs by exposure to 0.1 µM epoxomicin (apop^{ctrl}-DCs), which induced late apoptosis in 95% of DCs (Figure 1).

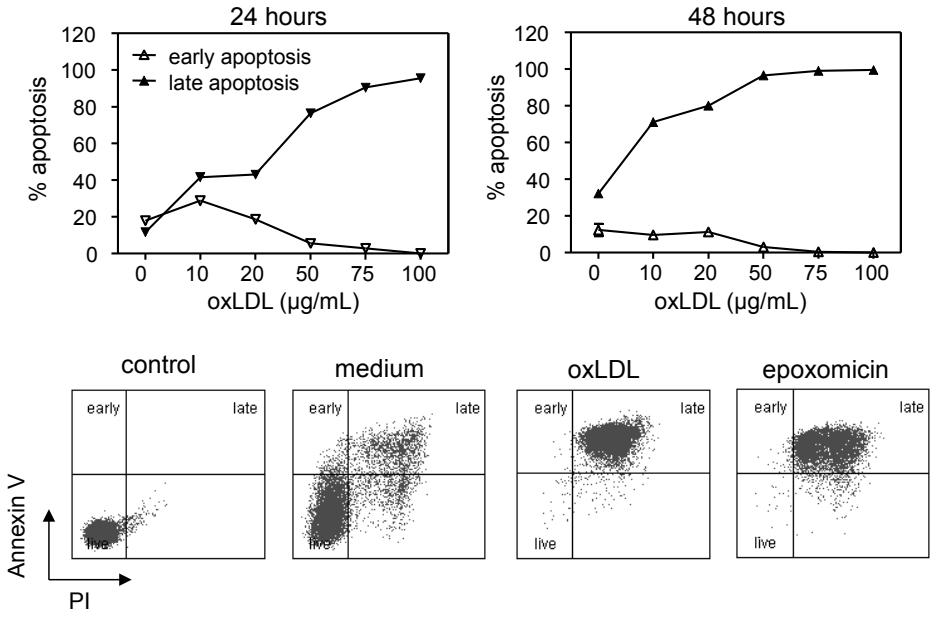


Figure 1. OxLDL and epoxomicin induce apoptosis in DCs. Quantification of apoptotis in DCs by flow cytometry. DCs were stimulated with indicated amounts of oxLDL. Early apoptosis was determined as AnnexinV$^+$PI$^-$ and late apoptosis as AnnexinV$^+$PI$^-$ by flow cytometry. Values are expressed as mean±SEM and are representative of three independent experiments. Representative dot plots of DCs treated with either medium, 50 µg/mL oxLDL or 0.1 µM epoxomicin for 48 hours are shown. Control indicates DCs without staining. Values are expressed as mean±SEM.

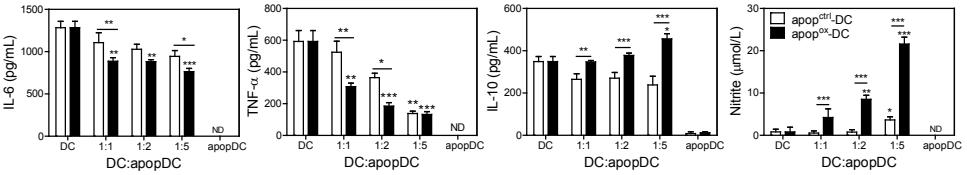


Figure 2. Apoptotic DC-exposure modulates cytokine responses by DCs in vitro. Apoptotic DC-exposure prevents LPS-induced inflammatory responses by DCs. OxLDL-induced apoptotic DCs (apop^{ox}-DCs; black bars) and control apoptotic DCs (apop^{ctrl}-DCs; white bars) were added to DCs at indicated ratios for 24 hours; viable DCs remained constant. After extensive washing, 100 ng/mL LPS was added for another 24 hours. IL-6, TNF-α, and IL-10 responses were determined by ELISA and nitrite by Griess reagent. All values are expressed as mean±SEM and are representative of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared DCs without apoptotic DCs or to apop^{ox}-DCs as indicated.

To assess whether these apoptotic DCs could modulate inflammatory responses by viable responder DCs (respDCs), we added different amounts of apop^{ox}-DCs and apop^{ctrl}-DCs to immature respDCs for 24 hours and subsequently stimulated them with 100 ng/mL LPS for another 24 hours. Both apop^{ox}-DCs and apop^{ctrl}-DCs decreased the release of inflammatory cytokines from respDCs: Pre-exposure to apop^{ox}-DCs resulted in a significant decrease of 40% in IL-6 and 80% in TNF-α responses, while

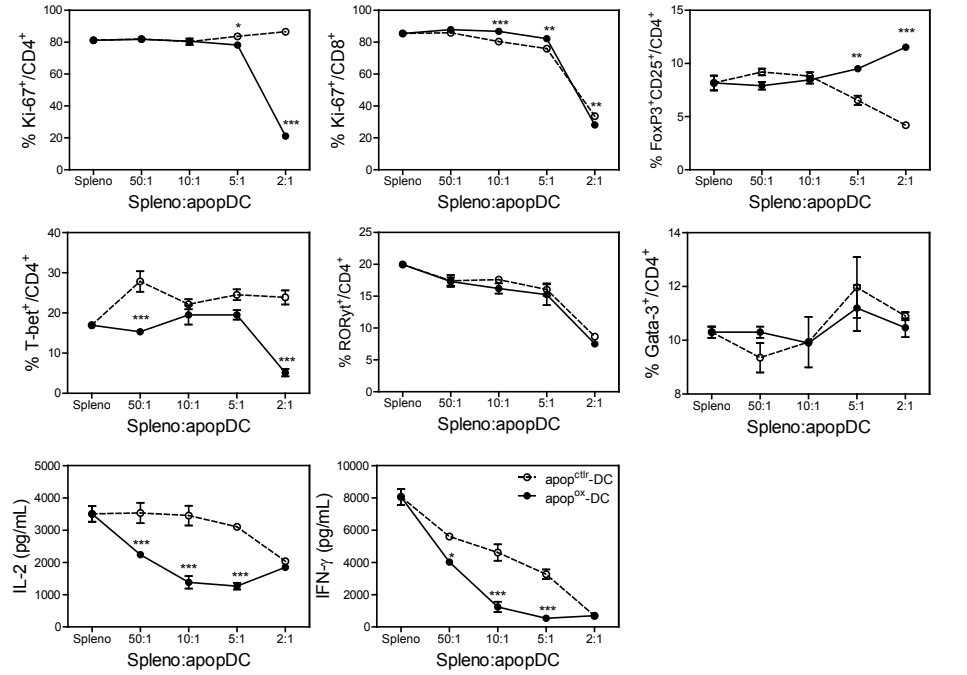


Figure 3. Apoptotic DC-exposure modulates T cell responses. Splenocytes from an LDLr$^{-/-}$ were co-cultured with increasing amounts of apop^{ox}-DCs (solid lines) and apop^{ctrl}-DCs (dotted lines) in the presence of αCD3/28 for 48 hours to induce T cell proliferation; splenocytes remained constant. Proliferation was assessed by Ki-67$^+$ cells of CD4$^+$ and CD8$^+$ T cells. Tregs (FoxP3$^+$CD25$^+$), Th1 (T-bet$^+$), Th2 (Gata-3$^+$), and Th17 (RORγt$^+$) cells were determined within CD4$^+$ T cells by flow cytometry. All values are expressed as mean±SEM and are representative of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to apop^{ox}-DCs.

pre-exposure to apop^{ctrl}-DCs resulted in a decrease of 26% in IL-6 and 77% in TNF-α responses of respDCs. The anti-inflammatory cytokine IL-10 was specifically increased, by 1.3 fold, when respDCs were pre-exposed to apop^{ox}-DCs, but not after pre-exposure to apop^{ctrl}-DCs. Nitrite production was robustly increased, by 26.7-fold, upon pre-exposure to apop^{ox}-DC, while pre-exposure to apop^{ctrl}-DCs only increased nitrite production by 4.5-fold (Figure 2).

We further established the immunomodulatory potential of apoptotic DCs by incubating them ex vivo with splenocytes from an LDLr$^{-/-}$ mouse. Increasing numbers of apop^{ox}-DCs induced a significant 74% reduction in CD4^{+} T cell proliferation, as assessed by the expression of the cell cycle marker Ki-67, while apop^{ctrl}-DCs had no effect. This was in line with a robust 41% increase in Tregs and a 70% reduction in Th1 cells by apop^{ox}-DCs. In contrast, apop^{ctrl}-DCs exposure resulted in a significant 51% reduction in Treg, while Th1 cells were unaffected. Th17 cells were reduced in both apop^{ox}-DC and apop^{ctrl}-DC-treated splenocytes cultures by 63% and 57%, respectively, while Th2 responses were not affected. Interestingly, CD8^{+} T cell proliferation was reduced by about 65% by both types of apoptotic DCs. Consistent with effects observed on T cell responses, IL-2 and IFN-γ production were significantly reduced by both apoptotic DCs (Figure 3).

Exposure to apop^{ox}-DCs more effectively induced IL-10 responses in DCs and induced Treg responses compared to apop^{ctrl}-DCs. Moreover, an increased nitrite production and a stronger inhibition of T cell responses were observed by apop^{ox}-DCs compared to apop^{ctrl}-DCs. To assess the different potential of apop^{ox}-DCs more closely, we checked differences in viable DCs that had been exposed to these apoptotic DCs. We found that apop^{ox}-DCs potently induced expression of ABCA1 and ABCG1, two main target genes of liver X receptor (LXR). In addition to an upregulation of IL-10 and a downregulation of IL-6, we also found a significant upregulation of the co-

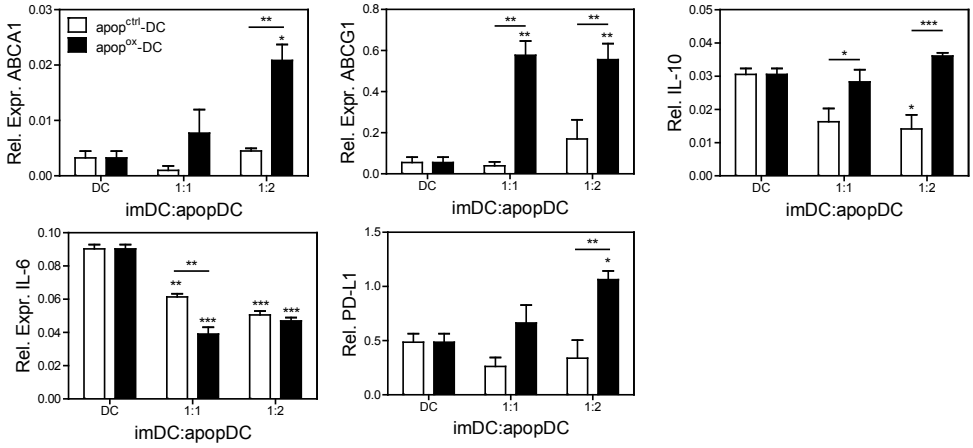


Figure 4. Increased LXR activation in viable DCs exposed to oxLDL-induced apoptotic DCs. OxLDL-induced apoptotic DCs (apop^{ox}-DCs; black bars) and control apoptotic DCs (apop^{ctrl}-DCs; white bars) were added to DCs at indicated ratios for 24 hours; viable DCs remained constant. After extensive washing, 100 ng/mL LPS was added for another 24 hours. Relative mRNA expression was determined by qPCR. All values are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001

inhibitory molecule PD-L1, indicating a more potent anti-inflammatory capacity of apop^{ox}-DC-exposed DCs (Figure 4).

OxLDL-presence in apoptotic DCs is needed to induce tolerogenic DCs and Treg responses in vivo

To assess whether apop^{ox}-DC treatment is beneficial for atherosclerosis, we administered apop^{ox}-DCs and apop^{ctrl}-DCs three times *intravenous* every other day prior to nine weeks WTD-feeding of LDLr$^{-/-}$ mice. One hour after the first injection, the majority of apoptotic DCs were cleared by marginal zone DCs and macrophages, while some apoptotic DCs might have been cleared by red pulp macrophages (Figure 5).

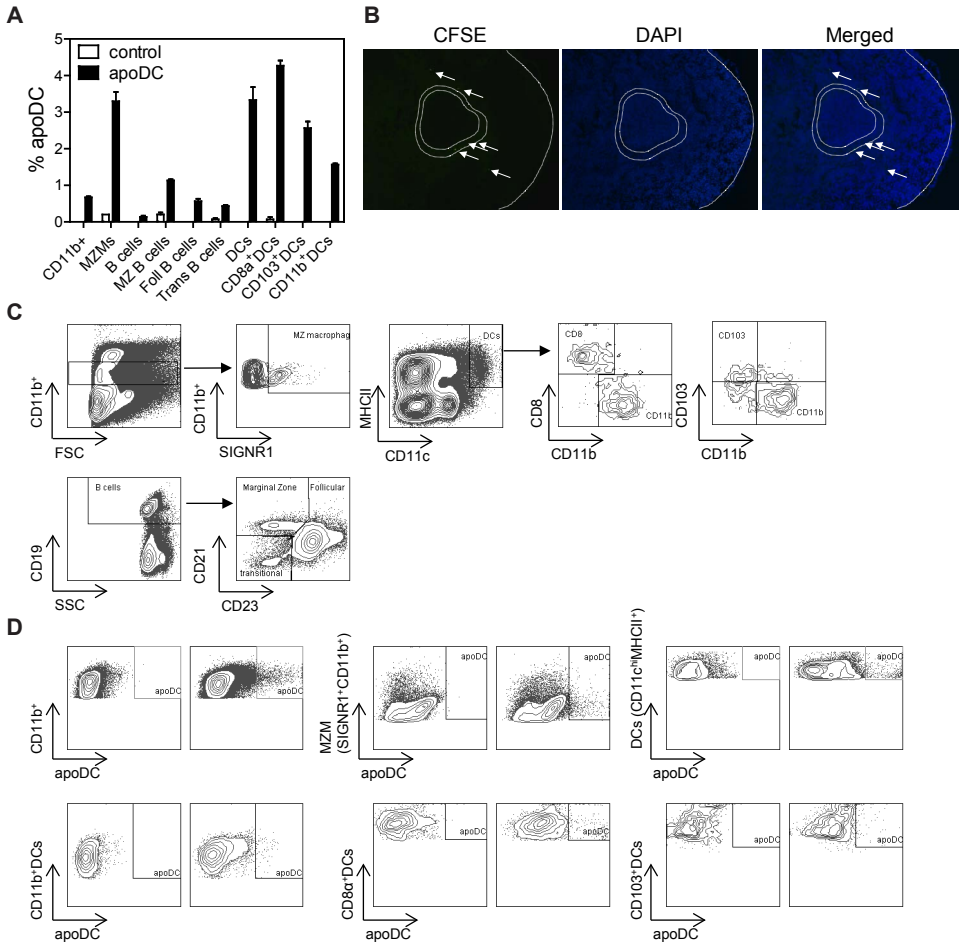


Figure 5. Uptake of apoptotic DCs in the spleen. OxLDL-induced apoptotic DCs were stained with CFSE. **A.** One hour after *i.v.* injection of 5 million apoptotic DCs, CFSE-positive cells were determined by flow cytometry within CD11b^{+} cells, Marginal zone macrophages (MZM), CD19^{+} cells (B cells), Marginal Zone (MZ) B cells, Follicular (Foll) B cells, Transitional (Trans) B cells and different subsets of DCs. Values are expressed as mean±SEM. **B.** Representative spleen sections stained with DAPI are shown. **C.** Gating strategies for different cell populations. **D.** Representative dot plots of macrophages and DCs that have taken up CFSE^{+} apoptotic DCs.

As splenic DCs efficiently clear apoptotic cells from the circulation[13], we analyzed these splenic DCs one week after apoptotic DC treatment. While absolute DC numbers remained unchanged, myeloid immunogenic CD8α$^-$CD11b$^+$ DCs were significantly decreased by 10% and 12% in apop^{ox}-DC- and apop^{ctrl}-DC-treated mice, respectively. No significant changes in lymphoid tolerogenic CD8α$^+$CD11b$^-$ DCs were observed. Interestingly, a significant 50% increase in anti-atherogenic CD103$^+$CD11b$^-$DCs was determined in the apop^{ox}-DC-treated-mice only (Figure 6A). To determine whether these DCs were tolerogenic, we isolated splenic CD11c$^+$ cells and co-cultured them with CD4$^+$ T cells. A significant 53% reduction in T cell proliferation, as determined by Ki-67 expression, was observed in co-cultures with DCs from apop^{ox}-DC treated mice. Co-cultures with DCs from apop^{ctrl}-DC-treated mice did not significantly affect T cell proliferation. Additionally, we observed a significant 78% increase in Tregs and a 2.5-fold increase in IL-10 production in co-cultures with DCs from apop^{ox}-DC-treated mice only. Nitrite production was low but increased by 1.7-fold and 1.5-fold in DCs from apop^{ox}-DC- and apop^{ctrl}-DC-treated-mice, respectively. Th1, Th2, and Th17 cells did not significantly differ (Figure 6B). Overall DCs from apop^{ctrl}-DC-treated mice only mildly affected T cell proliferation, indicating that oxLDL-presence in apoptotic DCs is necessary to potently modulate immune responses by increasing Tregs.

As efferocytosis can modulate T cell responses induced by DCs in vivo[28], we monitored CD4$^+$ and CD8$^+$ T cells in the circulation. While we did not observe effects on total CD4$^+$ and CD8$^+$ T cells (data not shown), apop^{ox}-DC-treatment potently induced

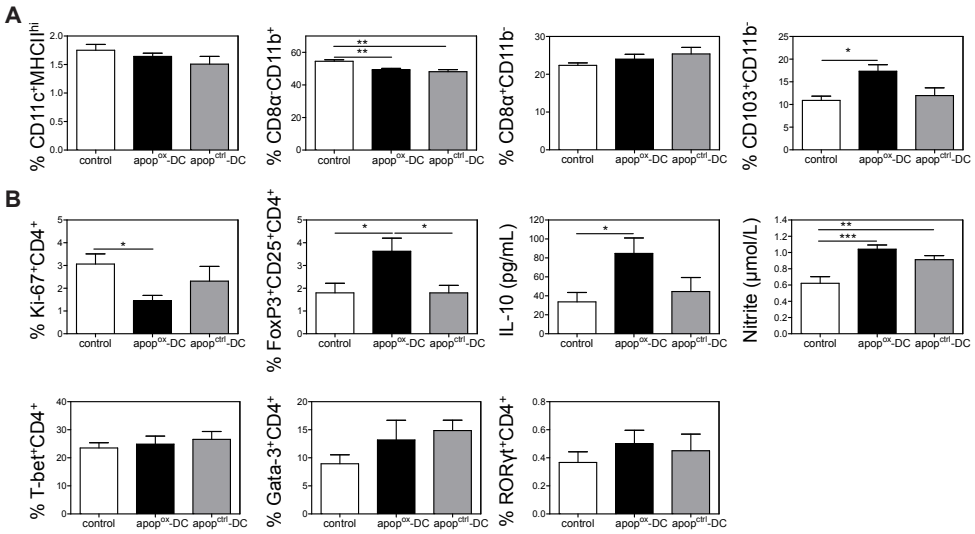


Figure 6. Apop^{ox}-DC treatment results in tolerogenic DCs and increased Tregs in initial atherosclerosis. A. The effect of apoptotic DC-treatment on splenic DC phenotype. After one week, CD11c^{hi}MHCII$^+$ DCs within Ly-6G- cells in the spleen and the percentage of myeloid CD8α$^-$CD11b$^+$DCs, lymphoid CD8α$^+$CD11b-DCs, and CD103$^+$CD11b$^-$DCs within these DCs were determined by flow cytometry. B. Splenic DCs of apop^{ox}-DC-treated mice induce Tregs and decrease T cell proliferation. We isolated splenic CD11c$^+$ cells and co-cultured them with CD4$^+$ T cells from LDLr$^{-/-}$ mice one week on WTD in a ratio of 1:10 in the presence of αCD3 for 72 hours. Specific T cell proliferation was determined by %Ki-67 expression divided by background. Tregs were determined as FoxP3$^+$CD25$^+$CD4$^+$ by flow cytometry. IL-10 production was determined by ELISA and nitrite production by Griess reagent. All values are expressed as mean±SEM and are representative of at least six mice.*P<0.05, **P<0.01, ***P<0.001.

Tregs in the circulation. A significant 24% increase was observed one week after apop^{ox}-DC treatment and remained mildly increased (10%, n.s.) nine weeks after treatment (Figure 7A). A similar pattern was observed in the spleen, where Tregs were robustly induced after one week (35%) and remained mildly increased (9%, n.s.) nine weeks after apop^{ox}-DC treatment (Figure 7B and data not shown). One week after treatment, the increase in circulating Tregs translated into significantly increased aortic Tregs only in the apop^{ox}-DC-treated group (Figure 7C). Apop^{ctrl}-DC-treatment did not induce circulating or aortic Tregs (Figure 7A and 7C), while a similar increase of splenic Treg percentages was observed after one week WTD (Figure 7B). However, this appeared to be related to an overall decrease of CD4$^+$ T cells and reduced subsets, resulting in an increased Treg percentage as absolute Treg numbers in the spleen were not changed and significantly less than in apop^{ox}-DC-treated mice (Figure 7D). After nine weeks WTD, apop^{ctrl}-DC treatment had no effect on T cell subsets (data not shown). Additionally, we determined T cell numbers in the aortic root after nine weeks and found a significant 68% and 52% reduction in apop^{ox}-DC-treated mice and apop^{ctrl}-DC-treated-mice, respectively (Figure 7E).

In line with the described effect of apoptotic cells on inflammation, we saw a dramatic 7-fold and 6.2-fold reduction of IL-6 expression in the aortic arch in apop^{ox}-

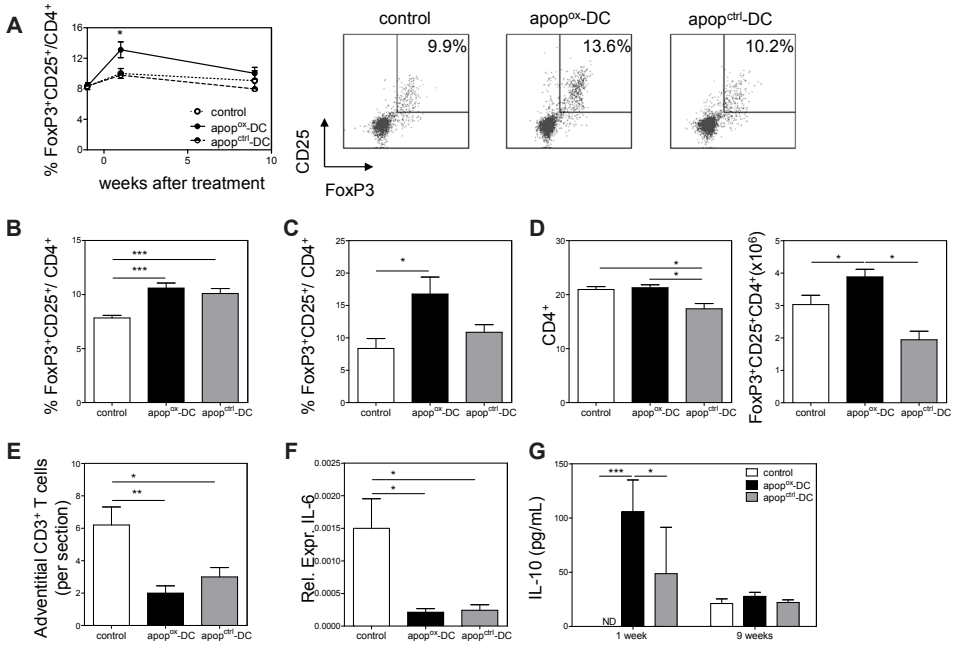


Figure 7. Apoptotic DC treatment reduces the inflammatory status in initial atherosclerosis. **A.** Treatment with apop^{ox}-DCs increases circulating Tregs, while treatment with apop^{ctrl}-DCs has no effect. During the experiment Tregs (FoxP3$^+$CD25$^+$ within CD4$^+$ T cells) were monitored in circulation. Representative dot plots are shown. **B.** Splenic Tregs and **C.** Aortic Tregs were determined by flow cytometry after one week WTD. We pre-gated on CD3$^+$ T cells within CD45$^+$ cells. **D.** CD4$^+$ T cell responses in the spleen as determined by flow cytometry and absolute amounts of Tregs after one week WTD. **E.** Quantification of CD3$^+$ T cells in the aortic root after nine weeks WTD. **F.** IL-6 expression in the aortic arch after nine weeks WTD was determined by qPCR. **G.** IL-10 in the serum was determined by ELISA. All values are expressed as mean±SEM and representative of at least six mice.*P<0.05, **P<0.01, ***P<0.001.

DC- and apop^{ctrl}-DC-treated mice, respectively (Figure 7F). We also observe an initial significant increase in IL-10 in the serum, corresponding to an induction of tolerance (Figure 7G).

OxLDL-presence in apoptotic DCs enhances beneficial effects on monocytes

Interestingly, while we aimed to modify DCs and subsequent T cell responses, major effects on circulating monocytes were observed by the apoptotic DC treatments. One week after treatment, a significant 40% and 36% reduction in circulating inflammatory Ly-6C^{hi} monocytes in apop^{ox}-DC-treated and apop^{ctrl}-DC-treated mice was determined, respectively. In the apop^{ox}-DC-treated mice also CCR2 expression of monocytes was significantly reduced one week after treatment. Nine weeks after treatment with apop^{ox}-DCs a 30% decrease in total monocyte numbers was observed, whereas apop^{ctrl}-DC-treated mice did not show a significant decrease in monocytes (Figure 8A). In line with these observations, the apop^{ctrl}-DC-treated mice only showed an initial decrease in CCL2 serum levels, while in apop^{ox}-DC-treated mice CCL2 levels were reduced by about half during the entire length of the experiment (Figure 8B).

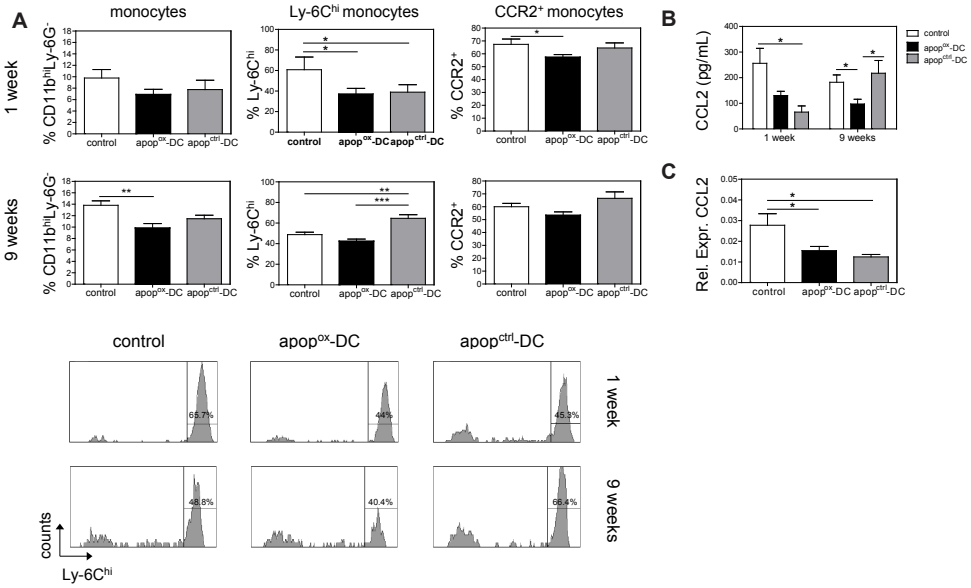


Figure 8. Apoptotic DC treatment reduces monocyte responses in initial atherosclerosis. A. Apoptotic DC-treatment reduces circulating inflammatory monocytes. After one week and nine weeks WTD, monocytes (CD11b^{hi}Ly-6G^{-}) in the circulation and inflammatory monocytes (Ly-6C^{hi} and CCR2^{+} within monocytes), were analyzed by flow cytometry. Representative histograms are shown. **B.** Quantification of CCL2 in the serum by ELISA. **C.** CCL2 mRNA expression in the aortic arch after nine weeks, as determined by qPCR. All values are expressed as mean±SEM and are representative of at least six mice.*P<0.05, **P<0.01, ***P<0.001.

These results clearly indicate that oxLDL presence in apoptotic DCs provides a long-term anti-inflammatory effect on monocytes. This was not a result of exposure to oxLDL by itself as treatment with oxLDL mildly increased CCL2 levels and significantly increased monocytes, as well as Ly-6C^{hi} monocytes, compared to apop^{ox}-DC-treated

mice (data not shown). Interestingly, CCL2 expression in the aortic arch was still 2-fold decreased in both apoptotic DC-treated groups at sacrifice (Figure 8C). The effects of apoptotic DCs on monocytes were not due to a difference in the number of progenitors in the bone marrow, determined as $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells (data not shown). Moreover no differences in monocyte proliferation, determined by Ki-67 expression, in bone marrow or spleen were observed; also no differences in the numbers of ($Ly\text{-}6C^{hi}$) monocytes, or levels of CCR2 expression on monocytes were observed (data not shown).

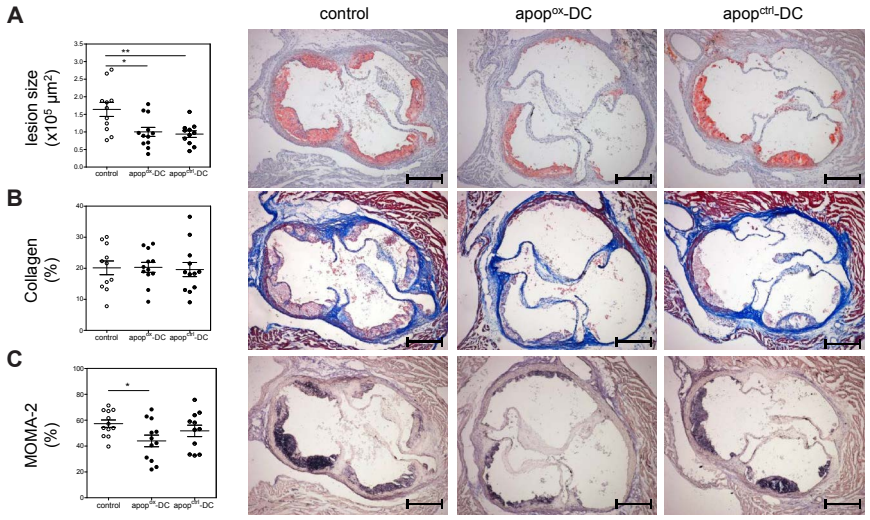


Figure 9. Apoptotic DC treatment reduces initial lesion development. **A.** Evaluation of lesion size in the aortic root after nine weeks WTD. Representative cross-sections stained with Oil-Red-O and haematoxylin are shown. **B.** Collagen content was determined by Masson's Trichrome staining as percentage of total lesion area. **C.** Macrophage content was assessed by MOMA-2 staining as percentage of total lesion area. **D.** CD68 expression in the aortic arch was determined by qPCR. All values are expressed as mean±SEM and are representative of all mice. Scale bar, 300 µm. *P<0.05, **P<0.01.

Apoptotic DC treatment reduces initial atherosclerotic lesion

Nine weeks after the apoptotic DC treatment, we analyzed aortic root lesion sizes and found a significant reduction of 39% in $apop^{ox}$-DC-treated-mice and 43% in $apop^{ctrl}$-DC-treated mice (Figure 9A). Lesion stability was not different between the groups as collagen content was on average 20% (Figure 9B). In line with the observed effects on monocyte numbers, a significant 24% decrease in the relative macrophage content was determined in the $apop^{ox}$-DC-treated-mice, whereas this was not affected in the $apop^{ctrl}$-DC-treated-mice (Figure 9C). Consistent with this, a significant 51% decrease in CD68 mRNA expression in the aortic arch of $apop^{ox}$-DC-treated-mice was observed. Also $apop^{ctrl}$-DC-treated mice showed a 55% reduction, in line with initial decreases of inflammatory monocytes (Figure 9D). The effects on lesion formation were not due to effects of apoptotic DCs on bodyweight or serum cholesterol levels as these did not differ between the groups (data not shown).

OxLDL-presence in apoptotic DCs reduces inflammatory responses in established atherosclerosis

As the apoptotic DC treatment proved highly effective in reducing initial atherosclerosis, we next determined whether it could reduce inflammation in pre-established atherosclerotic lesions. To this end, LDLr$^{-/-}$ mice that had been kept on a WTD for ten weeks, were treated three times with apoptotic DCs every other day and left for another nine weeks on WTD to assess lesion progression. We again did not observe any effects on bodyweight or serum cholesterol levels (data not shown). Similar to earlier observations, apop^{ox}-DC treatment was effective in modifying splenic DCs one week after apop^{ox}-DC-treatment: myeloid CD8α^{-}CD11b^{+}DCs were significantly decreased by 8% and CD103^{+}CD11b^{-}DCs were significantly increased by 17%. No significant effects were observed on lymphoid CD8α^{+}CD11b^{-}DCs or by the treatment with apop^{ctrl}-DCs (Figure 10A). Moreover, we again only observed a significant 22% increase in circulating Tregs in apop^{ox}-DC-treated mice one week after treatment (Figure 10B).

Interestingly, again only the apop^{ox}-DC-treatment resulted in a 35% reduction in monocytes and a 19% reduction in Ly-6C^{hi} monocytes nine weeks after treatment. In line with earlier observations, a significant 41% decrease in CCL2 serum concentrations in apop^{ox}-DC-treated mice was observed. No significant effects of apop^{ctrl}-DC treatment on immune responses in established atherosclerosis were found (Figure 10C).

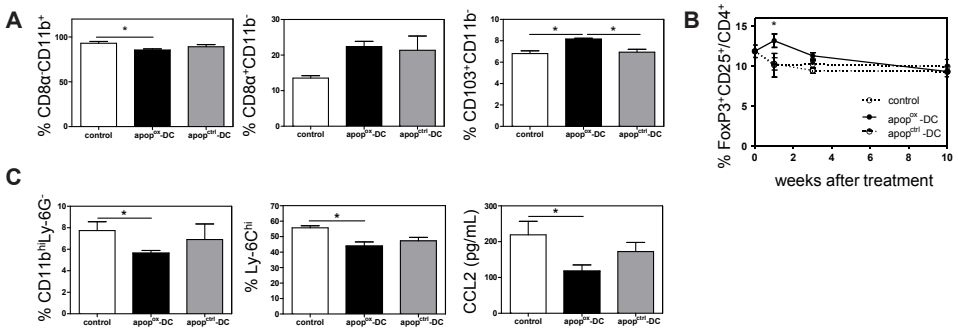


Figure 10. Apopox-DC treatment reduces inflammation in established atherosclerosis. A. The effect of apoptotic DC-treatment on splenic DC phenotype. The percentage of myeloid CD8α^{-}CD11b^{+}DCs, lymphoid CD8^{+}CD11b^{-}DCs, and CD103^{+}CD11b^{-}DCs of splenic CD11c^{hi}MHCII^{+}DCs (pregated on Ly-6G^{-} cells) was determined. **B.** Tregs in the circulation were determined as FoxP3^{+}CD25^{+} within CD4^{+} T cells. **C.** Apop^{ox}-DC treatment reduces monocyte responses at sacrifice. Circulating monocytes, CD11b^{hi}Ly-6G^{-}, and Ly-6C^{hi} monocytes within these were determined by flow cytometry. CCL2 levels in the serum were determined by ELISA. All values are expressed as mean±SEM and representative of at least six mice. *P<0.05.

OxLDL-presence in apoptotic DCs is needed for stabilization and reduction of lesion progression

Nine weeks after the last treatment, lesion sizes were compared to the baseline group, which was sacrificed at the time of apoptotic DC treatment. Atherosclerotic lesion progression in the aortic root was significantly attenuated by 38% in apop^{ox}-DC-treated

mice, whereas apop^{ctrl}-DC treatment had no significant effect on lesion progression (Figure 11A). Furthermore, we now observed a more stable lesion phenotype in apop^{ox}-DC-treated-mice: aortic root lesions were composed of 44.0±3.2% collagen, while those of control mice were composed of 33.2±3.9% and of apop^{ctrl}-DC-mice of 30.3±2.1% collagen (Figure 11B). Although inflammatory monocytes were again reduced, no effect on lesional macrophage content was observed (Figure 11C). The reduced T cell responses again resulted in a 64% decrease in T cells in the aortic root of apop^{ox}-DC-treated-mice. Interestingly also a 53% decrease of T cells was observed in apop^{ctrl}-DC-treated-mice, compared to control mice (Figure 11D).

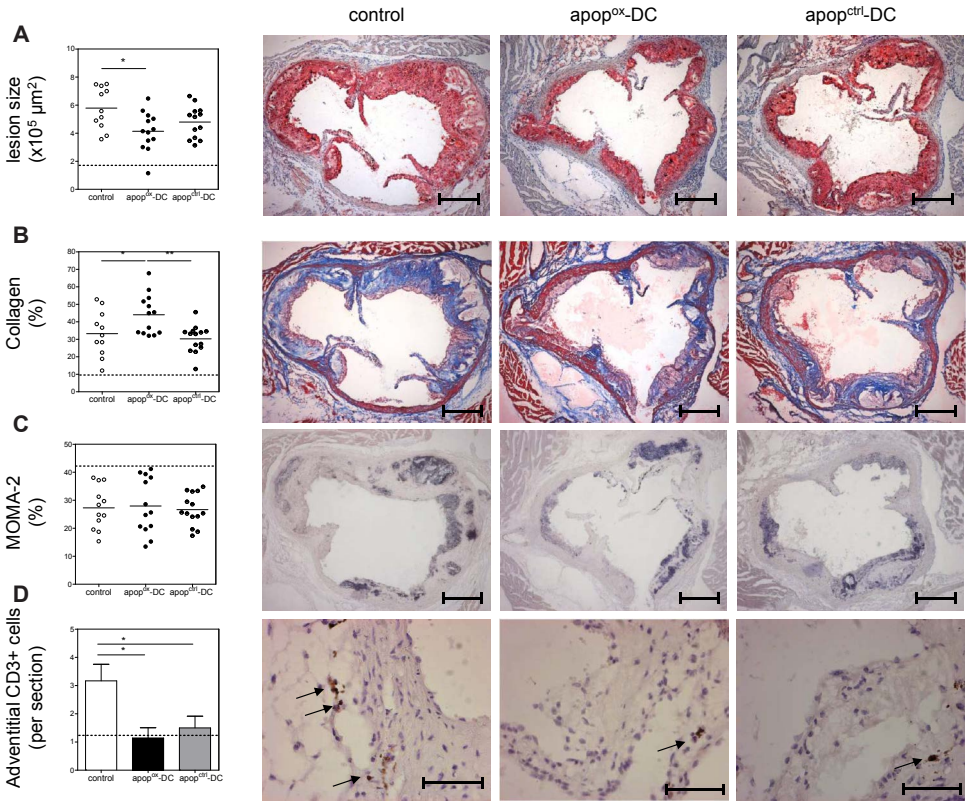


Figure 11. Apop^{ox}-DC-treatment reduces lesion progression and increases stability. A. At sacrifice, lesion size in the three valve area of the aortic root was determined; representative cross-sections stained with Oil-Red-O and hematoxylin are shown. **B.** Collagen content was determined by Massons' Trichrome staining. **C.** Macrophage content was determined by MOMA-2 staining. **D.** T cell numbers were determined by αCD3 staining. The dotted line indicates baseline values. All values are expressed as mean±SEM and are representative of all mice. Scale bar, 300 µm. *P<0.05, **P<0.01.

Discussion

Tolerogenic DC therapies have been investigated in autoimmune diseases for their potential to induce tolerance. In atherosclerosis, a study by Hermansson *et al.* demonstrated that adoptive transfer of apoB100-pulsed tolerogenic DCs, which induced Treg responses in vitro, could attenuate lesion development, by reducing $CD4^+$ T cell responses in vivo[29]. In this study we show that apoptotic DCs and in particular oxLDL-induced apoptotic-DCs may form an effective treatment for atherosclerosis by inducing tolerogenic DC responses in vivo. In addition, treatment with apoptotic DCs also dramatically modifies monocyte responses, which to our knowledge has not previously been described for apoptotic cell treatment. As oxLDL-induced apoptotic DCs contain oxLDL, the main antigen implicated in atherosclerosis, they are capable of transferring atherosclerosis-relevant antigenic peptides in the context of tolerogenic signals. While it needs to be established to what extent antigen-specific tolerance was induced, our results clearly show that oxLDL-induced apoptotic DCs are needed to modulate ongoing immune responses in established atherosclerosis.

We first established in vitro that uptake of apoptotic DCs modulates LPS responses by viable DCs and modulates T cell responses. However, only oxLDL-induced apoptotic DCs were able to increase IL-10 by viable DCs in response to LPS and induce Treg responses. While both oxLDL-induced and unpulsed apoptotic DCs reduced $CD8^+$ T cell proliferation and Th17 responses, only oxLDL-induced apoptotic DCs were able to decrease $CD4^+$ T cell proliferation and Th1 responses, indicating that the presence of oxLDL in apoptotic cells could more potently modulate T cell responses.

Next the effect of apoptotic DC-treatment on initial and advanced atherosclerotic lesions was assessed. In both initiation and progression of atherosclerosis, only $apop^{ox}$-DC-treated mice showed an induction of a tolerogenic phenotype in splenic DCs, resulting in an increase in Tregs and a decrease in T cell proliferation, likely via IL-10 and nitrite production. Similarly, we found increased Tregs in the circulation and the aorta of $apop^{ox}$-DC-treated-mice only. This translated into reduced numbers of T cells in the aortic root adventitia, indicating that inflammatory T cell responses and recruitment in $apop^{ox}$-DC-treated mice were attenuated. In $apop^{ctrl}$-DC-treated mice, a mild decrease in inflammatory splenic DCs was seen in initial atherosclerosis. This resulted in an overall reduced splenic T cell response as well as reduced adventitial T cells. However, the effects of $apop^{ctrl}$-DCs were milder compared to the $apop^{ox}$-DC-treated mice and an induction of Tregs was absent, indicating that presence of oxLDL potentiates the effect of apoptotic DCs on T cell responses and its presence in apoptotic DCs is needed to promote Treg responses.

Interestingly, $apop^{ox}$-DC-treatment affected both monocyte counts and inflammatory monocytes in initial and advanced atherosclerosis, while treatment with $apop^{ctrl}$-DCs only affected inflammatory monocytes in initial atherosclerosis. One week after treatment, Ly-$6C^{hi}$ monocytes were reduced, which was in line with a significant drop in CCL2 serum levels. However, only the $apop^{ox}$-DC-treatment resulted in a long-term decrease of Ly-$6C^{hi}$ and $CCR2^+$ monocytes, CCL2 serum levels, and decreased circulating monocytes. A study by Nahrendorf *et al.*[30] has shown that

both Ly-6C^{hi} and Ly-6C^{low} monocytes can ingest fluorescent nanoparticles, while a later study by Peng *et al.*[31] found that Ly-6C^{low} monocytes preferentially ingest apoptotic cells, although also Ly-6C^{hi} monocytes were found to ingest apoptotic cells. Ly-6C^{low} monocytes were found to migrate to the spleen where they differentiated into DCs, which were able to inhibit T cell responses[31]. However, it has been shown that Ly-6C^{hi} monocytes downregulate Ly-6C upon uptake of latex beads[32] and therefore also Ly-6C^{hi} monocytes could have substantially taken up apoptotic cells. Moreover, Ly-6C^{hi} and CCR2^{+} monocytes were found to be directly linked to the generation of CD103^{+} DCs in the lung[33]. This indicates that Ly-6C^{hi} and CCR2^{+} monocytes could also engulf apoptotic cells, migrate to the spleen and locally differentiate into CD103^{+} DCs, which seems to be augmented by oxLDL presence in apoptotic DCs. These studies indicate that apoptotic DC exposure and their uptake by monocytes may have directly affected Ly-6C expression. Indeed, our lab shows that monocytes co-cultured in vitro with apoptotic DCs downregulate Ly-6C (V. Frodermann and J. Kuiper, unpublished data). This, however, does not explain long-term effects on monocytes in apop^{ox}-DC-treated mice. Swirski *et al.*[34] and Tsou *et al.*[35] previously described the critical role of Ly-6C^{hi} and CCR2^{+} monocytes in atherosclerosis and their direct correlation with lesional macrophages. They show that the inflammatory environment prevents the production of Ly-6C^{low} monocytes[34]. On the other hand, CCL2$^{-/-}$ mice have decreased Ly-6C^{hi} monocytes[36]. Since we observed a decrease in the levels of inflammatory cytokines IL-6 and CCL2, this may explain the long-term reduction in circulating inflammatory Ly-6C^{hi} and CCR2^{+} monocytes in apop^{ox}-DC-treated mice. This long-term effect could also explain why we only see decreased lesional macrophages in apop^{ox}-DC-treated mice. The initial drop in inflammatory monocytes and CCL2 serum levels is sufficient to reduce initial lesion sizes in both apoptotic-DC-treated groups, which is in line with reduced lesions in CCL2$^{-/-}$ and CCR2$^{-/-}$ mice[36,37]. Apop^{ctrl}-DC treatment did not affect monocyte homeostasis in advanced atherosclerosis, again indicating that the presence of oxLDL is required to potentiate the effect on monocytes and to affect them in the advanced stages of disease.

However, reduced CCL2 serum levels and reduced monocyte responses do not affect macrophage content of pre-established lesions. Therefore, in contrast to early lesions, advanced lesions seem to be more affected by a general decrease in the inflammatory status and a decrease in T cell responses, while early lesions are more modulated by effects on monocyte responses. This is in line with earlier work from our group showing that CCR2 deficiency does not affect established lesions[38] and a recent study showing that macrophage content of established lesions is determined by local macrophage proliferation rather than influx of monocytes[39]. The pronounced effects of apop^{ox}-DCs on T cell responses and inflammation most likely contribute to the clear beneficial effects on pre-established lesions. Only apop^{ox}-DC-treatment increased lesion stability. This treatment induced an increased production of IL-10 by splenic DCs and initial increases of serum IL-10 levels were found upon treatment. IL-10 has been shown to promote lesion stability as both C57BL/6[40] and LDLr$^{-/-}$[41] mice deficient in IL-10 exhibit reduced collagen and lesion stability.

From our experiments, it is clear that oxLDL-induced apoptotic DCs are more

efficient in inducing anti-inflammatory responses than non-antigen-pulsed apoptotic DCs. Although our laboratory indicates that antigen processing and presentation by apoptotic DCs themselves are not required to reduce T cell responses, viable DCs could present antigens derived from oxLDL, transferred via apoptotic DCs, in a tolerogenic context, making these DCs more potent in inducing tolerance (V. Frodermann and J. Kuiper, unpublished data). Furthermore, it was found that both uptake of cholesterol and apoptotic cells results in a strong activation of LXR[42-44]. Indeed, we found that DC exposure to apop^{ox}-DCs resulted in a much stronger upregulation of ABCA1 and ABCG1, two target genes of LXR, than exposure to apop^{ctrl}-DCs. The activation of LXR has been shown to induce a tolerogenic phenotype in DCs, with decreased pro-inflammatory IL-12p70 and increased IL-10 production. Moreover, LXR-activated DCs were found to inhibit T cell activation and reduce IFN-γ production by T cells[45]. Interestingly, also ApoA-I activation of ABCA1 has been shown to reduce production of TNF-α and IL-6[46], which may further promote an anti-inflammatory phenotype of DCs. In addition to the increased IL-10 production by apop^{ox}-DC-exposed DCs, we also observed an upregulation of PD-L1 on these DCs. PD-L1 was shown to promote Treg development and maintenance[47] and this could contribute to the observed induction of Tregs preferentially by apop^{ox}-DC-treatment. Therefore, overall enhanced LXR activation through excess cholesterol might contribute to the enhanced anti-inflammatory effect of oxLDL-induced apoptotic DCs.

Apoptotic cell treatment was shown to be effective and safe in patients. In graft-versus-host disease, extracorporeal photopheresis has been employed to reduce immune responses. Up to 10% of the patient's circulating leukocytes are irradiated, resulting in apoptosis, and re-infused, which leads to Treg induction and disease amelioration, without inducing generalized immunosuppression[48]. Moreover, a recent phase I clinical trial in multiple sclerosis patients showed that treatment with myelin peptide cross-linked apoptotic splenocytes decreases antigen-specific T cell responses[49]. For atherosclerosis, we prove in this study that apoptotic DC treatment can significantly reduce lesion development, but that the presence of oxLDL in apoptotic DCs is needed to make them potent modulators of established atherosclerosis. Treatment with oxLDL-induced apoptotic DCs would offer a major advantage: LDL can be isolated from the patients, oxidized, and added to patient-derived DCs, providing tolerance induction by patient-specific LDL-derived peptides. This circumvents the search for relevant antigenic-peptides and ensures a patient-tailored treatment. Apop^{ox}-DC-treatment should therefore prove to be a very specific and efficient treatment of atherosclerosis.

References

1. Hansson, G. K. & Hermansson, A. The immune system in atherosclerosis. *Nat. Immunol.* 12, 204–12 (2011).
2. Angeli, V. *et al.* Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity* 21, 561–74 (2004).
3. Bobryshev, Y. V & Lord, R. S. Mapping of vascular dendritic cells in atherosclerotic arteries suggests their involvement in local immune-inflammatory reactions. *Cardiovasc. Res.* 37, 799–810 (1998).
4. Gallucci, S., Lolkema, M. & Matzinger, P. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* 5, 1249–55 (1999).
5. Albert, M. L. Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nat. Rev. Immunol.* 4, 223–31 (2004).
6. Ren, G. *et al.* Apoptotic cells induce immunosuppression through dendritic cells: critical roles of IFN-gamma and nitric oxide. *J. Immunol*. 181, 3277–84 (2008).
7. Patel, V. A. *et al.* Apoptotic cells, at all stages of the death process, trigger characteristic signaling events that are divergent from and dominant over those triggered by necrotic cells: Implications for the delayed clearance model of autoimmunity. *J. Biol. Chem.* 281, 4663–70 (2006).
8. Voll, R. E. *et al.* Immunosuppressive effects of apoptotic cells. *Nature* 390, 350–1 (1997).
9. Fadok, V. A. *et al.* Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* 101, 890–8 (1998).
10. Kim, S., Elkon, K. B. & Ma, X. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity* 21, 643–53 (2004).
11. Kushwah, R., Oliver, J. R., Zhang, J., Siminovitch, K. A. & Hu, J. Apoptotic dendritic cells induce tolerance in mice through suppression of dendritic cell maturation and induction of antigen-specific regulatory T cells. *J. Immunol.* 183, 7104–18 (2009).
12. McGaha, T. L., Chen, Y., Ravishankar, B., van Rooijen, N. & Karlsson, M. C. I. Marginal zone macrophages suppress innate and adaptive immunity to apoptotic cells in the spleen. *Blood* 117, 5403–12 (2011).
13. Liu, K. *et al.* Immune Tolerance After Delivery of Dying Cells to Dendritic Cells In Situ. *J. Exp. Med*. 196, 1091–1097 (2002).
14. Inaba, K. *et al.* Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* 188, 2163–73 (1998).
15. Ferguson, T. A. *et al.* Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. *J. Immunol.* 168, 5589–95 (2002).
16. Adler, A. J. *et al.* CD4+ T Cell Tolerance to Parenchymal Self-Antigens Requires Presentation by Bone Marrow-derived Antigen-presenting Cells. *J. Exp. Med.* 187, 1555–1564 (1998).
17. Perruche, S. *et al.* CD3-specific antibody-induced immune tolerance involves transforming growth factor-beta from phagocytes digesting apoptotic T cells. *Nat. Med.* 14, 528–35 (2008).
18. Elliott, M. R. & Ravichandran, K. S. Clearance of apoptotic cells: implications in health and disease. J. Cell Biol. 189, 1059–70 (2010).
19. Ait-Oufella, H. *et al.* Lactadherin deficiency leads to apoptotic cell accumulation and accelerated atherosclerosis in mice. *Circulation* 115, 2168–77 (2007).
20. Ait-Oufella, H. *et al.* Defective mer receptor tyrosine kinase signaling in bone marrow cells promotes apoptotic cell accumulation and accelerates atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 28, 1429–31 (2008).
21. Thorp, E., Cui, D., Schrijvers, D. M., Kuriakose, G. & Tabas, I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice. Arterioscler. Thromb. Vasc. Biol. 28, 1421–8 (2008).
22. Bhatia, V. K. *et al.* Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am. J. Pathol.* 170, 416–26 (2007).
23. Foks, A. C. *et al.* T-Cell Immunoglobulin and Mucin Domain 3 Acts as a Negative Regulator of Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 33, 2558–65 (2013).
24. Ren, Y. *et al.* Apoptotic cells protect mice against lipopolysaccharide-induced shock. *J. Immunol.* 180, 4978–85 (2008).
25. Turley, D. M. & Miller, S. D. Peripheral tolerance induction using ethylenecarbodiimide-fixed APCs uses both direct and indirect mechanisms of antigen presentation for prevention of experimental autoimmune encephalomyelitis. *J. Immunol.* 178, 2212–20 (2007).
26. Redgrave, T. G., Roberts, D. C. & West, C. E. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* 65, 42–9 (1975).

27. Van Berkel, T. J., De Rijke, Y. B. & Kruijt, J. K. Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. *J. Biol. Chem.* 266, 2282–9 (1991).
28. Huang, F. P. *et al.* A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J. Exp. Med.* 191, 435–44 (2000).
29. Hermansson, A. *et al.* Immunotherapy with tolerogenic apolipoprotein B-100-loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice. *Circulation* 123, 1083–91 (2011).
30. Nahrendorf, M. *et al.* The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J. Exp. Med*. 204, 3037–47 (2007).
31. Peng, Y., Latchman, Y. & Elkon, K. B. Ly6C(low) monocytes differentiate into dendritic cells and cross-tolerize T cells through PDL-1. *J. Immunol.* 182, 2777–85 (2009).
32. Tacke, F. *et al.* Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. J. Exp. Med. 203, 583–97 (2006).
33. Jakubzick, C. *et al.* Blood monocyte subsets differentially give rise to CD103+ and CD103- pulmonary dendritic cell populations. *J. Immunol.* 180, 3019–27 (2008).
34. Swirski, F. K. *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J. Clin. Invest.* 117, 195–205 (2007).
35. Tsou, C.-L. *et al.* Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest.* 117, 902–9 (2007).
36. Combadière, C. *et al.* Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation* 117, 1649–57 (2008).
37. Boring, L., Gosling, J., Cleary, M. & Charo, I. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 394, 894–897 (1998).
38. Guo, J. *et al.* Repopulation of apolipoprotein E knockout mice with CCR2-deficient bone marrow progenitor cells does not inhibit ongoing atherosclerotic lesion development. *Arterioscler. Thromb. Vasc. Biol.* 25, 1014–9 (2005).
39. Robbins, C. S. *et al.* Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat. Med.* 19, 1166–72 (2013).
40. Mallat, Z. et al. Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* 85, e17–24 (1999).
41. Potteaux, S. *et al.* Leukocyte-derived interleukin 10 is required for protection against atherosclerosis in low-density lipoprotein receptor knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 24, 1474–8 (2004).
42. Shiffman, D. *et al.* Large scale gene expression analysis of cholesterol-loaded macrophages. *J. Biol. Chem.* 275, 37324–32 (2000).
43. Kiss, R. S., Elliott, M. R., Ma, Z., Marcel, Y. L. & Ravichandran, K. S. Apoptotic cells induce a phosphatidylserine-dependent homeostatic response from phagocytes. *Curr. Biol.* 16, 2252–8 (2006).
44. A-Gonzalez, N. *et al.* Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity* 31, 245–58 (2009).
45. Geyeregger, R. *et al.* Liver X receptors regulate dendritic cell phenotype and function through blocked induction of the actin-bundling protein fascin. *Blood* 109, 4288–95 (2007).
46. Tang, C., Liu, Y., Kessler, P. S., Vaughan, A. M. & Oram, J. F. The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. *J. Biol. Chem.* 284, 32336–43 (2009).
47. Francisco, L. M. *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* 206, 3015–29 (2009).
48. Biagi, E. *et al.* Extracorporeal photochemotherapy is accompanied by increasing levels of circulating CD4+CD25+GITR+Foxp3+CD62L+ functional regulatory T-cells in patients with graft-versus-host disease. *Transplantation* 84, 31–9 (2007).
49. Lutterotti, A. *et al.* Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. *Sci. Transl. Med.* 5, 188ra75 (2013).

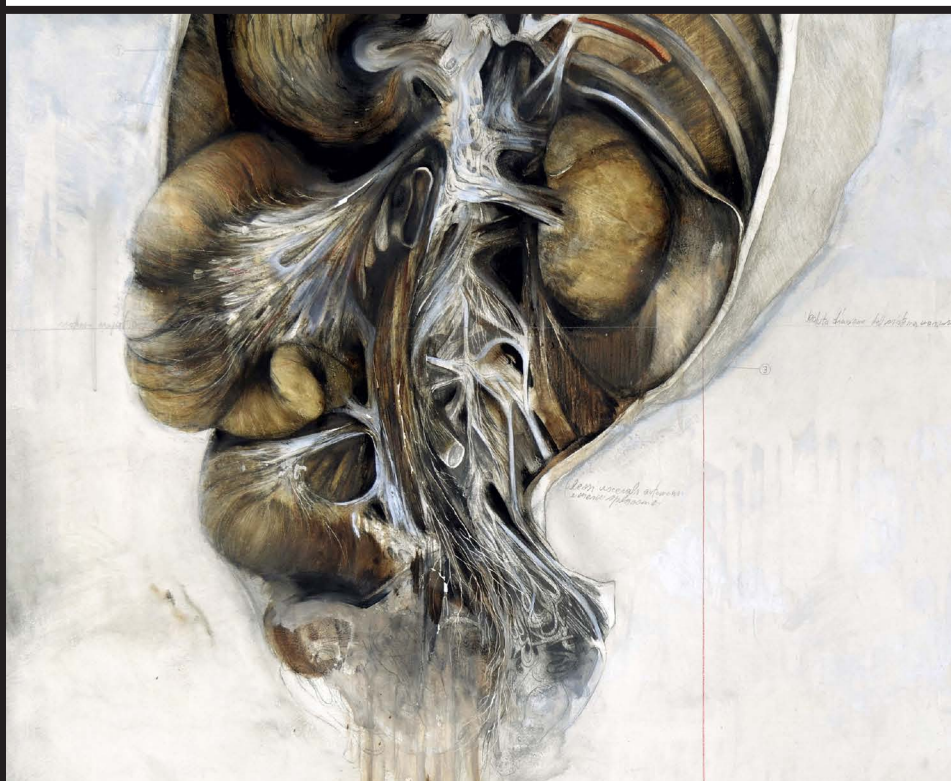
Vanessa Frodermann[1]
Julia Ober-Blöbaum[2,3]
Amanda C. Foks[1,4]
Gijs H.M. van Puijvelde[1]
Laura Wierts[1]
Peter J. van Santbrink[1]
Mariette N.D. ter Borg[1,5]
Björn Clausen[2,3]
Johan Kuiper[1]
Saskia C.A. de Jager[1,6]

3

[1] Division of Biopharmaceutics, LACDR, Leiden University, Leiden, The Netherlands
[2] Department of Immunology, Erasmus MC, Rotterdam, The Netherlands
[3] Present address: Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Germany
[4] Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, USA
[5] Present address: Department of Hematology, Erasmus University Medical Centre, Rotterdam, The Netherlands
[6] Present address: Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

β-Catenin Signaling in Dendritic Cells Reduces Atherosclerosis

Manuscript in preparation.



Abstract

Objective A driving force behind atherosclerotic disease is the chronic inflammatory response. It has been shown that immunomodulation by tolerogenic dendritic cell (DC) therapy is a feasible approach to reduce atherosclerosis. While it is mostly unclear what molecular pathways induce tolerogenic DCs, β-catenin has been implicated in this process. We therefore assessed whether induction of tolerogenic DCs, by means of β-catenin stabilization (CD11c-βcat^{EX3} DCs), can reduce atherosclerosis.

Methods and Results CD11c-βcat^{EX3} DCs possess similar antigen uptake capacities, but displayed enhanced CD40, CD86 and CCR7 expression and reduced production of pro-inflammatory cytokines TNF-α and IL-6 upon LPS exposure. Consequently, CD11c-βcat^{EX3} DCs inhibited T cell responses by 90% as a result of a 33% increased regulatory T cell (Treg) response.

To establish the effect on atherosclerosis, we generated CD11c-βcat^{EX3}/LDLr$^{-/-}$ bone marrow chimera and for a more clinical approach adoptively transferred CD11c-βcat^{EX3} DCs into LDLr$^{-/-}$ mice. Both CD11c-βcat^{EX3}/LDLr$^{-/-}$ chimeras and CD11c-βcat^{EX3} DC-treated mice experienced significantly less atherosclerosis (26% and 21% reduction, respectively). Additionally, CD11c-βcat^{EX3}/LDLr$^{-/-}$ chimeras had reduced necrotic cores and more stable collagen-rich lesions, while this was not observed after adoptive transfer of CD11c-βcat^{EX3} DCs. In both experiments we detected reduced splenic T cell proliferative capacity indicating reduced T cell responses. While CD11c-βcat^{EX3}/LDLr$^{-/-}$ chimeras showed an overall significant 1.6-fold increase of circulating Tregs, CD11c-βcat^{EX3} DC-treated mice only showed a significant 2-fold increase directly after treatment.

Conclusion We show here for the first time that β-catenin stabilization in DCs can effectively generate tolerogenic DCs that have the capacity to inhibit atherosclerotic lesion formation. This provides a new strategy to increase the potential of tolerogenic DC therapies for any inflammatory autoimmune disease.

Introduction

About two decades ago, cells with a Dendritic Cell (DC)-like morphology were found in human atherosclerotic lesions. DCs in the lesions were found in close proximity to other leukocytes, already indicating their role in ongoing inflammation[1]. During atherosclerosis development the amount of DCs increase in the lesions[2,3] and can eventually comprise about 10-30% of the total cell infiltrate[4]. DCs are actively involved in the uptake of antigens in the vessel wall, and facilitate an antigen-specific T cell response[5]. Lipid uptake has been shown to affect DC mobilization and could therefore impact DC-T cell interactions. It has been shown that under hypercholesterolemic conditions DCs cannot migrate out of the lesions[6] and will locally form clusters with T cells, a phenomenon associated with plaque destabilization[7]. Ex vivo studies have found that DCs isolated from aortas or DCs seeded in artificial human arteries were able to activate T cells[8,9]. On the other hand, it has also been recently shown that regulatory T cell (Treg)-inducing DCs are present in atherosclerotic lesions[10,11]. This implies that DCs present in the lesions can be involved in direct activation but also modulation of T cell responses. Moreover, it has been suggested that activation of T cells could also occur by DCs that have captured circulating atherosclerosis-specific antigens outside of atherosclerotic lesion, resulting in a systemic modulation of T cell responses[12].

Due to their critical role in initiating adaptive immune responses, we and others have addressed the possibility to modulate DCs in their capacity to induce T cell responses as a treatment option for atherosclerosis. Blocking antibodies for several co-stimulatory molecules were able to modulate T cell responses and reduce atherosclerosis[13,14]. Additionally we have shown that adoptive transfer of oxLDL-pulsed mature DCs reduces atherosclerosis by inducing oxLDL-specific antibodies[15] and that oxLDL-induced apoptotic DCs also reduce atherosclerosis by inducing tolerogenic DCs and Tregs and modulating monocyte responses[16]. Furthermore, Hermansson *et al.* showed that adoptive transfer of apoB100-pulsed tolerogenic DCs reduces atherosclerotic lesion development by induction of Treg responses [17]. Overall, these studies provide promising results and suggest that induction of a tolerogenic phenotype in DCs or the adaptive transfer of tolerogenic DCs is a promising approach to reduce atherosclerosis.

The term tolerogenic DCs is broad and describes a whole plethora of DCs with the capacity to induce tolerance, i.e. induction of Tregs and reduction of inflammatory responses. Multiple studies have established ways to induce or maintain a tolerogenic phenotype in DCs by culturing DCs with immunosuppressive components including cytokines[18], estriol[19], 1α,25-dihydroxyvitamin D3[20], or vasoactive intestinal peptide[21]. Interestingly, many of these factors are also produced in tissues under physiological conditions. Nonetheless, it remains unclear which exact intracellular signals are needed to promote and maintain the tolerogenic phenotype of DCs.

β-catenin was first discovered for its role in E-Cadherin cell-cell adhesion complexes[22]. While E-cadherin expression on DCs was found to mark inflammatory DCs in the intestine[23], disruption of these E-cadherin complexes was found to induce

a tolerogenic phenotype of DCs. This mechanical stimulation was shown to result in β-catenin release and a spontaneous upregulation of co-stimulatory molecules and chemokine receptors in DCs, without inflammatory cytokine production[24,25]. In mice, these tolerogenic DCs were able to induce peripheral tolerance and protect from experimental autoimmune encephalomyelitis[24]. Interestingly, β-catenin was also found to be crucially involved in maintaining the tolerogenic phenotype of intestinal DCs and thereby intestinal homeostasis. Mice with a CD11c-specific deficiency of β-catenin showed defects in Treg homeostasis and were more susceptible to inflammatory bowel disease[26]. All this indicates that β-catenin signalling is critical for the development of a tolerogenic phenotype in DCs.

In this study, we explored the potential of inducing tolerogenic DCs, by stable expression of β-catenin, to prevent atherosclerotic lesion development. For this we made use of CD11c-βcat^{EX3} mice, which have a constitutively active β-catenin in all CD11c^{+} cells, mostly DCs. We describe here that β-catenin stabilization in all CD11c^{+} cells of LDLr$^{-/-}$ mice, as well as adoptive transfer of CD11c-βcat^{EX3} DCs into LDLr$^{-/-}$, reduces lesion development by induction of Tregs and reduction of the overall inflammatory status in mice. Stabilization of β-catenin in DCs therefore has therapeutic potential and may help to improve DC-based therapies for atherosclerosis and other autoimmune diseases.

Material and Methods

Animals

LDLr$^{-/-}$ mice were originally obtained from The Jackson Laboratory and housed and bred at the animal facility of the Gorlaeus Laboratories. Bone marrow donors, CD11c-βcat^{EX3} and control non-transgenic littermates, were generated by breeding β-cat$^{EX3fl/fl}$ mice, carrying homozygous loxP site insertion flanking exon 3 of β-catenin gene, with CD11c-Cre transgenic mice on the C57BL/6 background. The offspring were genotyped for CD11c-Cre by PCR using the following Cre primers: 5'-GGACATGTTCAGGGATCGCCAGGCG-3' and 5'-GCATAACAGTGAAACAGCATTGCTG-3'. The floxed alleles for β–catenin were identified by PCR as previously described[27,28]. All mice were housed under standard laboratory conditions and were fed a regular chow diet or a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services). Diet and water were administered *ad libitum*. All animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University and performed at the animal facility of the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in compliance with Dutch government guidelines.

Atherosclerosis

To induce bone marrow aplasia, female LDLr$^{-/-}$ recipient mice of 8-10 weeks old were exposed to two doses of 4.5 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation with two hours break in between using an Andrex Smart 225 Röntgen source (YXLON International) with a 6-mm aluminum filter 1 day before the transplantation (n = 11

per group). Bone marrow was isolated by flushing the femurs and tibias from CD11c-βcat^{EX3} mice or control littermates with PBS. Single-cell suspensions were obtained by passing the cells through a 70 μm cell strainer (VWR International). Irradiated recipients received 5×10^6 bone marrow cells by *intravenous* injection. After an 8 week recovery period, mice were fed a WTD for another 8 weeks, after which animals were sacrificed. Body weight was monitored weekly and cholesterol levels were checked regularly throughout the study.

For adoptive transfer, female LDLr$^{-/-}$ mice were treated with 3 *intravenous* injections of PBS, 1.5 x 10^6 control DCs, or 1.5 x 10^6 CD11c-βcat^{EX3} DCs every other day prior to eight weeks WTD. DCs were loaded with oxLDL and matured with LPS as previously described[15].

Isolation, Loading and Stimulation of BM-derived and splenic DCs

DCs were cultured as previously described[15]. Briefly, bone marrow cells were isolated from the tibias and femurs of C57BL/6, CD11c-βcat^{EX3} mice or their littermates as indicated. The cells were cultured for ten days at 37°C and 5% CO_2 in 10 cm non-tissue culture treated petri dishes (Greiner Bio-One) in IMDM supplemented with 10% FCS, 100 U/mL penicillin/streptomycin, 2mM glutamax (all obtained from PAA) and 20 μm β-mercaptoethanol (Sigma-Aldrich) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). DC purity was assessed by CD11c expression (flow cytometry) and routinely found to be above 95%.

For in vitro DC cultures, 2 x 10^6 DCs were plated in 2 cm non-tissue culture treated petri dishes (Greiner Bio-One) to determine cytokine responses and flow cytometry analysis upon indicated amounts of LPS and for antigen uptake assessment. For visualization of ovalbumin uptake, DCs were cultured with the self-quenching DQ-Ovalbumin, which upon proteolytic digestion exhibits a bright green fluorescence (Molecular Probes), for 1 hour at 37ºC. For determination of unspecific background some cells were kept at 4ºC. Cells were extensively washed to remove extracellular DQ-Ovalbumin and uptake was determined by flow cytometry. For the assessment of oxLDL-uptake, DCs were cultured with indicated amounts of oxLDL for 24 hours and cytospins were made to determine Oil-red O positive area. Levels of IL-12p70, IL-6, TNF-α (all eBiosciences) and IL-10 (BD Biosciences) were measured in the supernatants of LPS-stimulated DCs by ELISA, according to manufacturer's protocol.

For isolation of splenic DCs, spleens were harvested after sacrifice and DCs were isolated with a Dendritic Cell Enrichment Set, according to manufacturer's protocol (BD Biosciences).

Proliferation assay and co-culture

For DC-T cell co-cultures (n=3 per group), 0.5 x 10^5 DCs/well were cultured with 2 x 10^5 T cells/well (1:5) in triplicate in 96-well round-bottom plates (Greiner Bio-One). CD4$^+$ T cells were isolated from a hyperlipidemic non-transplanted LDLr$^{-/-}$ mouse with a CD4$^+$ T Lymphocyte Enrichment Set, according to manufacturer's protocol (BD Biosciences). For splenocyte proliferation (n=6 per group), 2 x 10^5 splenocytes/well were cultured in quintuplicates in 96-well round-bottom plates (Greiner Bio-

One). Both cultures were done in the presence and absence of anti-CD3 (2 µg/mL, eBioscience) for 72 hours in complete RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2mM glutamax (all obtained from PAA) and 20 µm β-mercaptoethanol (Sigma-Aldrich). Proliferation of splenocytes was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences) for the last 16 hours of culture. 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). Responses are expressed as: stimulation index (SI) = mean dpm of quintuplicate cultures with stimulation/ mean dpm of quintuplicate cultures without stimulation. Parallel cultures in triplicates and DC-T cell co-cultures were used to determine the amount of T cell subsets by flow cytometry and to determine cytokine levels in the supernatant by Cytometric Bead Array.

Flow Cytometry

After the start of WTD, blood was drawn at indicated time points and at sacrifice spleen and heart lymph nodes were harvested. For flow cytometry analysis, single cell suspensions of n=6 mice per group were obtained by using a 70 µm cell strainer (VWR International). Red blood cells in the spleen and blood were lysed with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently 3 x 10^5 cells per sample were stained with CD4-PerCp (BD Biosciences, RM4-5), FoxP3-PE (eBioscience, FJK-16s), T-bet-eFluor 660 (eBioscience, eBio4B10), IL-10-APC (eBioscience, JES5-16E3), IL-12p40 (eBioscience, C17.8), CD86-APC (eBioscience, GL1), CD40-FITC (eBioscience, HM40-3), MHCII-FITC (eBioscience, M5/114.15.2), CCR7-APC (eBioscience, 4B12). For intracellular stainings, cells were fixed and permeabilized according to the manufacturer's protocol (eBioscience). FACS analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (BD Biosciences).

Real-time quantitative PCR

Gene expression was analyzed by real-time quantative PCR as previously described[29]. Briefly, mRNA was isolated from splenic DCs and total spleens using the guanidium isothiocyanate (GTC) method according to Chomczynski and Sacchi[30] and reverse transcribed using RevertAid M-MuLV reverse transcriptase. Quantitative gene expression analysis was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using SYBR green technology (Eurogentec). Ribosomal phosphoprotein 36B4, hypoxanthine phosphoribosyltransferase (HPRT) and ribosomal protein L27 (Rpl27) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of 36B4, HPRT and Rpl27 and raising 2 to the power of this difference. Genes that exhibited a Ct value of >35 were considered not detectable. The average Ct of the three housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the expression of the separate housekeeping genes. For used primer pairs refer to Table 1.

Gene	Forward	Reverse
36B4	CTGAGTACACCTTCCCACTTACTGA	CGACTCTTCCTTTGCTTCAGCTTT
CCR7	CGTGCTGGTGGTGGCTCTCCT	ACCGTGGTATTCTCGCCGATGTAGTC
CD40	GCACCAGCAAGGATTGCG	TCCATAACTCCAAAGCCAGGG
CD86	GTTAGAGCGGGATAGTAACGCTGA	TGCACTTCTTATTTCAGGCAAAGCA
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
IL-10	GGGTGAGAAGCTGAAGACCCTC	TGGCCTTGTAGACACCTTGGTC
IL-12 (p35)	CCAAACCAGCACATTGAAGA	CTACCAAGGCACAGGGTCAT
IL-4	GAACGAGGTCACAGGAGAAGGG	CCTTGGAAGCCCTACAGACGAG
IL-6	AGACAAAGCCAGAGTCCTTCAGAGA	GGAGAGCATTGGAAATTGGGGTAGG
IL-27	CGATTGCCAGGAGTGAACC	AGTGTGGTAGCGAGGAAGCA
MHCII	CTCACCTTCATCCCTTCTGACGA	CTGACATGGGGGCTGGAATCT
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
TGF-β	AGGGCTACCATGCCAACTTCT	GCAAGGACCTTGCTGTACTGTGT

Table 1. Primer Pairs. The expression of genes was determined relative to the average expression of the three household genes: ribosomal protein 36B4, hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). Abbreviations: CCR7, C-C chemokine receptor type 7; MHCII, major histocompatibility complex;TGF-β, transforming growth factor beta.

Histological analysis

To determine atherosclerotic plaque size, 10 μm cryosections of the aortic root were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). Corresponding sections were stained for collagen fibers using the Masson's Trichrome method (Sigma-Aldrich) or immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b, diluted 1:1000, Serotec). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100, Sigma-Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Dako) as enzyme substrates. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, ThermoScientific). BrightVision anti-rabbit-HRP was used as secondary antibody (Immunologic). The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. The section with the largest lesion and four flanking sections were analyzed for lesion size, collagen content, necrotic area, and MOMA-2, and two sections were analyzed for T cell content. The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2-positive area by the total lesion surface area. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

Statistical analysis

Values are expressed as mean±SEM. An unpaired two-tailed Student T-test was used to compare normally distributed data between two groups of animals. Data of three groups were analyzed with one-way ANOVA and data of two or more groups with more than one variable were analyzed by two-way ANOVA, both followed by Bonferroni *post hoc* testing. Statistical analysis was performed using the Instat3 software. Probability values of $P<0.05$ were considered significant.

Results

CD11c-βcat^{EX3} DCs are tolerogenic and induce Tregs in vitro

To determine the effect of β-catenin stabilization on DC phenotype and function, we cultured BM-derived DCs from CD11c-βcat^{EX3} mice and their littermates. We show here that CD11c-βcat^{EX3} DCs and control DCs possess similar phagocytotic capacities as they take up oxLDL and DQ ovalbumin to a similar extent (Figure 1A). Furthermore, we stimulated DCs with LPS to determine the effect of β-catenin stabilization on cell maturation. While we found no differences in the absolute amount of CD86, CD40, MHCII, and CCR7 positive DCs (data not shown), we did observe differences on cellular level. CD11c-βcat^{EX3} DCs showed a significant increase of average CD86, CD40 and CCR7 expression per cell as determined by flow cytometry (Figure 1B).

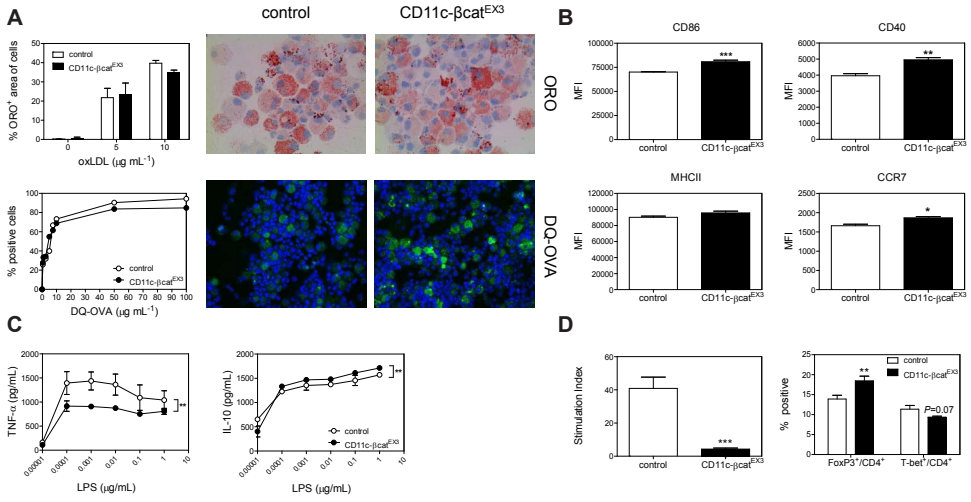


Figure 1. CD11c-βcatEX3 DCs are tolerogenic and induce Tregs in vitro. Bone marrow-derived DCs were generated from non-transgenic littermates (control) or from CD11c-βcat^{EX3} mice (CD11c-βcat^{EX3}). **A.** Antigen-uptake was determined by Oil-red-O staining (oxLDL) and flow cytometry (DQ-Ovalbumine). **B.** DCs were stimulated for 24 hours with 1 μg/mL LPS. Mean fluorescence intensity (MFI) of CD86, CD40, MHCII and CCR7 was determined by flow cytometry. **C.** TNF-α and IL-10 production were determined by ELISA. **D.** Splenocytes from control (n=3) or CD11c-βcat^{EX3} mice (n=3) were cultured in the presence of αCD3 for 72 hours. We pre-gated on CD4$^+$ T cells and determined Tregs (FoxP3$^+$) and Th1 cells (T-bet$^+$) by flow cytometry. Proliferation was assessed by ^{3}H-thymidine incorporation in dividing cells and is expressed as the stimulation index. All values are expressed as mean±SEM and are representative of three independent experiments done in triplicate. *P<0.05, **P<0.01, ***P<0.001.

We subsequently assessed cytokine production by the CD11c-βcat^{EX3} DCs. In a dose-dependent response to LPS, TNF-α levels were significantly decreased at all used LPS concentrations, with an average of 22% and IL-10 was slightly increased by 10% on average (Figure 1C). These results suggest that β-catenin stabilization results in a more phenotypically mature and tolerogenic DC phenotype upon activation.

Since we observed differences in cytokine production and amount of co-stimulatory molecules expressed per DC, we further wanted to determine whether this would translate into functional effects on T cell responses. Therefore, we cultured splenocytes from CD11c-βcat^{EX3} or control mice for 72 hours in the presence of αCD3, mimicking antigen-presentation by DCs, whereas the other two signals (co-stimulation and cytokines) needed for T cell expansion are intrinsic to the present DCs. We found that T cell proliferation was significantly reduced by 90% in the CD11c-βcat^{EX3} group compared to the control group. This was due to a significant increase in Treg by 33% and a concomitant trend to a decrease in Th1 induction (Figure 1D).

CD11c-βcat^{EX3} chimeras have reduced lesion sizes and show a trend to increased stability

To assess the effect of tolerogenic DCs with a β-catenin stabilization on atherosclerotic disease, we generated CD11c-βcat^{EX3}/LDLr$^{-/-}$ bone marrow chimeras (which we will refer to as CD11c-βcat^{EX3} mice) or as a control reconstituted LDLr$^{-/-}$ with bone marrow from their littermate controls. After a recovery period of eight weeks, mice were put on a Western-type diet (WTD) for an additional eight weeks to induce lesion formation. We confirmed the stabilization of β-catenin in CD11c^{+} cells by flow cytometry and found a highly significant overexpression of β-catenin when pre-gating on CD11c^{+} cells in the blood, draining lymph nodes of the heart (hLNs), and spleen, confirming the stabilization of β-catenin in CD11c-βcat^{EX3} mice (Figure 2).

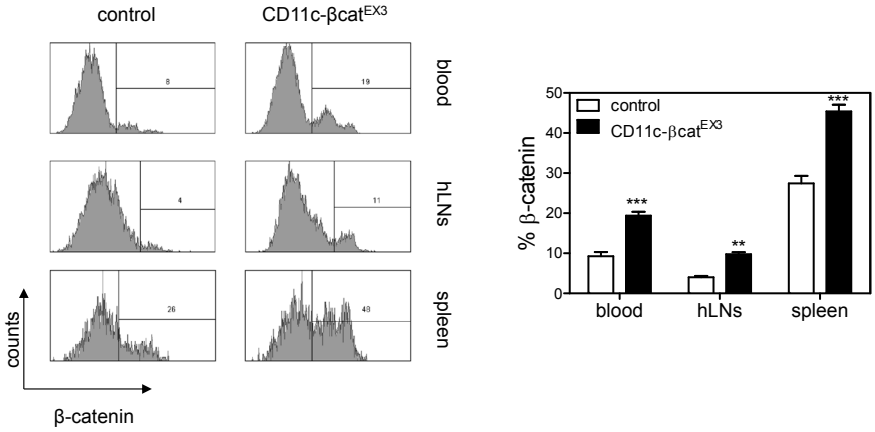


Figure 2. CD11c-βcat^{EX3} mice show increased β-catenin stabilization in CD11c^{+} cells. LDLr$^{-/-}$ mice reconstituted with either bone marrow from non-transgenic littermates (control, n=11) or from CD11c-βcat^{EX3} mice (CD11c-βcatEX3, n=11). After eight weeks recovery period, mice were put on a WTD for another eight weeks. β-catenin expression in CD11c^{+} cells was determined by flow cytometry in blood, hLN, and spleen at the end of the experiment. Representative histograms are shown. All values are expressed as mean±SEM and are representative of five mice.**P<0.01, ***P<0.001.

We observed in vitro that CD11c-βcat^{EX3} mice have overall more anti-inflammatory DCs, reduced amounts of CD4^{+} T cells, increased amounts of circulating Tregs and a reduction in Th1 responses. In line with this, we observed a significant decrease in aortic root lesion size of 26% in CD11c-βcat^{EX3} mice compared to control mice (control: 5.2 x 10^5 ± 0.3 x 10^5 µm versus CD11c-βcat^{EX3}: 3.8 x 10^5 ± 0.5 x 10^5 µm; Figure 3A). Moreover, lesions showed a trend towards an increased stability, with 25% more collagen (control: 26.6 ± 1.7% versus CD11c-βcat^{EX3}: 32.8 ± 2.8%; Figure 3B). The necrotic core size of lesions was significantly reduced by 46%, indicating an overall reduced inflammatory status of lesions in the CD11c-βcat^{EX3} mice (Figure 3C). Lesional macrophage content did not differ between the groups and was on average 40% (Figure 3D). Also the amount of lesional T cells did not differ, which could indicate increased recruitment of Tregs and reduced effector T cell recruitment (Figure 3E).

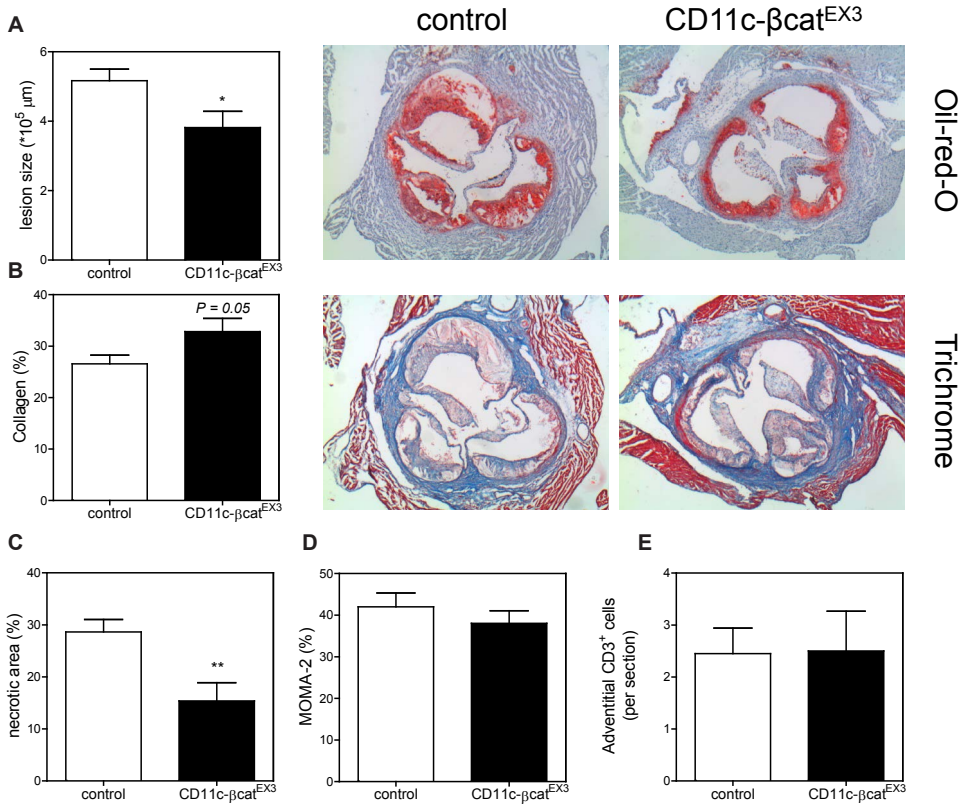


Figure 3. CD11c-βcat^{EX3} mice show reduced lesion formation, increased stability and reduced necrotic cores. A. Lesion formation in the three-valve area of the aortic root was assessed by Oil-Red-O and hematoxylin staining. Representative cross-sections are shown. **B.** Sections of the aortic root were stained for collagen fibers using Masson's trichrome staining. The percentage of collagen relative to lesion size was determined. Representative cross-sections are shown. **C.** The necrotic core was defined as the acellular, debris-rich plaque area as percentage of total plaque area in the trichrome staining. **D.** Macrophage content was assessed by MOMA-2 staining as percentage of total lesion area. **E.** T cell numbers were determined by αCD3 staining. All values are expressed as mean±SEM and are representative of all mice.*P<0.05, **P<0.01.

The reduction in lesion size was not due to differences in body weight or due to effects on lipid metabolism as total serum cholesterol levels and cholesterol distribution between the different lipoprotein fractions was not different between the groups (data not shown).

CD11c-βcat^{EX3} DCs are tolerogenic in vivo and can induce Tregs ex vivo

After eight weeks WTD, the phenotype of splenic DCs was assessed by flow cytometry. No striking phenotypical changes were observed in CD11c-βcat^{EX3} mice. The percentage of CD86 positive DCs was significantly increased, but no effect on CD40, MHCII and CCR7 DCs was observed (Figure 4A). However, when we isolated splenic DCs from CD11c-βcat^{EX3} mice, they showed reduced mRNA levels of pro-inflammatory cytokines compared to control DCs. IL-6 expression was significantly reduced by 85% and TNF-α was reduced although not significantly by 25%. Interestingly, IL-27 expression, which limits Tregs, was reduced by 33% (Figure 4B). No significant changes were observed in the expression of anti-inflammatory IL-10 and TGF-β (Figure 4C). This suggests that DCs in CD11c-βcat^{EX3} mice assume a less inflammatory phenotype and maintain a more tolerogenic phenotype compared to control DCs upon WTD.

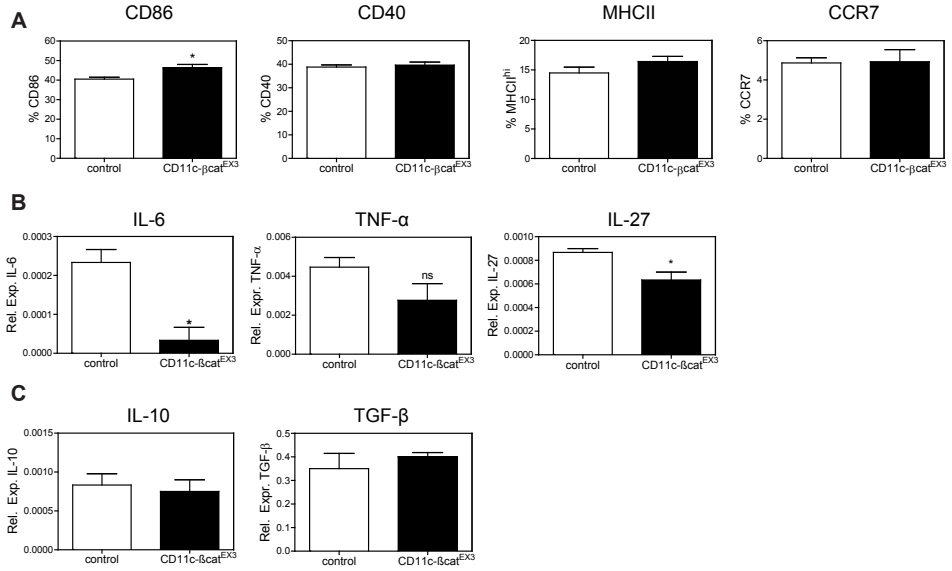


Figure 4. CD11c-βcat^{EX3} DCs assume a less inflammatory phenotype upon WTD. A. At sacrifice, splenic DCs were isolated and CD86, CD40, MHCII and CCR7 expression was determined by flow cytometry. **B.** Relative expression of pro-inflammatory IL-6, TNF-α and IL-27 was determined by qPCR. **C.** Relative expression of anti-inflammatory IL-10 and TGF-β was determined by qPCR. All values are expressed as mean±SEM and are representative of five mice.*P<0.05.

CD11c-βcat^{EX3} chimeras show decreased absolute CD4$^+$ T cell numbers and increased Tregs in the circulation

Once mice were put on a WTD, we monitored CD4$^+$ T cell and Treg numbers in the circulation to establish if these were affected by β-catenin stabilization in DCs. Four weeks after the start of WTD, when maximum cholesterol levels in the blood were

reached, a significant 48% drop in circulating CD4+ T cell numbers was observed in the CD11c-βcat^{EX3} mice. In parallel, a 62% increase of circulating Tregs in CD11c-βcat^{EX3} mice was seen, which likely is associated to the more tolerogenic phenotype of DCs in these mice (Figure 5A). After eight weeks of WTD, a significant 50% reduction of CD4+ T cells in the circulation (Figure 5A), a significant 17% reduction of CD4+ T cells in the spleen and a non-significant 13% decrease in the hLNs remained in CD11c-βcat^{EX3} mice compared to control mice (Figure 5B). We found a significant 1.5-fold induction of Tregs in the circulation, but no significant changes in the spleens and hLNs of CD11c-βcat^{EX3} mice (Figure 5A and C).

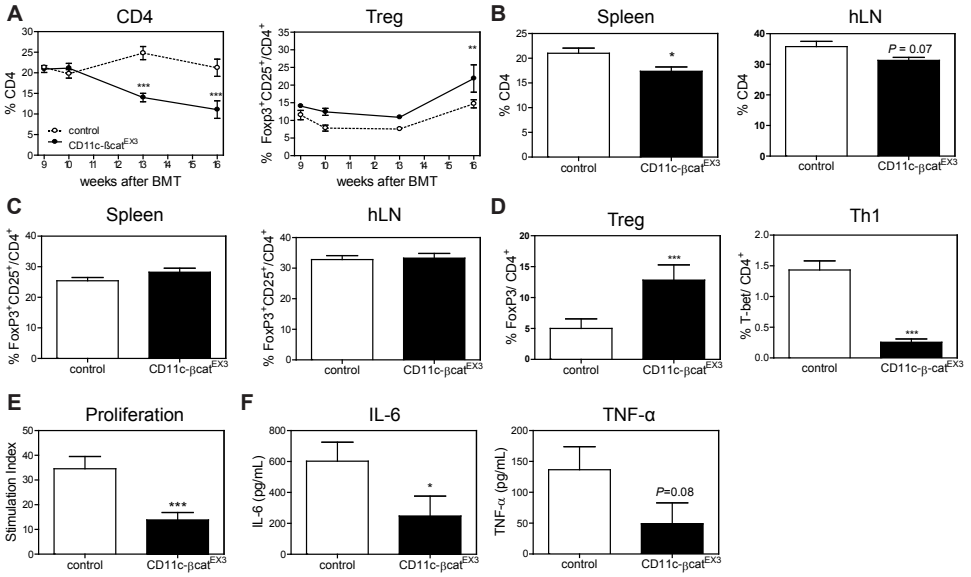


Figure 5. CD11c-βcat^{EX3} mice show decreased CD4+ T cell responses and increased Treg amounts. A. Circulating CD4+ T cells and Tregs (FoxP3+CD25+ within CD4+) were determined in the circulation throughout the experiment. **B.** At sacrifice, CD4+ T cells and **C.** Tregs were determined in the spleen and draining lymph nodes of the heart (hLN) by flow cytometry. **D.** Splenocytes were isolated and cultured for 72 hours in the presence of αCD3. Induction of Tregs (FoxP3+) and Th1 cells (T-bet+) was determined by flow cytometry. **E.** Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells and is expressed as the stimulation index (SI). **F.** IL-6 and TNF-α responses in splenocyte cultures were determined by cytometric bead array. All values are expressed as mean±SEM and are representative of at least five mice.*P<0.05, ***P<0.001.

To establish whether T cell responses were indeed modulated, we isolated splenocytes after eight week WTD and cultured them in the presence of αCD3 for 72 hours ex vivo. After stimulation, we found a significant 94% increase in Tregs and an almost complete inhibition of T cell differentiation to Th1 in CD11c-βcat^{EX3} mice (Figure 5D). This indicated that DCs present in the spleen were still able to modulate T cell responses and induce Tregs. The presence of Tregs resulted in a 60% reduction of proliferation of splenic CD11c-βcat^{EX3} T cells compared to the control (Figure 5E). This was in line with reduced CD4+ T cell numbers observed in the spleen in vivo, while likely Tregs migrate out of the spleen and are therefore not locally increased. Moreover, we found a significant 58% reduction in IL-6 production and a non-significant

64% reduction of TNF-α production by splenocytes (Figure 5F), indicating an overall reduced inflammatory response. This is in line with the reduced pro-inflammatory cytokine expression observed in isolated splenic DCs.

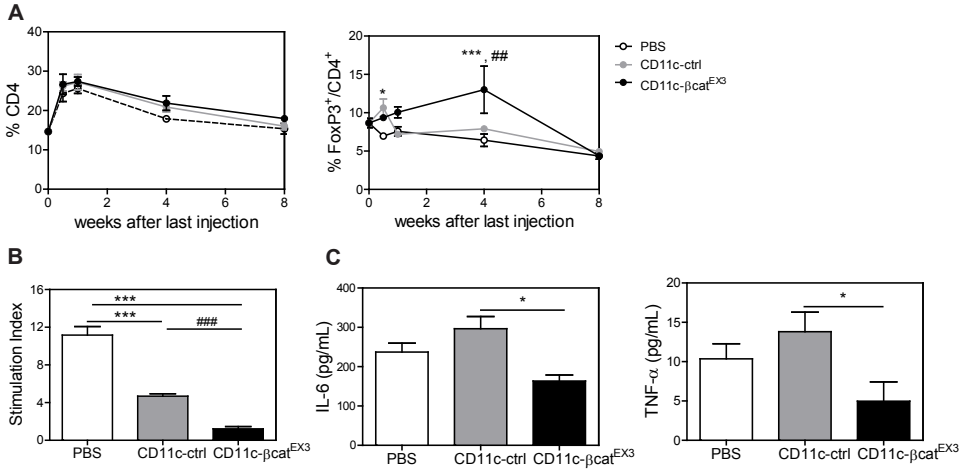


Figure 6. Reduced T cell responses after adoptive transfer of CD11c-βcat^{EX3} DCs. A. Circulating CD4$^+$ T cells and Tregs (FoxP3$^+$ within CD4$^+$ T cells) were determined in the circulation throughout the experiment. **B.** At sacrifice, splenocytes were isolated and proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells, expressed as stimulation index (SI). **C.** IL-6 and TNF-α production by proliferating splenocytes were determined by ELISA. All values are expressed as mean±SEM and are representative of at least five mice.*P<0.05, ***P<0.001 compared to PBS; ##P<0.01, ###P<0.001 compared to CD11c-ctrl.

Adoptive transfer of CD11c-βcat^{EX3} DCs reduces absolute CD4$^+$ T cell responses and induces Tregs

To determine if DCs with a β-catenin stabilization could be used as a potential therapy, we cultured BM-derived DCs of CD11c-βcat^{EX3} transgenic mice or their littermate controls and adoptively transferred them into LDLr$^{-/-}$ mice prior to eight weeks WTD. To determine if our treatment affected T cell responses, we monitored CD4$^+$ T cells and Tregs in the circulation. While no effect on circulating CD4$^+$ T cells was observed by any DC treatment, after four weeks CD11c-βcat^{EX3} DC-treated mice showed a significant 1.6-fold increase in circulating Tregs (Figure 6A). After eight weeks, we again isolated splenocytes and cultured them in the presence of αCD3 for 72 hours. We found a significant 2.4-fold decrease in proliferative capacity of T cells when splenocytes were isolated from mice treated with control DCs. However, when CD11c-βcat^{EX3} DCs had been adoptively transferred, a much stronger 9.2-fold inhibition of proliferation was observed. This was only 20% above background proliferation of unstimulated splenocytes, indicating that T cells were almost completely inhibited in their proliferative capacity. Moreover, only splenocytes of CD11c-βcat^{EX3} DC-treated mice showed a significant reduction of IL-6 and TNF-α production, indicating a reduced inflammatory response, in line with earlier observations (Figure 6C).

Adoptive transfer of CD11c-βcat^{EX3} DCs reduces lesion sizes

After eight weeks WTD, we determined whether the treatment with CD11c-βcat^{EX3} DCs could modulate atherosclerotic lesion development. No effect on cholesterol or weight during the entire experiment was observed (data not shown). We established a 21% reduction of aortic root lesion size in CD11c-βcat^{EX3} DC-treated mice compared to mice treated with control DCs and a 24% reduction compared to control mice (control: 5.3 x 10^5 ± 0.3 x 10^5 µm, CD11c-ctrl: 5.1 x 10^5 ± 0.4 x 10^5 µm, CD11c-βcat^{EX3}: 4.0 x 10^5 ± 0.3 x 10^5 µm; Figure 7A). Collagen content was on average 24% in all groups (Figure 7B). Moreover, no effect on necrotic core sizes and macrophage content was found (Figure 7C and D). However, in both DC treatment groups we observed a significant decrease of T cell numbers in lesions. Control DCs reduced lesional CD3$^+$ T cells by 47%, while again CD11c-βcat^{EX3} were more potent and decreased T cell numbers by 74% compared to mice who did not receive DC treatment (control: 5.7 ± 0.7 T cells, CD11c-ctrl: 3.0 ± 0.7 T cells, CD11c-βcat^{EX3}: 1.5 x 105 ± 0.4 T cells per section; Figure 7E).

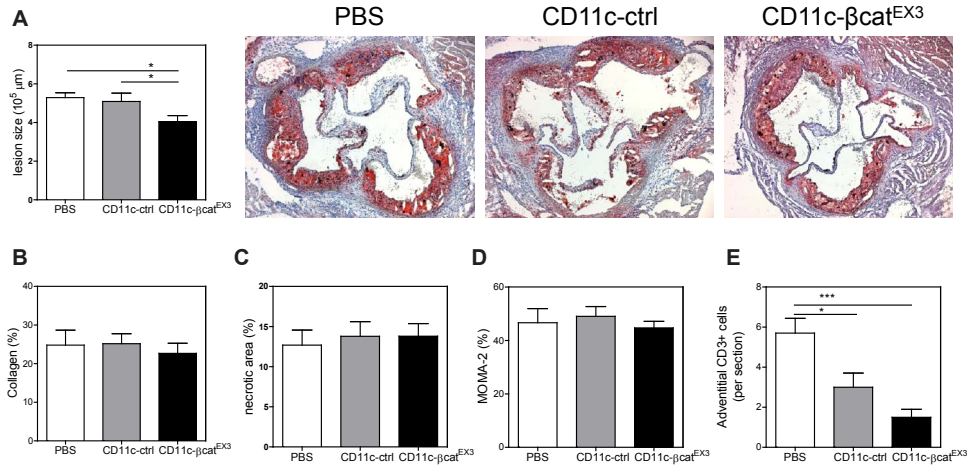


Figure 7. Adoptive transfer of CD11c-βcat^{EX3} DCs reduces atherosclerotic lesion formation. A. Lesion formation in the three valve area of the aortic root was assessed by Oil-Red-O and hematoxylin staining. Representative cross-sections of hearts are shown. **B.** Sections were stained for collagen fibers using Masson's trichrome staining. The percentage of collagen relative to the lesion size was determined. **C.** The necrotic core was defined as the acellular, debris-rich plaque area as percentage of total plaque area in the trichrome staining. **D.** Relative macrophage content was determined with MOMA-2 staining and quantified as percentage surface area relative to lesion size. **E.** T cell contant was determined by αCD3 staining. All values are expressed as mean±SEM and are representative of all mice.*P<0.05, ***P<0.001.

Discussion

Our study shows for the first time that β-catenin stabilization in DCs is beneficial in reducing atherosclerosis and can be a future strategy to treat the disease. We took two approaches in our study to determine the possible effect of β-catenin stabilization in DCs: First, we performed a bone marrow transplantation to generate LDLr$^{-/-}$ mice

with a CD11c-specific stabilization of β-catenin. Second, we adoptively transferred DCs with active β-catenin into LDLr$^{-/-}$ mice. In both cases we put the mice on a WTD for eight weeks and analyzed lesion formation and stability.

For our study we used CD11c-βcat^{EX3} mice, which have constitutive β-catenin signaling in CD11c^{+} cells. It has to be noted that while CD11c is highly expressed on DCs, it is also, although to lower extent, expressed on monocytes and macrophages[31], but can even be expressed in some T cell subsets[32], NK cells[33] and B cells[34] under specific disease circumstances. Under hypercholesterolemic conditions, an increased number of CD11c^{+} monocytes can be found, correlating with an increased migratory capacity of these cells into the lesions[35]. Furthermore, macrophages have been shown to increase their expression of CD11c upon uptake of oxLDL[36]. Therefore, β-catenin could also become stabilized in other mononuclear phagocytes during atherosclerosis. However, immature macrophages have almost undetectable levels of E-Cadherin and β-catenin and these only become upregulated by IL-4[37]. Therefore, we emphasize that the stabilization of β-catenin in our hypercholesterolemic model will only minimally affects monocytes and macrophages. This concurs with our findings that monocyte and macrophage responses were not affected. In DCs on the other hand the expression of E-Cadherin and β-catenin is found under immature as well as mature stages, and was shown to be important for the maintenance of cell clusters[24]. In addition, while macrophages can interact with T cells in the lesions, DCs are capable of shaping the initiated T cell responses. Therefore, the pronounced increase in Tregs and reduced T cell proliferation of CD4^{+} T cells in vivo is unlikely due to monocyte or macrophage contribution. Although we cannot rule out the contribution of monocytes and macrophages in our BMT setup, the potential of CD11c-βcat^{EX3} DCs becomes clear by the adaptive transfer performed in this study.

We provide conclusive evidence that CD11c-βcat^{EX3} DCs are potent inducers of Tregs. In a culture of total splenocytes from both transplanted and non-transplanted CD11c-βcat^{EX3} mice and a co-culture of CD11c-βcat^{EX3} DCs with T cells from a non-transplanted LDLr$^{-/-}$ mouse, we observed a significant induction of Tregs, while Th1 responses and general T cell proliferation were strongly reduced. CD11c-βcat^{EX3} chimera show significantly increased Tregs in the circulation and a general reduction of CD4^{+} T cells compared to control mice. At sacrifice, CD4^{+} T cell numbers were also decreased in the spleen and the hLNs indicating an overall reduction of T cell responses, although Treg numbers were not increased in spleen and hLNs. Nonetheless, when we isolated splenocytes and cultured them with αCD3, we did see a significant induction of Tregs, proving that CD11c-βcat^{EX3} DCs are able to potently induce Tregs. This could indicate that T cell responses are shifted to Tregs only when immune responses are induced and/or that Tregs migrate to the site of inflammation and are thus not locally increased in the spleen. After the adoptive transfer of CD11c-βcat^{EX3} DCs, an initial induction of circulating Tregs was observed, but in the long-term only overall T cell effector responses were reduced. Interestingly, while we saw an initial induction of Tregs in control DC-treated mice, CD11c-βcat^{EX3} DC-treated mice show a more pronounced and longer induction of Tregs, likely due to an increased lifespan of the CD11c-βcat^{EX3} DCs. We therefore show here that additional stabilization

of β-catenin can increase the potential of DC therapies.

It has to be noted that exon 3 mutations have been associated with e.g. colorectal carcinomas[38] and that with this approach we did take the risk of inducing a carcinogenic phenotype in DCs. However, during our studies we did not observe any tumor formation or excessive DC proliferation. We wanted to highlight the possibility of using β-catenin stabilization as a therapeutic approach for a DC therapy and eventually in the clinic a certain amount of DCs with a β-catenin stabilization would be injected and not all DCs in patients modulated, which further reduces the risk of tumors.

In both our experiments, we see an overall reduction of the inflammatory status in the CD11c-βcat^{EX3} mice, not only by induction of Tregs and reduction of Th1 cells, but also by reduction of pro-inflammatory cytokines, e.g. IL-6. β-catenin inhibits nuclear factor κB (NF-κB) mediated transcriptional activation of pro-inflammatory genes[39]. Also cluster disruption, which induces β-catenin signaling has been found to reduce pro-inflammatory cytokine responses[24]. Interestingly, β-catenin stabilization in Tregs extends their survival and enhances their protective effect in a Treg therapy for inflammatory bowel disease, compared to non-modulated Tregs[40]. This further emphasizes that β-catenin stabilization in anti-inflammatory cell therapies is promising. Activation of β-catenin signaling to induce tolerance is also exploited by the bacterium *Salmonella*, which produce an AvrA protein. AvrA induces β-catenin signaling and inhibits intestinal inflammation, enabling a chronic infection of the pathogen[41]. Recently, Fu *et al.* found that β-catenin in DCs inhibits their capacity for cross-priming due to increased IL-10 production, which results in reduced initiation of $CD8^+$ T cell responses; however β-catenin was also shown to be necessary to maintain $CD8^+$ T cell responses[41]. We did not observe any effect on the production of IL-10 by DC in vivo, but it seems that DCs remain in a more anti-inflammatory tolerogenic state when β-catenin is stabilized during atherosclerosis development as they produce less pro-inflammatory cytokines and increase Treg responses.

In contrast to earlier observations made by cluster disruption[24], we only found an upregulation of co-stimulatory molecules and chemokines receptors upon maturation of the DCs, but not due to β-catenin stabilization itself in immature DCs. While our model is based on CD11c-specific stabilization of β-catenin, previous studies have used the approach of cluster disruption of E-Cadherin/β-catenin complexes or inhibition of GSK3β, which phosphorylates β-catenin for proteasomal degradation[24]. These approaches could induce the concomitant upregulation of maturation markers through simultaneous activation of different signaling pathways, e.g. by effects on PI3K/Akt signaling or Rac1 signaling[42,43]. In our experiments we only see effects after LPS/TLR4 stimulation, indicating that indeed a second stimulus might be needed to induce upregulation of co-stimulatory molecules and chemokines receptors.

An alternative for stabilization of β-catenin by deletion of its phosphorylation site would be the treatment of DCs with LiCl[44], which is a potent inhibitor of GSK3β and would thus result in reduced degradation of β-catenin or activation of the Wnt signaling cascade[45], which induces β-catenin. Interestingly, these approaches have been implicated in inducing a tolerogenic phenotype of DCs. Another approach

could be cluster disruption[24], but it remains to be determined how controllable and reproducible this is for a clinical setting. However, since all these treatments only indirectly result in β-catenin accumulation and will inevitably have other effects, it needs to be established to what extent these approaches will resemble our β-catenin stabilization. A chemical stabilizer of β-catenin could circumvent this problem. SKL2001 e.g. has been shown to increase transcription of β-catenin but also to prevent its phosphorylation and degradation and it could be interesting to test its efficiency[46]. Moreover, direct administration of such a reagent and targeting it to DCs in vivo could prove as the best and least invasive treatment option.

In conclusion, we show here for the first time that DCs with a β-catenin stabilization are highly potent inducers of Tregs, inhibit Th1 responses and T cell proliferation, and remain tolerogenic under hypercholesterolemic conditions. These DCs decrease atherosclerotic lesion development and it will be interesting to determine their potential in inhibiting lesion progression. We predict that β-catenin stabilization in combination with other inducers of a tolerogenic phenotype in DCs, such as e.g. 1α,25-dihydroxyvitamin D3, will be a powerful DC therapy for atherosclerosis.

References

1. Bobryshev, Y. V & Lord, R. S. Ultrastructural recognition of cells with dendritic cell morphology in human aortic intima. Contacting interactions of Vascular Dendritic Cells in athero-resistant and athero-prone areas of the normal aorta. *Arch. Histol. Cytol.* 58, 307–22 (1995).
2. Chistiakov, D. A., Sobenin, I. A., Orekhov, A. N. & Bobryshev, Y. V. Dendritic cells in atherosclerotic inflammation: the complexity of functions and the peculiarities of pathophysiological effects. *Front. Physiol.* 5, 196 (2014).
3. Taghavie-Moghadam, P. L., Butcher, M. J. & Galkina, E. V. The dynamic lives of macrophage and dendritic cell subsets in atherosclerosis. *Ann. N. Y. Acad. Sci.* 1319, 19–37 (2014).
4. Shaposhnik, Z., Wang, X., Weinstein, M., Bennett, B. J. & Lusis, A. J. Granulocyte macrophage colony-stimulating factor regulates dendritic cell content of atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* 27, 621–7 (2007).
5. Lichtman, A. H., Binder, C. J., Tsimikas, S. & Witztum, J. L. Adaptive immunity in atherogenesis: new insights and therapeutic approaches. *J. Clin. Invest.* 123, 27–36 (2013).
6. Angeli, V. *et al.* Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity* 21, 561–74 (2004).
7. Bobryshev, Y. V & Lord, R. S. Mapping of vascular dendritic cells in atherosclerotic arteries suggests their involvement in local immune-inflammatory reactions. *Cardiovasc. Res.* 37, 799–810 (1998).
8. Choi, J.-H. *et al.* Identification of antigen-presenting dendritic cells in mouse aorta and cardiac valves. *J. Exp. Med.* 206, 497–505 (2009).
9. Han, J. W. *et al.* Vessel wall-embedded dendritic cells induce T-cell autoreactivity and initiate vascular inflammation. Circ. Res. 102, 546–53 (2008).
10. Choi, J.-H. *et al.* Flt3 signaling-dependent dendritic cells protect against atherosclerosis. Immunity 35, 819–31 (2011).
11. Subramanian, M., Thorp, E., Hansson, G. K. & Tabas, I. Treg-mediated suppression of atherosclerosis requires MYD88 signaling in DCs. *J. Clin. Invest.* 123, 179–88 (2013).
12. Subramanian, M. & Tabas, I. Dendritic cells in atherosclerosis. *Semin. Immunopathol.* 36, 93–102 (2014).
13. Foks, A. C. *et al.* Interruption of the OX40-OX40 ligand pathway in LDL receptor-deficient mice causes regression of atherosclerosis. *J. Immunol.* 191, 4573–80 (2013).
14. Foks, A. C. *et al.* Interference of the CD30-CD30L pathway reduces atherosclerosis development. *Arterioscler. Thromb. Vasc. Biol.* 32, 2862–8 (2012).
15. Habets, K. L. L. *et al.* Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc. Res.* 85, 622–30 (2010).
16. Frodermann, V. *et al.* Oxidized Low-Density Lipoprotein-Induced Apoptotic Dendritic Cells as a

Novel Therapy for Atherosclerosis. *J. Immunol.* (2015).
17. Hermansson, A. *et al.* Immunotherapy with tolerogenic apolipoprotein B-100-loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice. *Circulation* 123, 1083–91 (2011).
18. Torres-Aguilar, H. *et al.* Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4+ T cells. *J. Immunol.* 184, 1765–75 (2010).
19. Papenfuss, T. L. *et al.* Estriol generates tolerogenic dendritic cells in vivo that protect against autoimmunity. *J. Immunol.* 186, 3346–55 (2011).
20. Penna, G. & Adorini, L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol.* 164, 2405–11 (2000).
21. Gonzalez-Rey, E., Chorny, A., Fernandez-Martin, A., Ganea, D. & Delgado, M. Vasoactive intestinal peptide generates human tolerogenic dendritic cells that induce CD4 and CD8 regulatory T cells. *Blood* 107, 3632–8 (2006).
22. McCrea, P. D., Turck, C. W. & Gumbiner, B. A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. *Science* 254, 1359–61 (1991).
23. Siddiqui, K. R. R., Laffont, S. & Powrie, F. E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. *Immunity* 32, 557–67 (2010).
24. Jiang, A. *et al.* Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27, 610–24 (2007).
25. Vander Lugt, B. *et al.* TGF-β suppresses β-catenin-dependent tolerogenic activation program in dendritic cells. *PLoS One* 6, e20099 (2011).
26. Manicassamy, S. *et al.* Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* 329, 849–53 (2010).
27. Brault, V. *et al.* Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* 128, 1253–64 (2001).
28. Harada, N. *et al.* Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* 18, 5931–42 (1999).
29. Hoekstra, M., Kruijt, J. K., Van Eck, M. & Van Berkel, T. J. C. Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *J. Biol. Chem.* 278, 25448–53 (2003).
30. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. A*nal. Biochem.* 162, 156–9 (1987).
31. Geissmann, F., Gordon, S., Hume, D. A., Mowat, A. M. & Randolph, G. J. Unravelling mononuclear phagocyte heterogeneity. *Nat. Rev. Immunol.* 10, 453–60 (2010).
32. Chen, Z. *et al.* CD11c(high)CD8+ regulatory T cell feedback inhibits CD4 T cell immune response via Fas ligand-Fas pathway. *J. Immunol.* 190, 6145–54 (2013).
33. Aranami, T., Miyake, S. & Yamamura, T. Differential expression of CD11c by peripheral blood NK cells reflects temporal activity of multiple sclerosis. *J. Immunol.* 177, 5659–67 (2006).
34. Racine, R., Chatterjee, M. & Winslow, G. M. CD11c expression identifies a population of extrafollicular antigen-specific splenic plasmablasts responsible for CD4 T-independent antibody responses during intracellular bacterial infection. *J. Immunol.* 181, 1375–85 (2008).
35. Wu, H. *et al.* Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 119, 2708–17 (2009).
36. Cho, H. J. *et al.* Induction of dendritic cell-like phenotype in macrophages during foam cell formation. Physiol. Genomics 29, 149–60 (2007).
37. Van den Bossche, J. *et al.* Alternatively activated macrophages engage in homotypic and heterotypic interactions through IL-4 and polyamine-induced E-cadherin/catenin complexes. *Blood* 114, 4664–74 (2009).
38. Johnson, V. *et al.* Exon 3 beta-catenin mutations are specifically associated with colorectal carcinomas in hereditary non-polyposis colorectal cancer syndrome. *Gut* 54, 264–7 (2005).
39. Du, Q. & Geller, D. A. Cross-Regulation Between Wnt and NF-κB Signaling Pathways. *For. Immunopathol. Dis. Therap.* 1, 155–181 (2010).
40. Ding, Y., Shen, S., Lino, A. C., Curotto de Lafaille, M. A. & Lafaille, J. J. Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. *Nat. Med.* 14, 162–9 (2008).
41. Lu, R. *et al.* Consistent activation of the β-catenin pathway by Salmonella type-three secretion effector protein AvrA in chronically infected intestine. *Am. J. Physiol. Gastrointest.* Liver Physiol. 303, G1113–25 (2012).
42. Pece, S. Activation of the Protein Kinase Akt/PKB by the Formation of E-cadherin-mediated Cell-Cell Junctions. EVIDENCE FOR THE ASSOCIATION OF PHOSPHATIDYLINOSITOL 3-KINASE WITH THE E-CADHERIN ADHESION COMPLEX. *J. Biol. Chem.* 274, 19347–19351 (1999).

43. Nakagawa, M., Fukata, M., Yamaga, M., Itoh, N. & Kaibuchi, K. Recruitment and activation of Rac1 by the formation of E-cadherin-mediated cell-cell adhesion sites. *J. Cell Sci.* 114, 1829–1838 (2001).
44. Liu, K.-J. *et al.* Modulation of the development of human monocyte-derived dendritic cells by lithium chloride. *J. Cell. Physiol.* 226, 424–33 (2011).
45. Valencia, J. *et al.* Wnt5a skews dendritic cell differentiation to an unconventional phenotype with tolerogenic features. *J. Immunol.* 187, 4129–39 (2011).
46. Gwak, J. *et al.* Small molecule-based disruption of the Axin/β-catenin protein complex regulates mesenchymal stem cell differentiation. *Cell Res.* 22, 237–47 (2012).

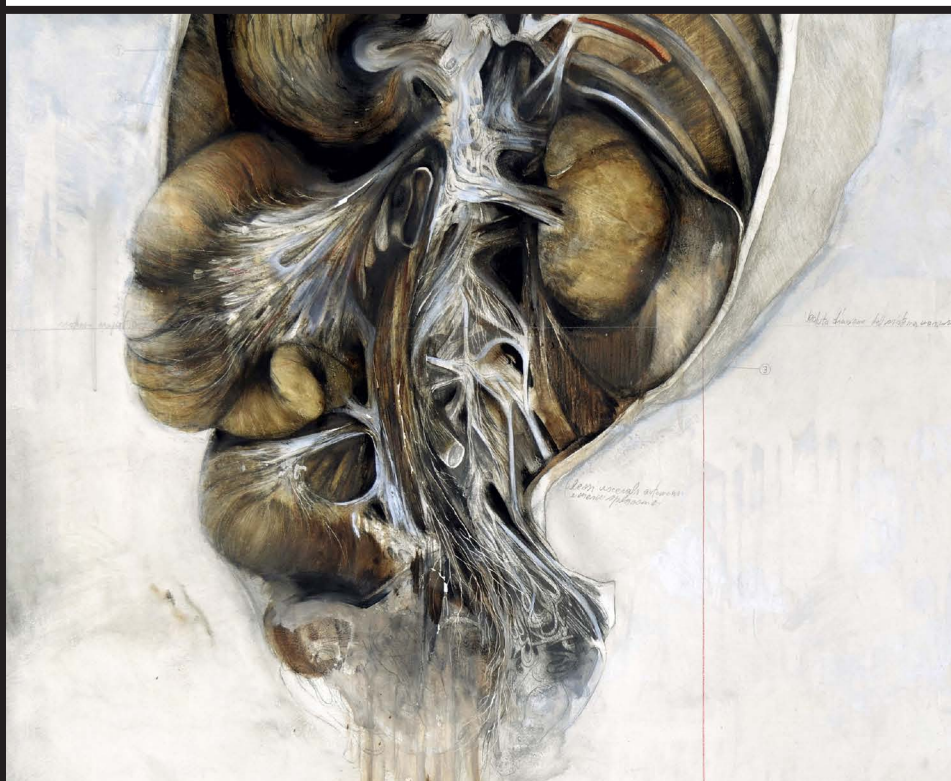
Vanessa Frodermann[1]
Janine van Duijn[1]
Melissa van Pel[2]
Peter J. van Santbrink[1]
Ilze Bot[1]
Johan Kuiper[1]
Saskia C.A. de Jager[1,6]

4

[1] Division of Biopharmaceutics, LACDR, Leiden University, Leiden, The Netherlands
[2] Department of Immunohematology & Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands
[3] Present address: Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

Mesenchymal Stem Cells Reduce Murine Atherosclerosis Development

Submitted.



Abstract

Objective Mesenchymal stem cells (MSCs) have regenerative properties, but have recently received increasing attention for their immunomodulatory capacities. As atherosclerosis is an immune-mediated disease, we investigated whether MSCs could beneficially affect atherosclerotic lesion development.

Methods and Results The immunomodulatory capacity of murine MSCs was first determined in vitro. In a co-culture of MSCs and dendritic cells, MSCs significantly reduced TNF-α and increased IL-10 production by dendritic cells in response to LPS. Moreover, MSCs dramatically inhibited $CD4^+$ and $CD8^+$ T cell responses in a co-culture with splenocytes or isolated $CD4^+$ T cells by preventing the differentiation of naïve T cells.

Next, we adoptively transferred MSCs into atherosclerosis-prone low-density lipoprotein-receptor deficient mice, which were fed a Western-type diet for eight weeks and subsequently assessed their effect on atherosclerotic lesion development. MSC-treated mice initially had higher levels of Tregs, while in the long-term, overall numbers of differentiated T cells were reduced by MSC treatment. Moreover, MSC-treated mice displayed a significant reduction in circulating monocytes and serum CCL2 levels. Interestingly, serum cholesterol levels were significantly reduced, due to a decreased VLDL production in the liver. Most importantly, MSCs induced a significant 33% reduction in aortic root lesion size and a 56% reduction in lesional macrophage content.

Conclusion We show here for the first time that MSC treatment significantly reduces atherosclerotic lesion development in mice and this may initiate future studies to assess the therapeutic capacity of MSCs in atherosclerosis patients.

Introduction

Mesenchymal stem cells (MSCs), also called bone marrow stromal cells or mesenchymal stroma cells, are multipotent cells that can give rise to cells of the mesodermal lineage, including adipocytes, osteocytes and myocytes[1]. They were first identified in the bone marrow, but can be isolated from other tissues such as umbilical cord, placenta and adipose tissue[2]. After isolation, MSCs can be easily expanded without losing their multipotency which renders them an interesting tool for therapeutic strategies[1]. MSCs were initially investigated for their ability to repair injured heart tissue after myocardial infarction[3,4]. They can migrate to sites of tissue damage and inflammation, where they can extravasate, engraft the tissues and reduce scar formation[4–8].

In recent years the immunomodulatory capacity of MSCs has been increasingly appreciated. Several studies have investigated the capacity of MSCs to modulate both adaptive and innate immune responses[2,9–14]. For instance, MSCs have been shown to reduce monocyte responses after myocardial infarction[10] and to skew macrophages to an anti-inflammatory IL-10-producing phenotype[10–13]. MSCs also inhibit the differentiation and maturation of dendritic cells (DCs)[14], by reducing the expression of co-stimulatory molecules and pro-inflammatory cytokines (TNF-α and IL-12), while increasing the production of anti-inflammatory cytokines (TGF-β and IL-10)[15,16], which indirectly suppresses T cell proliferation[16]. However, MSCs can also directly inhibit T cell proliferation[15–17], by inducing cell cycle arrest in all subsets, resulting in a quiescent state and decreased proliferation[18].

Inflammatory processes play a crucial role in all stages of atherosclerosis. Early in the disease process, entrapped oxidized low-density lipoprotein (oxLDL) leads to the activation of arterial endothelial cells and an ensuing recruitment of monocytes and T cells[19]. Upon recruitment, monocytes can differentiate to macrophages[20]. Macrophages can primarily promote T cell responses to local antigens, while DCs can activate naïve T cells in response to lesion-derived antigens in draining lymph nodes[21]. DCs are also present within lesions or can arise from blood-derived precursors. Both macrophages and DCs express scavenger receptors, enabling the uptake of oxLDL and foam cell formation, and toll-like receptors, which mediate maturation of the antigen-presenting cells and production of pro-inflammatory cytokines. $CD4^+$ T cells are crucially involved in the pathogenesis of atherosclerosis and their depletion results in a 70% reduction of lesion size[22]. The predominant subset in both human and murine atherosclerotic lesions is the Th1 subset[23,24], which produces a plethora of pro-inflammatory cytokines such as IFN-γ. IFN-γ promotes vascular inflammation by enhancing maturation and activation of antigen-presenting cells, increasing macrophage lipid uptake, reducing collagen production by SMCs, and enhancing expression of endothelial adhesion molecules, which subsequently stimulates leukocyte recruitment to the lesions[25]. The continuous recruitment of further leukocytes to atherosclerotic lesions results in a vicious self-maintaining progressive inflammatory cycle. Nonetheless, it has been shown that by altering the phenotype of macrophages and DCs, they can become atheroprotective[21,26,27]. Moreover, regulatory T cells (Tregs) have been clearly established as anti-atherogenic[28]. Tregs produce high amounts of

the anti-inflammatory cytokine IL-10 and inhibit ongoing inflammation.

Due to the key role of inflammatory processes in the initiation and progression of atherosclerosis, adoptive transfer of MSCs, which have the capacity to modulate and reduce inflammation, may be a therapeutic approach to treat atherosclerosis. Preclinical studies have already shown that adoptively transferred MSCs are capable of modulating immune responses and they can prevent allograft rejection[17,29] and alleviate autoimmune diseases[30–32]. Moreover, in a phase II clinical trial, it was found that MSCs can reduce graft-versus-host disease[33]. We now investigated the capacity of murine MSCs to affect the inflammatory process of atherosclerosis and thereby atherosclerotic lesion development.

Materials and Methods

Animals

C57BL/6 and LDLr$^{-/-}$ mice were originally obtained from the Jacksons Laboratory, kept under standard laboratory conditions, and administered food and water *ad libitum*. All animal work was approved by the Ethics Committee for Animal Experiments of Leiden University and conforms to Dutch government guidelines.

DC culture

For DC cultures, bone marrow cells were isolated from the tibias and femurs of C57BL/6 mice. The cells were cultured for ten days at 37°C and 5% CO_2 in 10 cm^2 petri dishes in IMDM supplemented with 10% FCS, 1% penicillin/streptomycin (all obtained from PAA), 1% glutamax (Thermo Fisher Scientific) and 20 μM β-mercaptoethanol (Sigma Aldrich) in the presence of 20 ng/mL granulocyte-macrophage colony-stimulating factor (Peprotech). DC purity was assessed by CD11c expression (flow cytometry) and routinely found to be above 95%.

MSC isolation, phenotyping and labelling

Bone marrow cells were obtained by flushing femurs with washing medium (RPMI supplemented with 2% FCS, penicillin, streptomycin and L-glutamine). Next the bone marrow cells were cultured in a 75 cm^2 flask containing αMEM (Life Technologies) supplemented with 10% FCS (Greiner Bio-One), 2% penicillin/streptomycin (Life Technologies) and 1% L-Glutamine (Life Technologies; MSC medium). Subsequently, plastic adherent MSCs were cultured to 95% confluency in a fully humidified atmosphere at 37°C and 5% CO_2, harvested using trypsin and further expanded until sufficient numbers were obtained.

The MSCs that were used throughout this study were of passage six to eight; for in vivo MSC treatment cells of passage eight were used. MSC were phenotyped using the following antibodies: TER119 (clone TER119), CD31 (clone MEC 13.3), CD45.2 (clone 104), CD90.2 (clone 53-2.1), CD29 (clone HMb1-1), Sca-1 (Clone D7), CD105 (clone MJ7/18), CD44 (clone IM7) and CD106 (clone 429). All antibodies were obtained from BD Biosciences.

For tracking experiments, MSCs were labelled with 10 μM Carboxyfluorescein

succinimidyl ester (CFSE) according to manufacturer's protocol (Thermo Fisher Scientific). Briefly, MSCs were resuspended in prewarmed PBS/0.1 % BSA at a concentration of 1 x 10^6 cells/mL and incubated with dye at 37°C for 10 minutes in the dark. Afterwards, the staining was quenched by adding ice-cold media and incubating for another 5 minutes on ice, followed by three subsequent washing steps to remove excess CFSE.

Co-cultures

For DC-MSC co-cultures, 1 x 10^6 DCs were plated in 2 cm^2 non-tissue culture treated petri dishes (Greiner Bio-One) with indicated ratios of MSCs for 24 hours in MSC medium. DCs were stimulated with 100 ng/mL LPS to determine cytokine responses.

For splenocytes-MSC co-cultures, single cell suspensions of spleens from $LDLr^{-/-}$ mice were obtained by using a 70 µm cell strainer (VWR International). Red blood cells were lysed with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.2). 1 x 10^5 splenocytes were added per well with indicated amounts of MSCs. For T cell-MSC co-cultures, $CD4^+$ T cells (>95% purity) were isolated from splenocytes by using the BD IMagTM mouse CD4 T lymphocyte enrichment set according to manufacturer's protocol (BD Biosciences). 1 x 10^5 T cells were added per well with indicated amounts of MSCs. Both splenocytes and T cell-MSC co-cultures were cultured in quintuplicates in 96-well round-bottom plates (Greiner Bio-One) in the presence or absence of αCD3/28 (2 µg/mL, eBioscience) for 72 hours in complete RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2mM L-glutamine (all obtained from PAA) and 20 µm β-mercaptoethanol (Sigma Aldrich). Proliferation was measured by Ki-67 expression by flow cytometry or addition of ^{3}H-thymidine (0.5µCi/well, Amersham Biosciences) for the last 16 hours of culture. 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). T cell subsets were determined by flow cytometry.

For splenocytes and T cell cultures in the presence of MSC culture supernatant, MSC culture supernatant was added in different concentration of total medium added. MSC supernatant was filtered using a 0.2µm filter to remove residual cells before use.

Atherosclerosis

Atherosclerosis was induced in 16 weeks old male $LDLr^{-/-}$ mice by feeding a Western-type diet (WTD) (0.25% cholesterol and 15% cocoa butter; Special Diet Services). Mice received 3 *i.v.* injections of PBS or 0.5 x 10^6 MSCs every other day prior to eight weeks WTD.

Flow Cytometry

Mice were sacrificed and subsequently, blood and spleen were harvested. White blood cells were obtained as described above. 3 x 10^5 cells per sample were stained with the appropriate FACS antibodies. The following antibodies were used: Ly-6G (clone 1A8; BD Biosciences), CD11b (clone M1/70), CD11c (clone N418), CD4 (clone RM4-5; BD Biosciences), CD8 (clone 53-6.7; BD Biosciences), FoxP3 (clone FJK-

16s), Gata-3 (clone TWAJ), Ki-67 (clone SolA15), MHCII (clone AF6-120.1), RORγt (clone AFKJS-9), and T-bet (clone eBio4B10). All antibodies were purchased from eBioscience, unless stated otherwise. For intracellular staining, cells were fixed and permeabilized according to the manufacturer's protocol (eBioscience). FACS analysis was performed on the FACS Canto II and data were analysed using FACS Diva software (BD Biosciences).

In Vivo Imaging Systems (IVIS)

For MSC tracking experiments, 1 x 10^6 MSCs were stained with CFSE and injected *i.v*. Ex vivo imaging was performed by placing either whole animals or organs in the IVIS Lumina Imaging System (Xenogen) at indicated time points and analyzing fluorescence based on the manufacturer's recommendations. Fluorescence intensity was quantified as photons/sec/cm^2 by Living Image software (Xenogen).

Histological analysis

To determine plaque size, 10 μm cryosections of the aortic root were stained with Oil-Red-O and haematoxylin (Sigma Aldrich). Corresponding sections were stained for collagen fibers using the Masson's Trichrome staining (Sigma Aldrich) or immunohistochemically with an antibody against a macrophage-specific antigen (MOMA-2, polyclonal rat IgG2b, 1:1000, Serotec Ltd.). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100; Sigma Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrate. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, Thermo Scientific). BrightVision anti-rabbit-HRP was used as secondary antibody (Immunologic). The section with the largest lesion and four flanking sections were analyzed for lesion size and collagen content, two sections were analyzed for T cell content. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). The percentage of collagen in the lesions was determined by dividing the collagen-positive area by the total lesion surface area.

Real-time PCR

mRNA was isolated from the liver using the guanidium isothiocyanate method and reverse transcribed (RevertAid Moloney murine leukemia virus reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast real-time PCR system (Applied Biosystems) using SYBR Green technology. The expression was determined relative to the average expression of three housekeeping genes: succinate dehydrogenase complex, Subunit A (SDHA), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). The following primer pairs were used:

Scd1:	*5′- TACTACAAGCCCGGCCTCC-3′*	and *5′-CAGCAGTACCAGGGCACCA-3′*
SREBP-1c:	*5′-TCTGAGGAGGAGGGCAGGTTCCA-3′*	and 5′-GGAAGGCAGGGGGCAGATAGCA-3′
SREBP-2:	*5′-TGAAGCTGGCCAATCAGAAAA-3′*	and *5′-ACATCACTGTCCACCAGACTGC-3′*
SDHA:	*5′-TATATGGTGCAGAAGCTCGGAAGG-3′*	and *5′-CCTGGATGGGCTTGGAGTAATCA-3′*
HPRT:	*5′-TACAGCCCCAAAATGGTTAAGG-3′*	and *5′-AGTCAAGGGCATATCCAACAAC-3′*
Rpl27:	*5′-CGCCAAGCGATCCAAGATCAAGTCC-3′*	and 5′*-AGCTGGGTCCCTGAACACATCCTTG-3′*

Cytokines
IL-10, TNF-α, IFN-γ and CCL2 were determined by ELISA (BD Biosciences) according to manufacturer's protocol.

Serum cholesterol levels
During the experiment, mice were weighed and blood samples were obtained by tail vein bleeding. Serum concentrations of total cholesterol were determined by enzymatic colorimetric assays (Roche Diagnostics). Precipath (standardized serum; Roche Diagnostics) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 µl of serum using a Superose 6 column (3.2 x 300 mm, Smart-System; Pharmacia). Total cholesterol content of the effluent was determined as described above.

Statistical analysis
Values are expressed as mean±SEM. Data of two groups were analysed with a two-tailed Student's T-test. Data of three or more samples were compared by one-way ANOVA and data of two groups with two variables were analyzed by two-way ANOVA, both followed by Bonferroni post-testing. Statistical analysis was performed using Prism (GraphPad). Probability values of $P<0.05$ were considered significant.

Results

MSCs were generated from the bone marrow of male C57BL/6 mice and their phenotype was confirmed by flow cytometry analysis. All MSCs expressed Sca-1, CD29, CD44, CD105 and CD106, while CD45, CD31 and TER119 were not expressed (Figure 1).

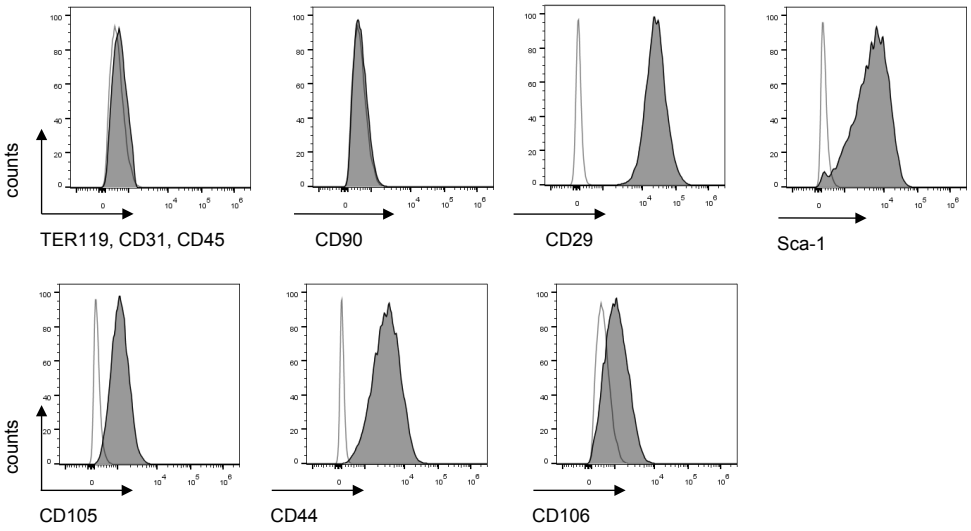


Figure 1. MSC Phenotype. MSCs were generated from the bone marrow of male C57BL/6 mice and the expression of surface markers was analyzed by flow cytometry. Representative histograms are shown.

To determine their immunomodulatory capacity, MSCs were co-cultured with DCs for three hours at different ratios, and subsequently stimulated with LPS. Twenty-four hours after LPS stimulation, we did not observe effects of MSCs on the expression of the co-stimulatory molecules CD40, OX40L and CD30L by DCs. The percentage of DCs positive for the co-stimulatory molecule CD86 increased by 12% and the percentage of DCs expressing the negative co-stimulatory molecule PD-L2 increased by 22%. The mean expression of CD86 per cell was however not significantly affected, whereas the mean expression of PD-L2 was decreased by 18% at higher MSC to DC ratios. The mean expression of the co-stimulatory molecule CD80 on DCs was significantly increased by 34% upon co-culture with MSCs (Table 1). CD80/CD86 function to induce T cell activation but can also promote Treg development, while PD-L2 functions to inhibit T cell activation, indicating that DCs might adopt a more tolerogenic phenotype after exposure to MSCs. In line with this, MSCs significantly affected the cytokine production of DCs in response to LPS. Pro-inflammatory TNF-α release was reduced by 57% while anti-inflammatory IL-10 production was increased by 45% (Figure 2A).

	DCs only	½ MSC per DC	1 MSC per DC	2 MSCs per DC	
CD80	95.5 ± 0.3	97.5 ± 0.4	98.2 ± 0.1	98.2 ± 0.1	Percentages
CD86	68.1 ± 0.7	73.8 ± 0.3 (***)	76.0 ± 0.1 (***)	76.5 ± 0.1 (***)	
CD40	88.9 ± 0.3	92.1 ± 2.0	90.1 ± 0.8	93.43 ± 0.2	
OX40L	35.5 ± 0.8	47.6 ± 3.7 (*)	43.2 ± 1.1	44.1 ± 0.4	
CD30L	18.2 ± 1.9	17.6 ± 1.2	19.5 ± 1.2	21.1 ± 1.5	
PD-L2	50.9 ± 0.6	56.5 ± 2.3	61.8 ± 0.9 (**)	62.5 ± 0.15 (**)	
CD80	12666 ± 118	15568 ± 407 (***)	16351 ± 321 (***)	16961 ± 272 (***)	MFI
CD86	49906 ± 446	55290 ± 957 (***)	52075 ± 55	49102 ± 75	
CD40	12447 ± 177	12065 ± 1148	11825 ± 287	12622 ± 12	
OX40L	7439 ± 78	7655 ± 221	8196 ± 139	8135 ± 91	
CD30L	2943 ± 41	2953 ± 53	2924 ± 32	3018 ± 59	
PD-L2	5469 ± 105	5012 ± 100	4695 ± 211	4534 ± 76 (*)	

Table 1. MSCs only mildly affect co-stimulatory molecule expression of LPS-stimulated DCs. DCs were co-cultured with indicated ratios of MSCs for 3 hours prior to addition of 100 ng/mL LPS for 24 hours. DC numbers remained constant. Co-stimulatory molecules on DCs, determined as $CD11c^{+}MHCII^{+}$, were determined by flow cytometry. The percentage and mean fluorescence intensity (MFI) is shown. All values are expressed as mean±SEM and representative of two independent experiments done in triplicate. *** P<0.001

Since MSCs have been shown to affect T cell responses, we co-cultured MSCs with splenocytes from atherosclerosis-prone $LDLr^{-/-}$ mouse in the presence of αCD3/CD28 for 72 hours. MSCs potently inhibited T cell proliferation by 99%, as measured by ^{3}H-thymidine incorporation, compared to αCD3/CD28 stimulated splenocytes in the absence of MSCs. $CD4^{+}$ T cell proliferation was reduced by 92% and $CD8^{+}$ T cell proliferation by 87%, as measured by Ki-67 expression. Polarization of all $CD4^{+}$ T cell subsets was inhibited to a similar extent (Th1 and Th2 more than 99% and Tregs by 89%; Figure 2B). Cytokine responses indicated similar effects: IL-10 production was not detectable, IFN-γ was reduced by 99%, and TNF-α showed a 92% reduction upon co-incubation with MSCs (Figure 2C). Co-cultures of isolated $CD4^{+}$ T cells and MSCs

showed comparable, but milder effects: proliferation of T cells was on average reduced by 80%, the skewing towards specific T cell subsets was reduced by an average of 85% (Figure 2D and E). Interestingly, MSC culture supernatant had no effect on T cell proliferation, suggesting that cell-cell contact is crucial (data not shown).

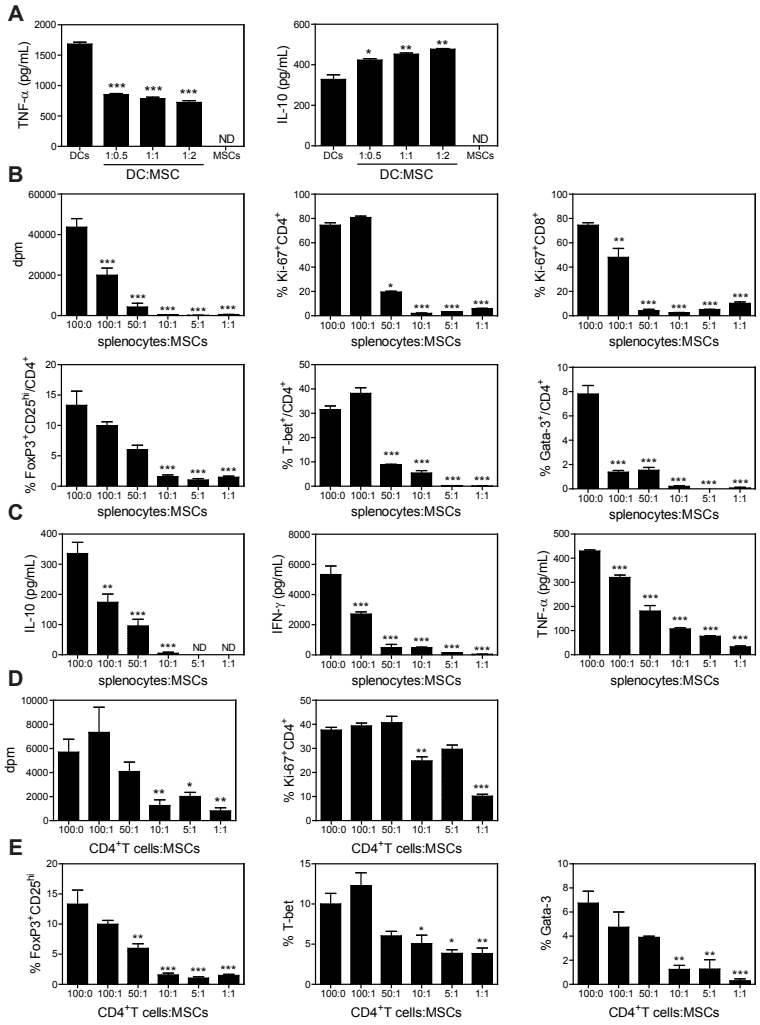


Figure 2. MSCs affect innate and adaptive immune responses. A. DCs were co-cultured with indicated ratios of MSCs for 3 hours prior to addition of 100 ng/mL LPS for 24 hours. DCs numbers remained constant. Cytokine responses were determined by ELISA. **B.** Splenocytes from an LDLr$^{-/-}$ mouse were co-cultured with indicated ratios of MSCs in the presence of αCD3/CD28 for 72 hours. Proliferation was assessed by ^{3}H-thymidine incorporation and Ki-67 expression by flow cytometry. Treg (FoxP3^{+}CD25^{hi}), Th1 (T-bet^{+}) and Th2 (Gata-3^{+}) within CD4^{+} T cells were determined by flow cytometry. Splenocyte numbers remained constant. **C.** Cytokine responses by splenocytes were determined by ELISA. **D.** Proliferation of CD4^{+} T cells from an LDLr$^{-/-}$ mouse in the presence of MSCs was assessed by ^{3}H-thymidine incorporation and by Ki-67 expression by flow cytometry. CD4^{+} T cell numbers remained constant. Proliferation was assessed by ^{3}H-thymidine incorporation and Ki-67 expression by flow cytometry. **E.** Treg (FoxP3^{+}CD25^{hi}), Th1 (T-bet^{+}) and Th2 (Gata-3^{+}) within CD4^{+} T cells were determined by flow cytometry. All values are expressed as mean±SEM and representative of at least two independent experiments done in triplicate. *P<0.05, **P<0.01, ***P<0.001. ND defines not determined.

Before testing the effect of MSCs on atherosclerosis, we fluorescently labelled MSCs with CFSE (Figure 3A) and established the fate of fluorescently labelled MSCs after *i.v.* injection in LDLr$^{-/-}$ mice on a cholesterol rich diet (WTD) and determined to which organs they migrated. MSCs initially accumulated primarily in the lungs and then slowly migrated out of the lungs (Figure 3B). One to three hours aft er injection, we found that MSCs had migrated to the liver, the heart, the draining lymph nodes of the heart and the aorta. Surprisingly, only few MSCs were recovered in the spleen (Figure 3C).

To test whether MSCs are able to modulate immune responses and thereby atherosclerosis, LDLr$^{-/-}$ mice were treated with three *i.v.* injections of MSCs every

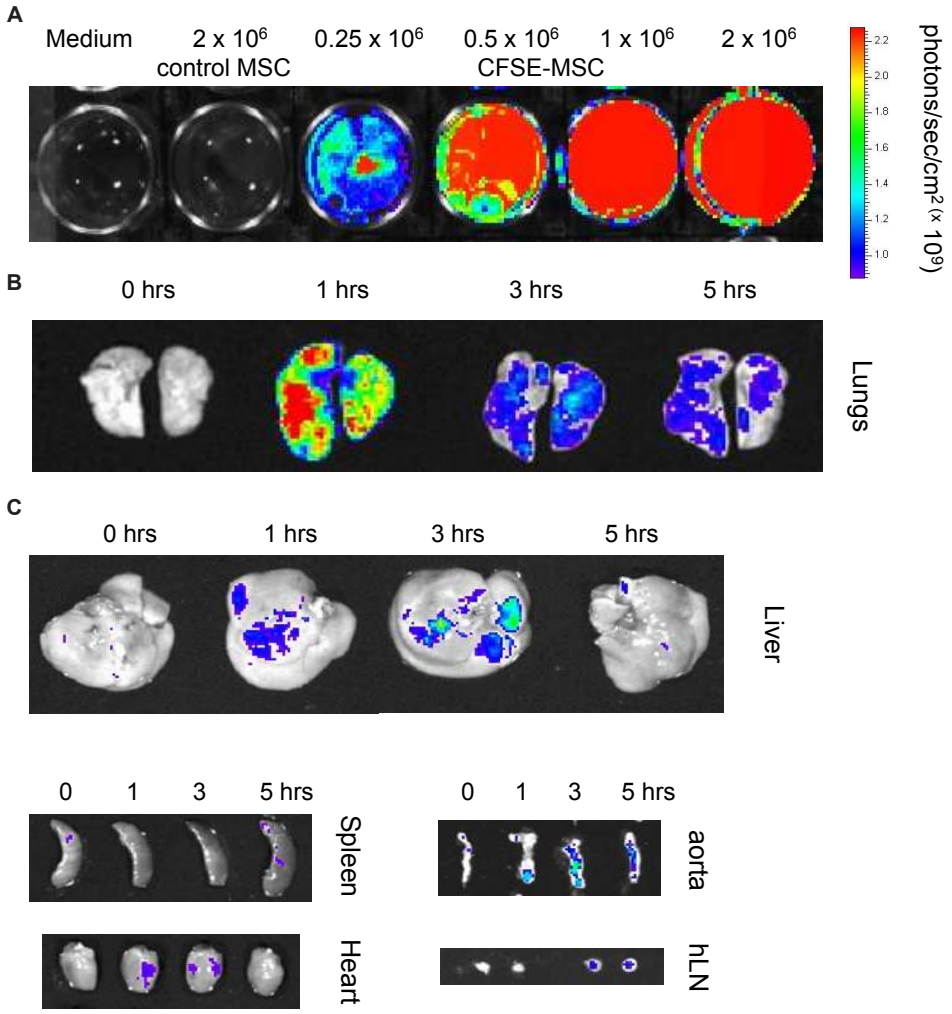


Figure 3. CFSE-labelled MSCs migrate preferentially to lung, liver, and the vasculature. A. MSCs were labelled with 10μM CFSE. Signal intensity on IVIS correlates with amount of cultured CFSE^{+} MSCs. Control MSCs indicates non-labelled MSCs. **B.** MSC presence in the lung 1-5 hrs after injections. **C.** Organ distribution of MSCs as determined by IVIS 1-5 hrs after injection of MSCs.

other day prior to induction of atherosclerosis by WTD feeding. One day after the start of WTD, we found a significant 38% drop in circulating CD4$^+$ T cells (8.7% vs 14.1% for MSC vs control, respectively). Interestingly, we also observed an initial 51% increase in circulating Tregs (13.9% vs 9.2% for MSC vs control, respectively; Figure 4A). Eight weeks after inducing atherosclerosis, no difference in absolute white blood cell counts (data not shown) and percentage of circulating and splenic CD4$^+$ T cells was found (Figure 4A and B). But a significant 18% decrease in splenic effector CD4$^+$ T cells was still observed (Figure 4C). Both Th1 and to a lesser extent Tregs were significantly reduced in the circulation (44% and 10%, respectively), whereas this effect was not observed in the spleen. No significant effects of MSC therapy on Th2 cells were observed (Figure 4C).

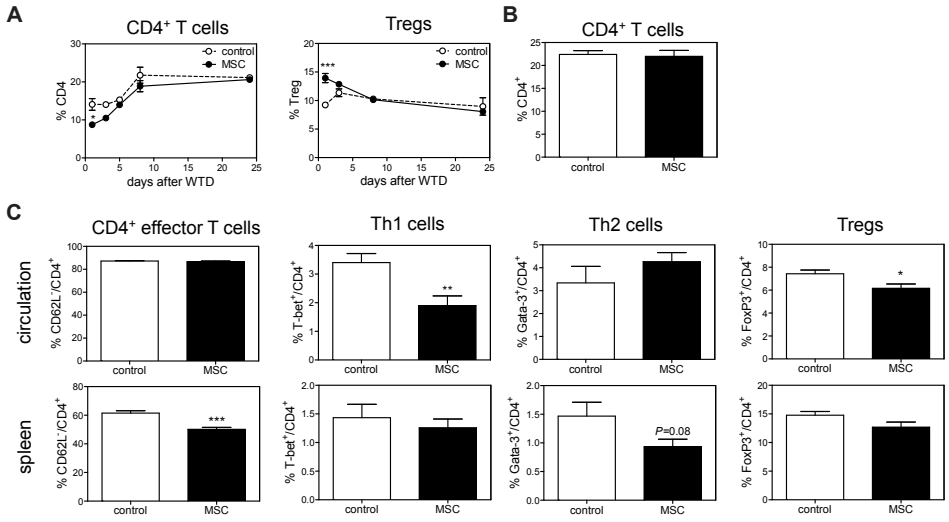


Figure 4. MSC-treatment affects CD4$^+$ T cell responses in vivo. Male LDLr$^{-/-}$mice received three *i.v.* injections of either PBS (control) or 0.5 x 10^6 MSCs (MSC) and were then fed a Western-type diet (WTD) for eight weeks. **A.** CD4$^+$ T cells, as well as the percentage of FoxP3$^+$ regulatory T cells (Treg) within CD4$^+$ T cells, were measured in the circulation throughout the entire experiment by flow cytometry. **B.** After eight weeks, CD4$^+$ T cells were determined in the spleen. **C.** After eight weeks, effector CD4$^+$ T cells, determined as CD62L$^-$ within CD4$^+$ T cells, as well as T cell subsets of CD4$^+$ T cells in the circulation and spleen were determined by flow cytometry. All values are expressed as mean±SEM and representative of six mice. * P<0.05, ** P<0.01, *** P<0.001.

Total CD8$^+$ T cell numbers were not affected by MSC-treatment in the circulation throughout the entire experiment (Figure 5A) and were also not affected in the spleen after eight weeks of WTD (Figure 5B). However, we again found a 25% decrease in effector CD62L$^-$/CD8$^+$ T cells in the circulation and spleen after eight weeks WTD upon MSC-treatment (Figure 5C). To evaluate the proliferative capacity of T cells after MSC therapy, we isolated splenocytes eight weeks after induction of atherosclerosis by WTD feeding and cultured them in the presence of αCD3/CD28. In line with our in vitro data, splenocytes obtained from MSC-treated recipients showed a significant 30% decrease in T cell proliferation (Figure 5D). Furthermore, MSC treatment also significantly reduced circulating monocytes by 33%, which was directly associated

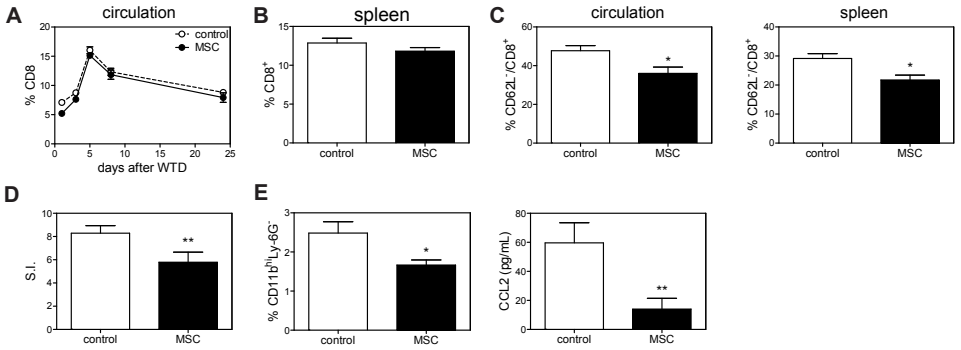


Figure 5. MSC-treatment affects CD8⁺ T cell and monocyte responses in vivo. A. $CD8^+$ T cells were measured in the circulation throughout the entire experiment by flow cytometry. **B.** After eight weeks, splenic $CD8^+$ T cells were determined by flow cytometry. **C.** Effector $CD8^+$ T cells, determined as $CD62L^-$ within $CD8^+$ T cells, in the circulation and spleen were determined by flow cytometry. **D.** Splenocytes were isolated and stimulated with αCD3/CD28 for 72 hours. Proliferation was assessed by the amount of ^{3}H-thymidine incorporation. Proliferation is normalized for proliferation of controls (without stimulation) and expressed as the stimulation index (S.I.). **E.** Circulating monocytes, determined as $CD11b^{hi}Ly\text{-}6G^-$, were analyzed by flow cytometry. CCL2 levels in serum were determined by ELISA. All values are expressed as mean±SEM and representative of six mice. * P<0.05, ** P<0.01.

with a 77% reduction in serum CCL2 levels (Figure 5E), again suggesting a reduced inflammatory status of the MSC-treated mice.

Interestingly, we found a significant 33% reduction in serum cholesterol levels in MSC-treated mice after eight weeks WTD, but no effect on bodyweight (Figure 6A). The decrease in cholesterol levels was mainly a result of a decreased VLDL as assessed by FPLC analysis (Figure 6B). The effect on VLDL levels likely originated from reduced VLDL production, because liver mRNA expression of the rate-limiting enzyme

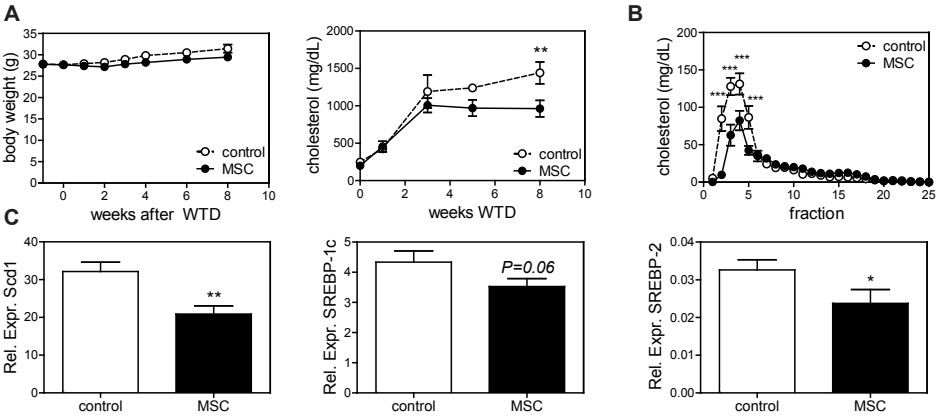


Figure 6. MSC treatment reduces VLDL production. A. Body weight and cholesterol levels were monitored throughout the entire experiment. **B.** Cholesterol distribution among plasma lipoprotein subclasses was determined by FPLC analysis after eight weeks WTD. For FPLC analysis serum of three mice was pooled. **C.** Liver mRNA expression of Scd1, SREBP-1c and SREBP-2 is shown, relative to the expression of three housekeeping genes (SDHA, HPRT and Rpl27). All values are expressed as mean±SEM and representative of all mice. *P<0.05, **P<0.01, ***P<0.001.

in fatty acid synthesis (Stearoyl-CoA desaturase-1, Scd1) was significantly reduced by 35%. Moreover transcription factors regulating fatty acid and cholesterol synthesis, sterol regulatory element-binding proteins 1c and 2 (SREBP-1c and SREBP-2) were reduced by 19% and 24%, respectively (Figure 6C).

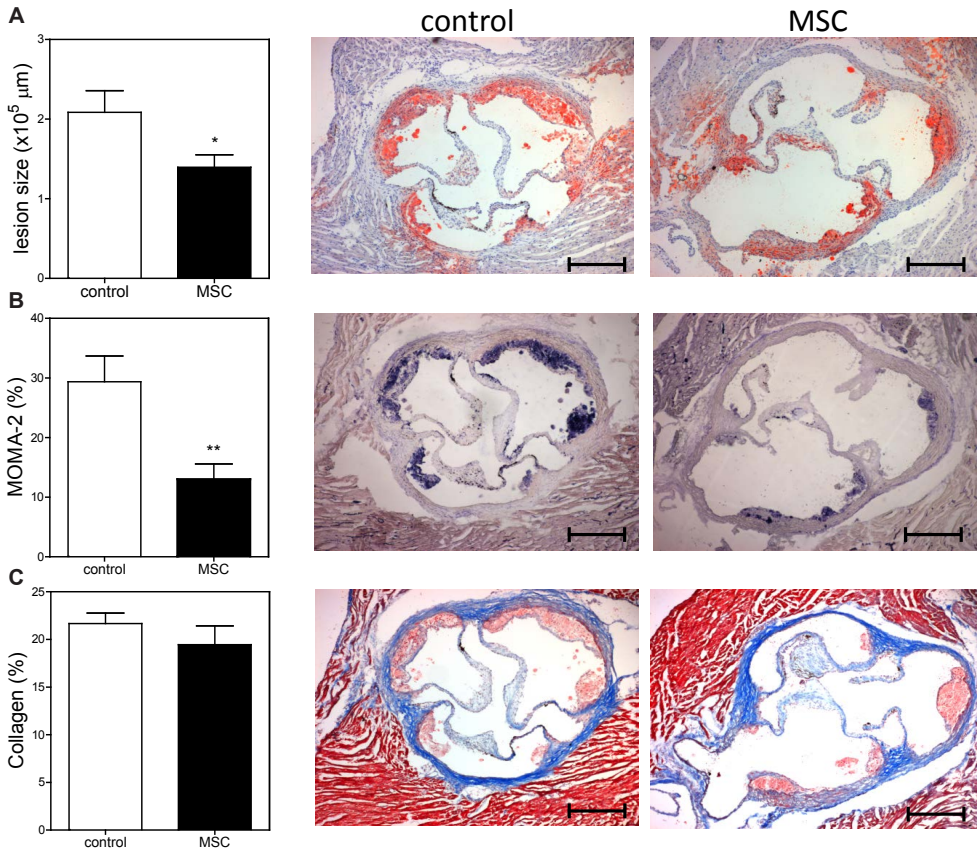


Figure 7. MSC treatment reduces lesion development. A. Lesion size in the three valve area of the aortic root was determined; representative cross-sections stained with Oil-Red-O and hematoxylin are shown. **B.** Macrophage content was determined by MOMA-2 staining as percentage of total lesion area. **C.** Collagen content was determined by Masson's Trichrome staining as percentage of total lesion area. Values are expressed as mean±SEM and representative of all mice. Scale bar, 300 µm. *P<0.05, **P<0.01.

The beneficial effects of MSC-treatment both on immune responses and cholesterol metabolism cumulated in a significant 33% decrease in atherosclerotic lesion size in MSC-treated mice (1.4 x 10^5 ± 0.2 x 10^5 µm), compared with control mice (2.1 x 10^5 ± 0.3 x 10^5 µm; Figure 7A). Additionally, we determined a significant 56% reduction in relative macrophage positive area of total lesion size (control: 29.4 ± 4.3 % vs. MSC: 13.0 ± 2.6 %), indicating a reduced inflammatory status of the lesions (Figure 7B). Lesion stability was not affected as the collagen content, determined by Masson's trichrome staining, was not significantly different between the groups (control: 21.7 ± 1.1 % vs. MSC: 19.4 ± 2.0 %; Figure 7C).

Discussion

Here we show that MSC treatment may be a promising strategy to reduce atherosclerotic lesion development. In agreement with previous studies[1,15,17,18], we describe that MSCs can reduce the production of pro-inflammatory cytokines by DCs and dramatically inhibit T cell proliferation. We further show that adoptive transfer of MSCs into LDLr$^{-/-}$ mice results in an initial drop in circulating CD4^{+} T cell numbers. While this reduction was only observed initially after MSC transfer and induction of atherosclerosis by WTD feeding, a significant decrease in effector CD62L^{-}/CD4^{+} and CD62L^{-}/CD8^{+} T cells in the spleen was observed after eight weeks WTD, clearly indicating a reduced differentiation of naïve T cells, which is consistent with effects observed in vitro. In agreement, a significant decrease in circulating Th1 cells as well as Tregs was observed eight weeks after MSC transfer, although we did observe an initial increase in circulating Tregs. Since we observed an overall significant inhibition of T cell differentiation both in vivo and in vitro, we hypothesized that additional processes and cell types are needed for this initial increase in Tregs. It has been suggested that MSCs can induce apoptosis of T cells, which may explain the initial drop in CD4^{+} T cells observed in vivo, and that these apoptotic T cells are cleared by phagocytes, which in turn may induce Tregs[34]. Therefore in vivo Tregs could initially be induced indirectly, while later the overall inhibition of T cell differentiation reduces Treg numbers. Additionally, MSC therapy significantly reduced circulating monocytes and serum CCL2 levels, again clearly suggesting reduced immune responses. This is in line with a previous study showing reduced monocytes after MSC therapy for myocardial infarction[10]. The reduced monocytes and their reduced recruitment to the lesions resulted in a significant 56% reduction in lesional macrophages.

Unexpectedly, we found significantly lower plasma cholesterol levels in MSC-treated mice, due to a reduction of VLDL levels, which is to our knowledge an effect that was not previously described upon MSC treatment. The effects of MSC therapy on plasma cholesterol levels only emerged around four to five weeks after treatment indicating that the effects of MSCs could be indirect, e.g. by modulation of other cell types. Previous studies have found a link between immune cells and cholesterol metabolism. For example lymphotoxins, which are expressed by CD4^{+} T cells and DCs, play a role in the homeostasis of these cells, but can also contribute to metabolic disease[35,36]. CD4^{+} T cell expression of LIGHT, another ligand of the lymphotoxin receptor, increases plasma cholesterol levels, again showing a direct link between immune cells and cholesterol metabolism[35]. Furthermore, an increased lifespan of DCs[37] and the adoptive transfer of mature DCs was found to correlate with decreased serum cholesterol levels[38]. Hence a modulation of DCs by MSCs could also indirectly affect cholesterol metabolism. We hypothesize that the overall reduced inflammatory environment in MSC-treated mice, due to the immunomodulatory effects of MSCs on immune cells, affected VLDL synthesis, by downregulation of Scd1, SREBP-1c and SREBP-2. For example, TNF-α, which is downregulated upon MSC and splenocyte co-culture, has been shown to upregulate SREBP-1c[39], thereby increasing VLDL synthesis. Moreover, IL-10 overexpression has been shown to result in reduced plasma

cholesterol, mostly due to reduced VLDL, in LDLr$^{-/-}$ mice[40].

Overall MSC therapy was highly effective in reducing both immune responses and improving dyslipidemia, the two driving forces behind atherosclerosis, and resulted in a significant 33% reduction in aortic root lesion sizes. Future studies will need to show the effect of MSCs on different stages of atherosclerosis, but our observations suggest that due to a combined effect on inflammation and plasma cholesterol levels a beneficial effect may be anticipated. For example, an increased stability of advanced lesions can be expected as IL-10 has been shown to promote lesion stability[41,42]. The fact that we do not observe an effect on lesion stability is likely associated with early atherosclerotic lesions which contain a low amount of vascular smooth muscle cells. Recently, allogeneic MSCs were evaluated for their potential to repair ruptured lesions and were shown to increase regeneration of the inner endothelial lining and collagen fiber formation in the vessel wall[43], implying their potential for the treatment of progressed lesions. Moreover, it could for instance prove interesting to repeatedly administer MSCs to ensure a more effective treatment for long term intervention. Moreover a modulation of MSCs to enhance their anti-inflammatory capacities and/or their lifespan could prove interesting.

The drawback of some studies[9,10] assessing the effect of MSCs on inflammation is that they employ human MSCs in SCID mice, which lack T and B cells. As these do not have a fully functional immune system it is difficult to examine the effect of MSCs on immune responses. Additionally, there have been reports that allogeneic MSCs can be rejected by recipient mice[9]. For these reasons, we performed an adoptive transfer of MHC-matched mouse MSCs into LDLr$^{-/-}$ mice in our study to avoid host immune responses to the grafted MSCs and to be able to do the experiment in mice with a functional immune system.

To translate our findings to clinical application, we anticipate to replicate our studies using human MSCs in humanized mouse models for atherosclerosis, which can be humanized in their immune system, e.g. by transfer of human hematopoietic stem cells into SCID, NOG or NSG mice[44,45]. Also, a more humanized cholesterol metabolism, such as in the ApoE*3/CETP-Leiden mice[46], would enable a confirmation of effects observed on VLDL metabolism. Taken together, our study provides first evidence that a MSC-based treatment strategy for atherosclerosis may be beneficial.

References

1. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–7 (1999).
2. Bernardo, M. E. & Fibbe, W. E. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 13, 392–402 (2013).
3. Toma, C., Pittenger, M. F., Cahill, K. S., Byrne, B. J. & Kessler, P. D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 105, 93–8 (2002).
4. Mangi, A. A. *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat. Med. 9, 1195–201 (2003).
5. Barbash, I. M. et al. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* 108, 863–8 (2003).
6. Amado, L. C. *et al.* Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem

cells after myocardial infarction. *Proc. Natl. Acad. Sci. U. S. A.* 102, 11474–9 (2005).
7. Silva, G. V *et al.* Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. *Circulation* 111, 150–6 (2005).
8. Houtgraaf, J. H. *et al.* Intracoronary infusion of allogeneic mesenchymal precursor cells directly after experimental acute myocardial infarction reduces infarct size, abrogates adverse remodeling, and improves cardiac function. *Circ. Res.* 113, 153–66 (2013).
9. Uccelli, A., Moretta, L. & Pistoia, V. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 8, 726–36 (2008).
10. Dayan, V. *et al.* Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. *Basic Res. Cardiol.* 106, 1299–310 (2011).
11. Németh, K. *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat. Med.* 15, 42–9 (2009).
12. Melief, S. M., Geutskens, S. B., Fibbe, W. E. & Roelofs, H. Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. Haematologica 98, 888–95 (2013).
13. Melief, S. M. *et al.* Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem Cells* 31, 1980–91 (2013).
14. Nauta, A. J., Kruisselbrink, A. B., Lurvink, E., Willemze, R. & Fibbe, W. E. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. *J. Immunol.* 177, 2080–7 (2006).
15. Aggarwal, S. & Pittenger, M. F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 105, 1815–22 (2005).
16. Beyth, S. *et al.* Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 105, 2214–9 (2005).
17. Bartholomew, A. *et al.* Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp. Hematol.* 30, 42–8 (2002).
18. Glennie, S., Soeiro, I., Dyson, P. J., Lam, E. W.-F. & Dazzi, F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 105, 2821–7 (2005).
19. Hansson, G. K. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 352, 1685–95 (2005).
20. Weber, C., Zernecke, A. & Libby, P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat. Rev. Immunol.* 8, 802–15 (2008).
21. Taghavie-Moghadam, P. L., Butcher, M. J. & Galkina, E. V. The dynamic lives of macrophage and dendritic cell subsets in atherosclerosis. *Ann. N. Y. Acad. Sci.* 1319, 19–37 (2014).
22. Emeson, E. E., Shen, M. L., Bell, C. G. & Qureshi, A. Inhibition of atherosclerosis in CD4 T-cell-ablated and nude (nu/nu) C57BL/6 hyperlipidemic mice. A*m. J. Pathol.* 149, 675–85 (1996).
23. Frostegård, J. *et al.* Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 145, 33–43 (1999).
24. Zhou, X., Paulsson, G., Stemme, S. & Hansson, G. K. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J. Clin. Invest.* 101, 1717–25 (1998).
25. Voloshyna, I., Littlefield, M. J. & Reiss, A. B. Atherosclerosis and interferon-γ: new insights and therapeutic targets. *Trends Cardiovasc. Med.* 24, 45–51 (2014).
26. Murray, P. J. & Wynn, T. A. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* 11, 723–37 (2011).
27. Chistiakov, D. A., Sobenin, I. A., Orekhov, A. N. & Bobryshev, Y. V. Dendritic cells in atherosclerotic inflammation: the complexity of functions and the peculiarities of pathophysiological effects. *Front. Physiol.* 5, 196 (2014).
28. Foks, A. C., Lichtman, A. H. & Kuiper, J. Treating Atherosclerosis With Regulatory T Cells. *Arterioscler. Thromb. Vasc. Biol.* (2014). doi:10.1161/ATVBAHA.114.303568
29. Reinders, M. E. J. *et al.* Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl. Med.* 2, 107–11 (2013).
30. Zappia, E. *et al.* Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106, 1755–61 (2005).
31. Augello, A., Tasso, R., Negrini, S. M., Cancedda, R. & Pennesi, G. Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum.* 56, 1175–86 (2007).
32. Fan, H. *et al.* Pre-treatment with IL-1β enhances the efficacy of MSC transplantation in DSS-

induced colitis. *Cell. Mol. Immunol.* 9, 473–81 (2012).
33. Le Blanc, K. *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 371, 1579–86 (2008).
34. Akiyama, K. *et al.* Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* 10, 544–55 (2012).
35. Lo, J. C. *et al.* Lymphotoxin beta receptor-dependent control of lipid homeostasis. *Science* 316, 285–8 (2007).
36. Upadhyay, V. & Fu, Y.-X. Lymphotoxin signalling in immune homeostasis and the control of microorganisms. *Nat. Rev. Immunol.* 13, 270–9 (2013).
37. Gautier, E. L. *et al.* Conventional dendritic cells at the crossroads between immunity and cholesterol homeostasis in atherosclerosis. *Circulation* 119, 2367–75 (2009).
38. Habets, K. L. L. *et al.* Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc. Res.* 85, 622–30 (2010).
39. Ruan, H. *et al.* Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. *Diabetes* 51, 3176–88 (2002).
40. Von Der Thüsen, J. H. *et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J.* 15, 2730–2 (2001).
41. Mallat, Z. *et al.* Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* 85, e17–24 (1999).
42. Potteaux, S. *et al.* Leukocyte-derived interleukin 10 is required for protection against atherosclerosis in low-density lipoprotein receptor knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 24, 1474–8 (2004).
43. Fang, S.-M. *et al.* Allogeneic bone marrow mesenchymal stem cells transplantation for stabilizing and repairing of atherosclerotic ruptured plaque. *Thromb. Res.* 131, e253–7 (2013).
44. Ito, M. *et al.* NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 100, 3175–82 (2002).
45. Shultz, L. D. *et al.* Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J. Immunol.* 174, 6477–89 (2005).
46. Van den Maagdenberg, A. M. *et al.* Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 268, 10540–5 (1993).

Vanessa Frodermann[1]
Janine van Duijn[1]
Gijs H.M. van Puijvelde[1]
H. Maxime Lagraauw[1]
Peter J. van Santbrink[1]
Margreet R. de Vries[2,3]
Paul H.A. Quax[2,3]
Ilze Bot[1]
Amanda C. Foks[1,4]
Saskia C.A. de Jager[1,5]
Johan Kuiper[1]

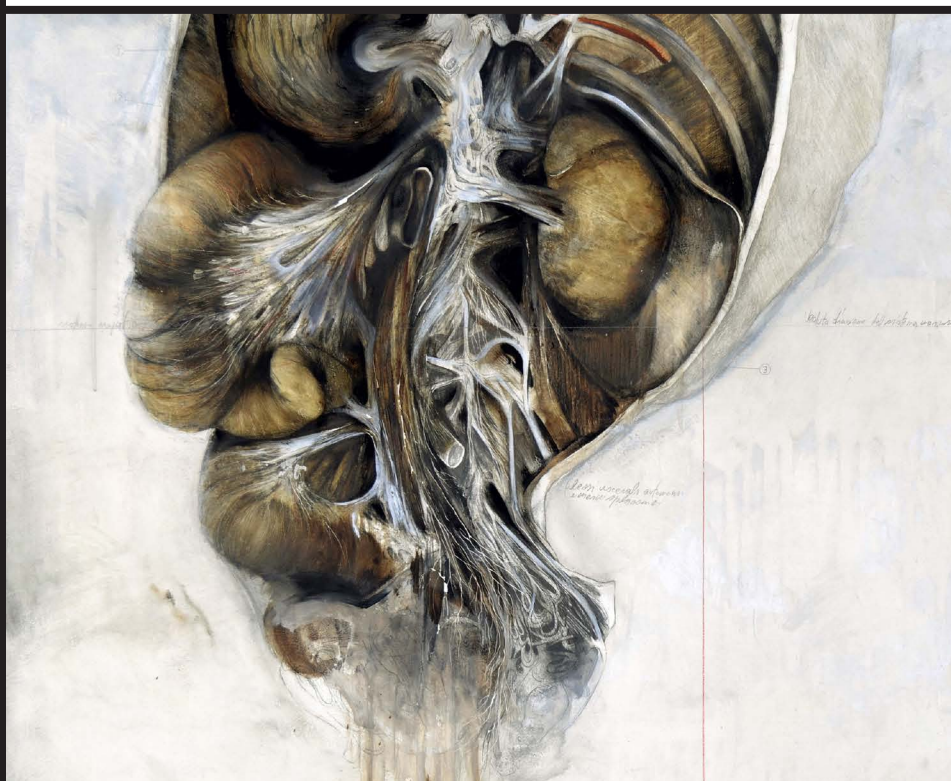
5

[1] Division of Biopharmaceutics, LACDR, Leiden University, Leiden, The Netherlands
[2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands
[3] Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands
[4] Present address: Department of Pathology, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts, USA.
[5] Present address: Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

Modulation of Macrophages in Atherosclerosis by Heat-Killed *S. aureus*

Identification of a Novel Anti-Atherogenic Function for TLR2/PI3K-Signaling

Submitted



Abstract

Objective *Staphylococcus aureus* cell wall components can induce IL-10 responses by immune cells, which may result in atheroprotection. Here we thus investigated whether heat-killed *Staphylococcus aureus* (HK-SA) could reduce atherosclerosis by modulation of macrophage function.

Methods and Results We administered HK-SA twice weekly *intraperitoneally* to LDL receptor-deficient mice, which were subsequently put on a Western-type diet for six weeks. HK-SA administration resulted in an immediate significant 1.6-fold increase in IL-10 production by peritoneal cells and splenocytes, and a 12-fold increase in serum IL-10 levels. Moreover, aortic plaque ICAM-1, VCAM-1 and CCL2 expression were significantly downregulated by 40%. HK-SA-treated mice had reduced numbers of inflammatory Ly-6C^{hi} monocytes as well as Th1 and Th17 cells in circulation and spleen, respectively. Subsequently, attenuated leukocyte recruitment resulted in a significant inhibition of macrophage and T cell infiltration in atherosclerotic plaques, culminating in a significant 34% reduction of aortic root lesion sizes.

To determine the effects of *intraperitoneal* HK-SA treatment, we stimulated macrophages with HK-SA in vitro. This resulted in a significant TLR2-dependent increase in IL-10, arginase-1, iNOS, TNF-α, PD-L1, CCL22, and IDO expression. PI3K was found to crucially determine the balance of pro- and anti-inflammatory gene expression. The HK-SA-induced macrophage phenotype resembles M2b-like immunoregulatory macrophages.

Conclusion In contrast to the currently accepted pro-atherogenic role of TLR2 and PI3K, our data suggest that combined TLR2/PI3K-dependent signaling induced by HK-SA in macrophages is atheroprotective.

Introduction

Macrophages are found in all stages of atherosclerotic lesion development and outnumber any other cell type in the lesion[1,2]. They play a crucial role in clearing debris in the arterial wall, but are also involved in shaping immune responses. Therefore modulation of macrophages towards an anti-inflammatory phenotype is an attractive therapeutic goal.

The macrophage phenotype is determined by its local microenvironment, e.g. by cytokines and Toll-like receptor (TLR) agonists. IFN-γ and LPS induce classically activated M1 macrophages, characterized by e.g. iNOS and IL-12p70 expression[3,4]; while e.g. IL-4 can induce alternatively activated M2 macrophages, characterized by an upregulation of arginase-1 and IL-10 production[3,4]. The specific role of each macrophage subset in atherosclerosis is still under investigation. However, due to the production of anti-inflammatory mediators, such as IL-10, M2 macrophages are currently considered to be anti-atherogenic[5]. Indeed, we and others have previously confirmed that IL-10 can significantly protect against atherosclerosis[6,7]. This is due to the wide-ranging beneficial effects of IL-10, such as a reduction of CCL2 levels[8], reduced lesional macrophages[9], decreased lesional IFN-γ-producing T cells[6] and inhibition of T cell activation and proliferation[10].

Macrophages, as well as DCs, recognize pathogen- and danger-associated molecules in their microenvironment via their TLRs. This results in the maturation and activation of antigen-presenting cells (APCs) with subsequent production of pro-inflammatory cytokines. While mature DCs are vital for the activation of naïve T cells, both APCs can shape adaptive T cell responses and ensure long-lasting and specific responses.

TLRs recognize various highly conserved molecules and TLR2 and TLR4 signaling in the atherosclerotic plaque has been mainly associated with pro-inflammatory cytokine production by lesional macrophages[11,12]. TLR2 and TLR4 both recruit intracellular myeloid differentiation primary-response protein 88 (MyD88), and the MyD88 adaptor-like protein (MAL), which result in downstream activation of nuclear factor (NF)-κB and activating protein-1 (AP-1). TLR4 additionally results in TIR domain-containing adaptor protein inducing IFNβ (TRIF)/ TRIF-related adaptor molecule (TRAM)-dependent induction of NF-κB and AP-1, resulting in pro-inflammatory responses[13]. Another adaptor molecule, B-cell adaptor for PI3K (BCAP), was found to link TLRs to PI3K and was shown to reduce pro-inflammatory signaling in response to TLRs, e.g. in B cells and macrophages[13].

After pathogen elimination, it is vital to terminate the immune response to ensure a return to immune homeostasis without inducing damage to the affected tissue. LPS/TLR4 stimulation of monocytes results in a late anti-inflammatory IL-10 response to downregulate the primary induction of pro-inflammatory cytokines[14]. Similarly, it has been found that TLR2 can induce anti-inflammatory IL-10 responses[15]. Animal studies have established that certain bacteria, such as *Porphyromonas gingivalis* and *Yersinia* bacteria, induce a TLR2-dependent IL-10 production to evade the host's immune system, which can inhibit IFN-γ production by T cells[16,17]. Moreover,

administration of heat-killed *Staphylococcus epidermis* was found to potently induce IL-10 responses in APCs, which effectively inhibited massive T cell activation and toxic shock syndrome in mice[18]. This was attributed to peptidoglycan-embedded molecules in the *Staphylococcal* cell wall[18]. Indeed, peptidoglycan induced an IL-10 response in human macrophages via TLR2/PI3K, which resulted in the inhibition of T cell proliferation. However, stimulation of DCs with peptidoglycan resulted in an induction of T helper (Th) 1 and Th17 responses, indicating that not only the ligand and its kinetics, but also the cell type involved determines the type of immune response[19].

In this study we established that exposure of macrophages to heat-killed *Staphylococcus aureus* (HK-SA), which contains cell wall components such as peptidoglycan that are recognized by TLR2, induced anti-inflammatory IL-10-producing macrophages that protected LDL receptor-deficient ($LDLr^{-/-}$) mice from atherosclerosis development.

Material and Methods

Animals

C57BL/6, $LDLr^{-/-}$ and $TLR2^{-/-}$ mice were originally obtained from the Jacksons Laboratory, kept under standard laboratory conditions, and administered food and water *ad libitum*. All animal work was approved by the Ethics Committee for Animal Experiments of Leiden University and conforms to Dutch government guidelines.

Macrophage and peritoneal macrophage stimulations

Bone marrow cells were isolated from the tibias and femurs of C57BL/6 mice. The cells were cultured for seven days in RPMI supplemented with 10% FCS, 100 U/mL penicillin/streptomycin, 0.1mM nonessential amino acids, 1% pyruvate (all obtained from PAA), 2mM L-glutamine (Thermo Fisher Scientific) in the presence of 10 ng/mL macrophage colony-stimulating factor (Peprotech) to obtain macrophages. Macrophage purity was assessed by CD11c, CD11b and F4/80 expression (flow cytometry) and routinely found to be above 90%. Peritoneal cells were isolated by peritoneal lavage, washed with PBS and left to adhere on plates for 2 hours at 37°C and 5% CO_2. Non-adherent cells were removed by aspiration and replaced with fresh RPMI, supplemented with 10% FCS, 100 U/mL penicillin/streptomycin and 2mM L-glutamine.

For in vitro stimulations, 1 x 10^5 cells per well were plated in 96-well flat-bottom plates (Greiner Bio-One) for analysis of cytokine production by ELISA or 5 x 10^5 per well in 24-well plates (Greiner Bio-One) for mRNA isolation. Cells were stimulated with indicated amounts of heat-killed *Staphylococcus aureus* (tlrl-hksa, Invivogen) for 24 hours.

Atherosclerosis

Atherosclerosis was induced in 10-12 weeks old female $LDLr^{-/-}$ mice by feeding a Western-type diet (WTD; 0.25% cholesterol and 15% cocoa butter; Special Diet Services) for six weeks. Mice were treated twice weekly *intraperitoneally* with 10^8 colony-forming units of HK-SA (tlr-hksa, Invivogen) or PBS as a control. Treatment

was started one week prior to WTD and continued during the entire experiment.

Flow Cytometry

At sacrifice, blood and spleen were harvested. Single cell suspensions of spleens from $LDLr^{-/-}$ mice were obtained by using a 70 µm cell strainer (VWR International). Red blood cells were lysed with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently, 3 x 10^5 cells per sample were stained with the appropriate antibodies. The following antibodies were used: CD11b-eFluor450 (clone M1/70), CD11c-FITC (clone clone N418), CD4-PerCP (clone RM4-5; BD Biosciences), CD25-FITC (clone eBio3C7), FoxP3-APC (clone FJK-16s), Gata-3-PE (clone TWAJ), Ly-6C-PerCP (clone HK1.4), Ly-6G-FITC (clone 1A8; BD Biosciences), RORγt-PE (clone AFKJS-9), and T-bet-APC (clone eBio4B10). All antibodies were purchased from eBioscience, unless stated otherwise. For intracellular staining, cells were fixed and permeabilized according to the manufacturer's protocol (eBioscience). Flow cytometry analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (BD Biosciences).

Histological analysis

To determine plaque size, 10 µm cryosections of the aortic root were stained with Oil-Red-O and haematoxylin (Sigma Aldrich). Corresponding sections were stained for collagen using Sirius Red (Sigma Aldrich) or immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b, 1:1000, Serotec Ltd.). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100; Sigma Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrate. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, ThermoScientific). BrightVision anti-rabbit-HRP was used as secondary antibody (Immunologic). The section with the largest lesion and four flanking sections were analyzed for lesion size and collagen content, two flanking sections were analyzed for macrophage and T cell content. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2-positive area by the total lesion surface area.

Real-time PCR

mRNA was isolated from macrophages and the aortic arch using the guanidium isothiocyanate method and reverse transcribed (RevertAid Moloney murine leukemia virus reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast real-time PCR system (Applied Biosystems) using SYBR Green technology. The expression was determined relative to the average expression of three household genes: Succinate dehydrogenase complex, subunit A, flavoprotein (Sdha), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). For used primer pairs refer to Table 1.

Gene	Forward	Reverse
Arg1	TGGCAGAGGTCCAGAAGAATGG	GTGAGCATCCACCCAAATGACAC
CCL22	TCTTGCTGTGGCAATTCAGA	GAGGGTGACGGATGTAGTCC
CCL2	CTGAAGCCAGCTCTCTCTTCCTC	GGTGAATGAGTAGCAGCAGGTGA
CD3	TCTGCTACACACCAGCCTCAAA	ATGACCATCAGCAAGCCCAGA
CD4	CAAAGTTCTCTCCATGTCCAACCTA	CACTCTTATAGGCCGTGATAGCTG
CD68	TGCCTGACAAGGGACACTTCGGG	GCGGGTGATGCAGAAGGCGATG
CD163	CAGTGCCCCTCGTCACCTTG	GATCTCCACACGTCCAGAACAGTC
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
ICAM-1	GTCCGCTTCCGCTACCATCAC	GGTCCTTGCCTACTTGCTGCC
IDO	CACTGCACGACATAGCTACCAGTC	TCCAGCCAGACAGATATATGCGGA
IL-10	GGGTGAGAAGCTGAAGACCCTC	TGGCCTTGTAGACACCTTGGTC
iNOS	CCTGGTACGGGCATTGCT	GCTCATGCGGCCTCCTTT
p35	CCAAACCAGCACATTGAAGA	CTACCAAGGCACAGGGTCAT
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
Sdha	TATATGGTGCAGAAGCTCGGAAGG	CCTGGATGGGCTTGGAGTAATCA
TGF-β	AGGGCTACCATGCCAACTTCT	GCAAGGACCTTGCTGTACTGTGT
TNF-α	GCCTCTTCTCATTCCTGCTTGTG	ATGATCTGAGTGTGAGGGTCTGG
VCAM-1	AGACTGAAGTTGGCTCACAATTAAGAAG	AGTAGAGTGCAAGGAGTTCGGG

Table 1. Primer Pairs used for qPCR analysis. The relative expression of genes was determined relative to the average expression of the three household genes: succinate dehydrogenase complex, subunit A, flavoprotein (Sdha), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). Abbreviations: Arg1, arginase-1; IDO, indole 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor α; VCAM-1; Vascular cell adhesion protein 1.

Cytokine and analysis

IL-12p40 (eBiosciences), IL-10, IL-12p70, TNF-α, and CCL2 (all BD Biosciences) were determined by ELISA, according to manufacturer's protocol.

Serum cholesterol levels

Serum concentrations of total cholesterol were determined by enzymatic colorimetric assays (Roche Diagnostics). Absorbance was read at 490 nm. Precipath (standardized serum; Roche Diagnostics) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 µl of serum of each mouse using a Superose 6 column (3.2 x 300 mm, Smart-System; Pharmacia). Total cholesterol content of the effluent was determined as described above.

Statistical analysis

Values are expressed as mean±SEM. Data of two groups were analyzed by Student's

T-test, data of three groups were analyzed by one-way ANOVA and data of two or more groups with more than one variable were analyzed by two-way ANOVA, followed by Bonferroni post-testing. Statistical analysis was performed using Prism (GraphPad). Probability values of $P<0.05$ were considered significant.

Results

HK-SA potently induces IL-10 responses upon intraperitoneal administration

Peptidoglycan-embedded lipopeptides and glycopolymers in the *Staphylococcus aureus* cell wall have been shown to induce IL-10 responses[18,19]. We therefore determined whether *intraperitoneal* injections with 10^8 colony-forming units HK-SA could induce IL-10 responses in LDLr$^{-/-}$ mice. Indeed, two days after the injection, serum IL-10 levels were significantly increased by 12-fold in mice treated with HK-SA compared to controls, while IL-12 was not affected (Figure 1A).

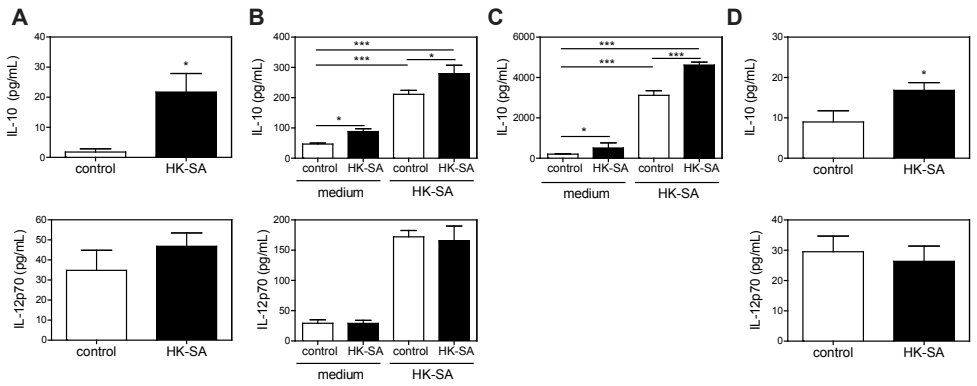


Figure 1. *Intraperitoneal* injection of HK-SA increases IL-10 responses in LDLr$^{-/-}$ mice. A. Two days after the injection of HK-SA cytokine levels in the plasma were measured. **B.** Splenocytes and **C.** peritoneal cells were isolated two days after injection of HK-SA and cultured in the presence of medium or HK-SA. **D.** After six weeks Western-type diet, cytokine levels in the plasma were measured. All cytokine responses were determined by ELISA. All values are expressed as mean±SEM and are representative of at least five mice.

We additionally isolated splenocytes and peritoneal macrophages two days after the HK-SA injection and cultured them ex vivo. We observed a significant 1.8-fold higher basal IL-10 production in cultured splenocytes in the HK-SA group (Figure 1B) and a 2.5-fold higher IL-10 production in cultured peritoneal macrophages from HK-SA-treated mice (Figure 1C). Upon re-stimulation with HK-SA, IL-10 production was significantly increased in splenocytes and peritoneal macrophages isolated from both groups. However, IL-10 responses in the HK-SA treated group were 1.6-fold higher than in the control group (Figure 1B and C). IL-12p70 responses were unaffected in splenocytes, while undetectable in peritoneal macrophages (Figure 1B and data not shown). Overall, these results suggest a significant in vivo IL-10 response upon HK-SA treatment.

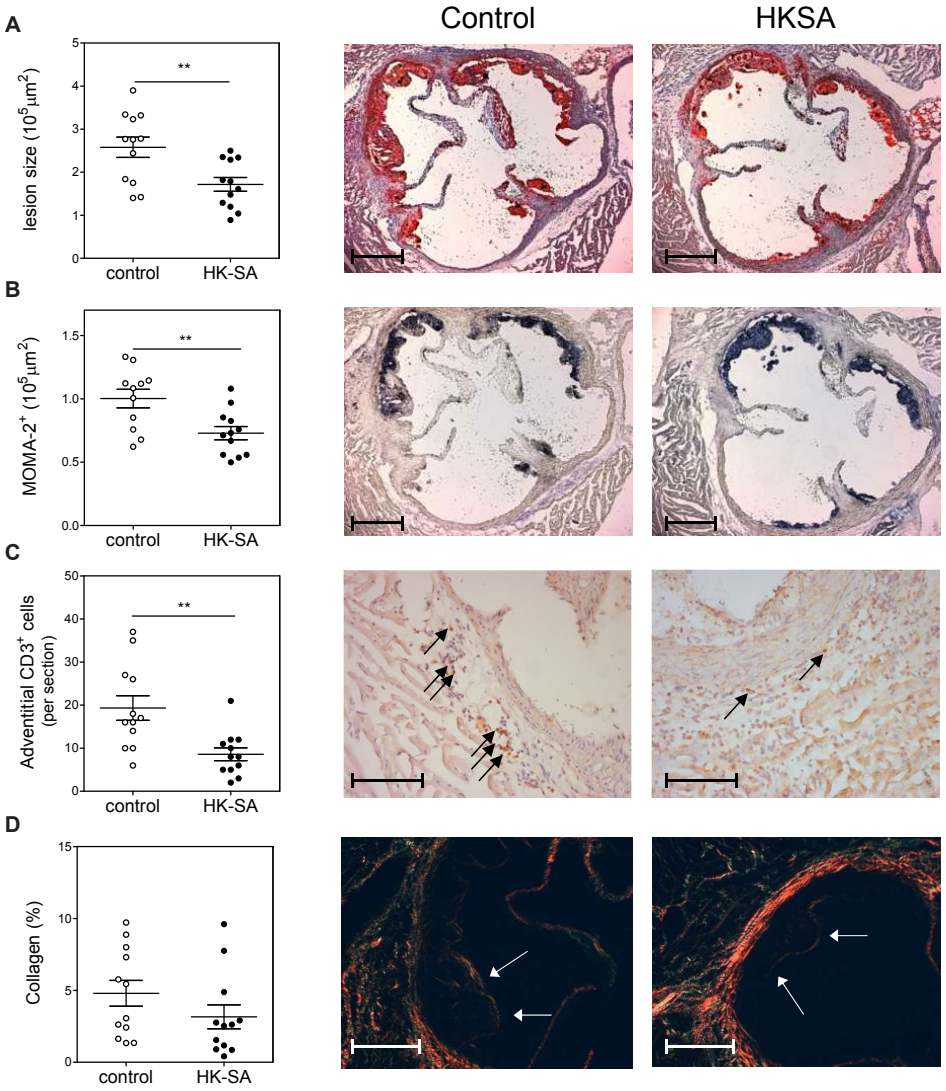


Figure 2. ***Intraperitoneal*** **injections of HK-SA reduce atherosclerotic lesion development. A.** After six weeks Western-type diet, lesion size in the three valve area of the aortic root was determined; representative cross-sections stained with Oil-Red-O and hematoxylin are shown. Scale bar, 300 µm. **B.** Macrophage positive area was determined by MOMA-2 staining. Scale bar, 300 µm. **C.** $CD3^+$ T cells were determined by αCD3 staining. Arrows indicate T cells. Scale bar, 100 µm. **D.** Collagen positive area was determined by Sirius Red staining under polarized light. Arrows indicate collagen-positive area. Scale bar, 200 µm. All values are expressed as mean±SEM and are representative of six mice. * P<0.05, ** P<0.01.

HK-SA treatment reduces atherosclerotic lesion development

We further determined whether *intraperitoneal* injections of HK-SA would be able to prevent atherosclerotic lesion development. We thus injected female $LDLr^{-/-}$ mice with 10^8 CFU of HK-SA twice weekly and after the first two injections we fed the mice a Western-type diet (WTD) for six weeks to induce atherosclerotic lesion development.

After six weeks, we still found a significant 2-fold increase of serum IL-10 levels in HK-SA-treated mice, suggesting increased IL-10 levels throughout our experiment, while IL-12 levels were not affected (Figure 1D).

Strikingly, HK-SA treatment resulted in a significant 34% reduction of aortic root lesion sizes compared to control mice (control: 2.6 x 10^5 ± 0.2 x 10^5 µm versus HK-SA: 1.7 x 10^5 ± 0.2 x 10^5 µm; Figure 2A). Additionally we observed a significant 27% reduction of macrophage positive area in the aortic root (control: 1.0 x 10^5 ± 0.1 x 10^5 µm versus HK-SA: 0.7 x 10^5 ± 0.1 x 10^5 µm; Figure 2B). Similarly, a 56% decrease in adventitial $CD3^+$ T cells in HK-SA-treated mice compared to control mice was observed (control: 19.3 ± 2.8 versus HK-SA: 8.6 ± 1.5 T cells per section; Figure 2C). No significant differences in collagen content between the two groups were observed (control: 4.7 ± 0.9% vs HK-SA: 3.2 ± 0.8%; Figure 2D). The observed effects were not due to effects on cholesterol or weight, as these did not differ between the groups (Figure 3).

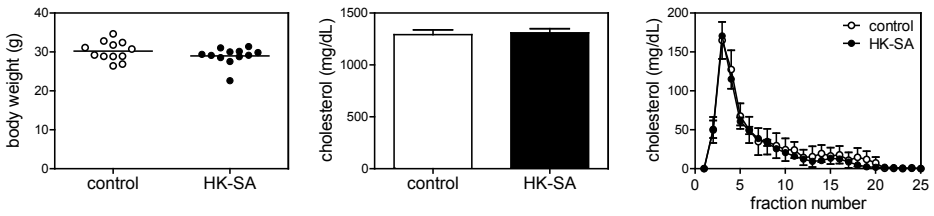


Figure 3. *Intraperitoneal* injections of HK-SA do not affect weight and cholesterol. After six weeks, mice were weighed and cholesterol levels were detected in the serum of mice. All values are expressed as mean±SEM and are representative of twelve mice. For FPLC analysis 3 mice per group were pooled.

HK-SA treatment reduces monocyte and T cell recruitment

To elucidate what caused the reduced macrophage and T cell content of lesions, we assessed whether vascular adhesion molecules were affected by the treatment after six weeks WTD. Indeed we observed a significant 3.7-fold reduction in ICAM-1 and a 1.8-fold reduction in VCAM-1 expression in the aortic arch upon HK-SA treatment (Figure 4A). Furthermore CCL2 expression was significantly reduced by 2.3-fold upon HK-SA treatment, while only a modest non-significant 19% reduction in serum CCL2 levels was observed (Figure 4B).

Because CCL2 plays a crucial role in the recruitment of monocytes to atherosclerotic lesions, we assessed monocyte responses. Overall monocyte numbers were not affected, but the number of inflammatory Ly-$6C^{hi}$ monocytes, which predominantly give rise to lesional macrophages[20], was significantly reduced by 31% (Figure 3C). Indeed, not only the amount of aortic root macrophages, but also CD68 expression in the aortic arch was significantly reduced by 81% in HK-SA-treated mice compared to controls, indicating a reduction of macrophages (Figure 4D).

ICAM-1 and VCAM-1 are also involved in diapedesis of T cells, possibly explaining low T cell numbers found in aortic root lesions. Similarly, we found that the expression of CD3 and CD4 were significantly decreased by 67% in the aortic arch (Figure 4E).

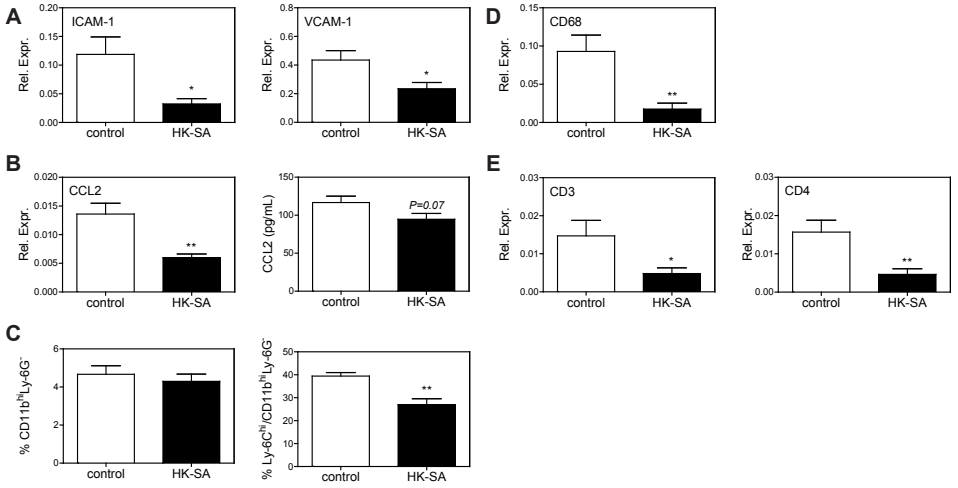


Figure 4. ***Intraperitoneal*** **injections of HK-SA decrease monocyte and T cell recruitment in LDLr mice. A.** Relative ICAM-1 and VCAM-1 mRNA expression in the aortic arch was determined by qPCR. **B.** CCL2 expression in the aortic arch was determined by qPCR and serum CCL2 levels were determined by ELISA. **C.** Circulating monocytes were determined as $CD11b^{hi}Ly\text{-}6G^{-}$ by flow cytometry. Inflammatory $Ly\text{-}6C^{hi}$ monocytes were determined as the percentage of total monocytes. **D.** Relative mRNA expression of CD68 and **E.** CD3 and CD4 in the aortic arch was determined by qPCR. Relative expression was determined compared to housekeeping genes (Sdha, HPRT, Rpl27). All values are expressed as mean±SEM and are representative of at least six mice.* P<0.05, ** P<0.01.

HK-SA treatment reduces Th1, Th2 and Th17 responses in vivo

Lesional T cell responses are determined by induced T cell subsets and their proliferation. We thus monitored T cell responses in the circulation during the entire experiment and found no effects on Tregs or Th1 cells. However, Th2 numbers after three weeks WTD were decreased by 38% and Th17 cells were decreased by 37% after six weeks WTD (Figure 5A). Six weeks after induction of atherosclerosis, splenic $CD4^+$ T cell responses showed a significant 48% reduction in Th1 and a 31% reduction in Th17 responses, while no effects on Th2 and Treg responses were observed (Figure 5B). Next, we isolated splenocytes from control and HK-SA-treated mice after six weeks WTD and stimulated them ex vivo to assess their proliferative capacity. Upon treatment with HK-SA proliferation of splenocytes was decreased by 81% in HK-SA-treated mice, while upon stimulation with oxLDL and αCD3/αCD28 proliferation was decreased by 73% and 64%, respectively (Figure 5C). This indicates an overall reduced proliferative capacity of T cells in mice treated with HK-SA.

HK-SA induces an IL-10-producing immunoregulatory M2b-like phenotype in macrophages

To further delineate the effects of HK-SA on macrophages we cultured bone marrow-derived macrophages and treated these with increasing amounts of HK-SA (up to 10^8 colony-forming units). Indeed, we found a significant dose-dependent IL-10 production in response to increasing amount of HK-SA (Figure 6A). As atherosclerosis

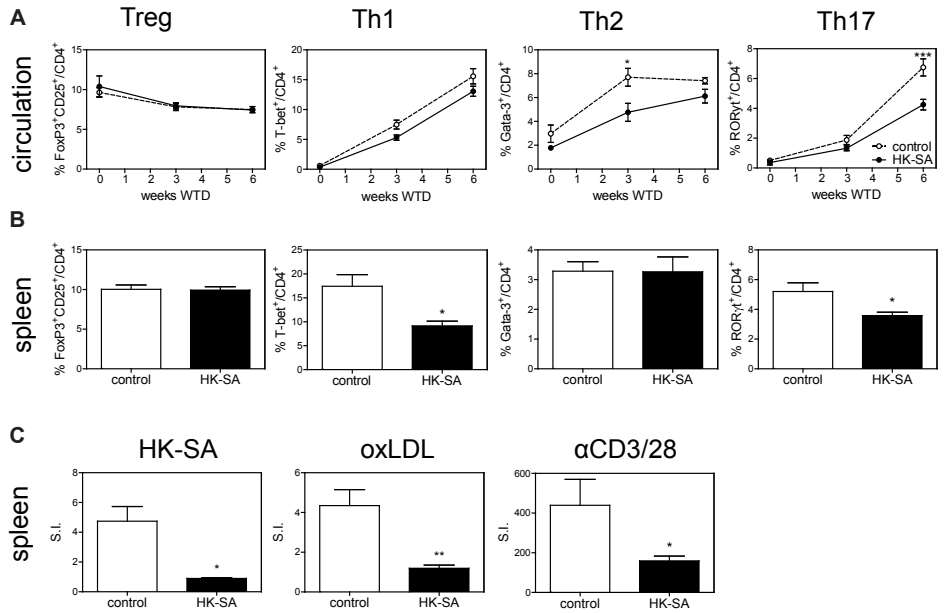


Figure 5. Intraperitoneal injections of HK-SA decrease inflammatory T cell responses. A. Treg ($FoxP3^+CD25^+$), Th1 ($T\text{-}bet^+$), Th2 ($Gata\text{-}3^+$), and Th17 ($ROR\gamma t^+$) cells within $CD4^+$ T cells were measured in the circulation throughout the entire experiment and **B.** in the spleen after six weeks WTD by flow cytometry. **C.** After six weeks WTD, splenocytes were isolated and cultured in the presence of 10^8 CFU of HK-SA, 5 µg/mL oxLDL, or 1 µg/mL αCD3/αCD28 for 72 hours. Proliferation was assessed by the amount of ^{3}H-thymidine incorporation during the last 16 hours of culture. Proliferation is normalized for proliferation of controls (splenocytes without stimulation) and expressed as the stimulation index (S.I.). All values are expressed as mean±SEM and are representative of six mice.* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

is a predominantly Th1-driven disease, we determined whether HK-SA induced IL-12 production and thereby could exacerbate Th1 responses. We found that macrophages did produce large amounts of IL-12p40, which can heterodimerize to form IL-12 and IL-23, or form homodimers to block IL-12 function (Figure 6A). However, no IL-12 or IL-23 production could be observed (data not shown). This clearly indicates that treatment of macrophages with HK-SA could indeed be responsible for the strong IL-10 responses and the lack of IL-12 responses observed in vivo.

As recognition of pathogens is associated with macrophage activation and phenotype changes, we determined whether HK-SA exposure induced a specific macrophage subset. Exposure to HK-SA induced some markers of inflammatory M1 macrophages: a significant upregulation of iNOS (220-fold) and TNF-α (5-fold) was observed (Figure 6B). TNF-α and iNOS have also been described to be produced, in parallel with IL-10, by immunoregulatory M2b macrophages[21,22] and indeed we saw an 18-fold upregulation of IL-10 and a 45-fold upregulation of arginase-1, an M2 marker[23,24], upon treatment with 10^8 colony-forming units HK-SA (Figure 6C). Additionally we observed a 6-fold upregulation of CCL22, a 30-fold upregulation of IDO and a 6-fold upregulation of PD-L1; all anti-inflammatory markers[25,26] (Figure 6D). Other markers of M2 subsets, such as CD163 (M2a,c) and TGF-β (M2c)[23,24] were

significantly downregulated or not affected, respectively (Figure 6E). This expression profile indicates that macrophages are skewed towards immunoregulatory M2b macrophages with an overall anti-atherogenic phenotype upon HK-SA exposure.

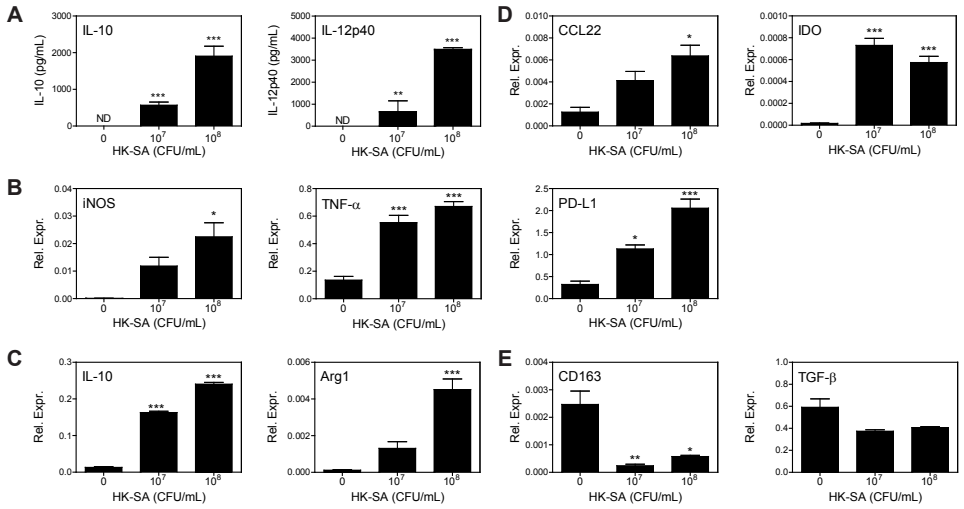


Figure 6. HK-SA induces a distinct macrophage phenotype. Bone marrow-derived macrophages were stimulated with indicated colony-forming units (CFU) of HK-SA. **A.** IL-10 and IL-12p40 responses were determined by ELISA. **B.** Markers for M1 macrophages, **C.** M2(b) macrophages, **D.** anti-inflammatory markers and **E.** M2a and M2c macrophages were determined by qPCR, relative to housekeeping genes (Sdha, HPRT, Rpl27). All values are expressed as mean±SEM and are representative of three experiments. * P<0.05, ** P<0.01, *** P<0.001.

IL-10 responses to HK-SA are TLR2/PI3K-dependent

The *Staphylococcus aureus* cell wall contains TLR2 ligands and we therefore tested whether the observed anti-inflammatory IL-10 response was dependent on TLR2 ligation. Indeed, upon stimulation with 10^8 colony-forming units of HK-SA, macrophages of TLR2$^{-/-}$ mice produced 74% less IL-10 than wild-type controls. Moreover, deficiency of TLR2 also reduced TNF-α responses by 81% (Figure 7A), confirming that TLR2 recognition is needed for cytokine responses by macrophages to HK-SA. Other TLR2 ligands, including peptidoglycan, Pam3CSK4 and FSL-1 also showed a TLR2-dependent IL-10 induction (Figure 7B).

TLR2-induced IL-10 responses have been linked to PI3K activation upon receptor ligation[13]. We therefore treated macrophages with 10μM of the PI3K inhibitor wortmannin. We observed that PI3K inhibition dramatically reduced IL-10 responses by 80% in bone marrow-derived macrophages. On the other hand, PI3K inhibition significantly increased TNF-α and iNOS expression, and interestingly also induced a significant expression of IL-12. In line with IL-10 responses, also arginase-1 was significantly reduced. Surprisingly, IDO was however upregulated by PI3K inhibition (Figure 7C). Therefore, it seems that anti-inflammatory IL-10 responses are clearly PI3K-dependent and correlate with arginase-1 expression of macrophages. Interestingly, M1 markers and the anti-inflammatory enzyme IDO are however under

negative feedback control of PI3K, suggesting a central role for PI3K in determining the macrophage phenotype upon HK-SA treatment.

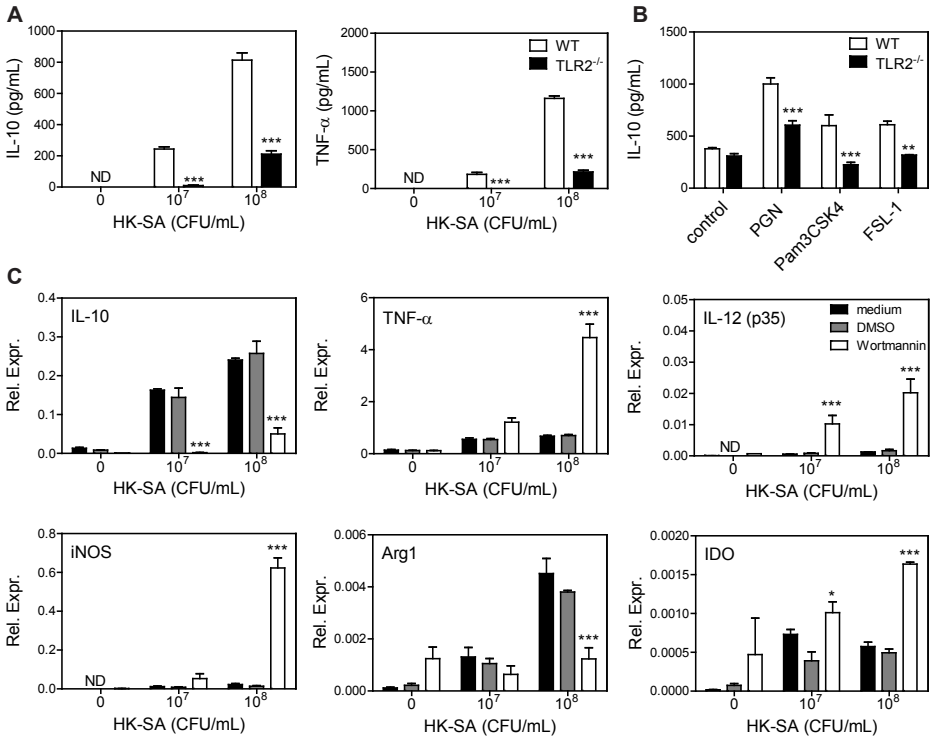


Figure 7. HK-SA-induced IL-10 responses are TLR2/PI3K-dependent. A. Bone marrow-derived macrophages from wild type (WT) or TLR2$^{-/-}$ mice were stimulated with indicated colony-forming units (CFU) of HK-SA or **B.** 10μg/mL of other TLR2 ligands. Cytokine responses are measured by ELISA. **C.** Bone marrow-derived macrophages were stimulated in the presence of the PI3K inhibitor Wortmannin (10μM) or vehicle control (DMSO). Relative expression was determined by qPCR, relative to housekeeping genes (Sdha, HPRT, Rpl27). All values are expressed as mean±SEM and are representative of at least three experiments. * P<0.05, ** P<0.01, *** P<0.001. ND defines not determined.

Discussion

In this study we exploited the finding that exposure to HK-SA induces a strong anti-inflammatory IL-10 response in macrophages to treat atherosclerosis. We show that HK-SA treatment is a potent therapy to reduce both inflammation and atherosclerotic lesion development.

We found that in vitro treatment of murine macrophages with HK-SA induced a strong IL-10 response, while IL-12 and IL-23 responses were lacking, suggesting that exposure of macrophages to HK-SA in an inflammatory environment such as the atherosclerotic lesion may reduce inflammation. To determine whether HK-SA induced a distinct macrophage phenotype, we looked at the gene expression signature of macrophages upon HK-SA stimulation. As expected, recognition of HK-SA induced

the expression of some pro-inflammatory genes such as TNF-α and iNOS, considered as M1 markers. Interestingly, while nitric oxide together with IL-12 has been found to promote Th1[27], it can also induce Tregs in the absence of IL-12[28]. Moreover, nitric oxide expression by macrophages suppresses T cell proliferation[29,30]. Similarly, TNF-α can also have anti-inflammatory effects by promoting the survival and suppressive function of Tregs and by reducing effector T cell activation upon chronic exposure[31]. As such, both high levels of TNF-α and iNOS expression in combination with high IL-10 levels and absence of IL-12p70, as observed after HK-SA-exposure of macrophages, have been described as a hallmark of M2b macrophages[21,22]. M2b macrophages have been shown to be potent immunoregulators and can even protect mice from LPS-induced septic shock[22]. Additionally, the M2 marker arginase-1 is induced by HK-SA treatment. After stimulation with HK-SA, we find that macrophages also express anti-inflammatory IDO, PD-L1 and CCL22. IDO expression is known to suppress $CD4^+$ T cell responses and induce Tregs[25]. PD-L1 also plays a crucial role in inducing and maintaining Tregs[26], while CCL22 plays a role in Treg recruitment. We therefore conclude that HK-SA is capable of inducing a specific anti-inflammatory M2b-like macrophage signature.

The observed anti-inflammatory IL-10 response was largely dependent on TLR2. However, as IL-10 responses are not completely inhibited in $TLR2^{-/-}$ macrophages, other receptors (including scavenger receptors, NOD-like receptors, or other TLRs) recognizing HK-SA components likely contribute to induce IL-10 responses. For example, NOD-like receptor signaling is induced by peptidoglycan and triggers RIP2-dependent activation of NF-κB. The purified gram positive *Streptococcus pneumonia*, which mostly consists of peptidoglycan, was found to induce TLR2-dependent IL-10 responses, which were also largely dependent on NOD2-signaling[32]. However, *intraperitoneal* administration of only muramyl dipeptide, a NOD2-ligand and a component of peptidoglycan, increased atherosclerotic lesion development[33], indicating that combined signals might be needed.

Interestingly, we find that TLR2-induced IL-10 responses upon HK-SA exposure are largely dependent on PI3K. Indeed, previous studies found that TLR2 activation results in IL-10 responses that are partially PI3K-dependent[19,34,35]. Furthermore, TLR3, TLR7 and TLR9 have also been shown to recruit PI3K[13,36], indicating that other TLRs could possibly recognize components of HK-SA, e.g. single-stranded CpG-DNA, and contribute to IL-10 responses to HK-SA. Moreover, we confirm previous observations that PI3K exhibits a negative feedback on the induction of pro-inflammatory cytokine responses by TLRs[19,37–39]. We show that PI3K inhibition by wortmannin significantly increases TNF-α, IL-12p70 and iNOS responses of macrophages in response to HK-SA. Surprisingly PI3K also inhibits anti-inflammatory IDO expression by macrophages, suggesting that it plays a crucial role in balancing several pro- and anti-inflammatory responses to HK-SA in macrophages. Interestingly, PI3K has been suggested to be involved in determining the macrophage phenotype and increased PI3K signaling has been found to skew macrophages to an M2 phenotype[40]. In the context of atherosclerosis, only class Ib PI3K deficiency has been investigated and no effect on macrophage polarization was found, while atherosclerosis was significantly

attenuated[41,42]. Our study suggests that it will be interesting to determine the effect of targeted activation of (class Ia/Ib) PI3K in lesional macrophages.

In general TLR2 is known for the induction of pro-inflammatory responses. For example blocking TLR2 promotes graft acceptance upon renal transplantation[43] and reduces myocardial infarct sizes[44], due to reduced inflammation and tissue necrosis. In atherosclerosis, studies have shown that administration of Pam3Csk4 (TLR2/TLR1 agonist) and MALP2 (TLR2/TLR6 agonist) result in increased atherosclerotic lesion formation[45–47]. Deficiency of either TLR1 or TLR6 was found to have no effect on atherosclerosis in LDLr$^{-/-}$ mice[47]. Interestingly, the pro-atherogenic effect of TLR2 in mice was found to be associated to TLR2 signaling in non-bone marrow-derived cells[46,48].

In line with our findings, preclinical studies have shown that TLR2 signaling can also be exploited to reduce inflammation. *Intraperitoneal* injections of peptidoglycan and Pam3Csk4 were found to reduce ischaemia/reperfusion injury in a PI3K-dependent way[49]. Moreover, *intranasal* Pam3Csk4 administration suppressed asthma development[50], while *intraperitoneal* administration reduced type 1 diabetes[51], both via induction of IL-10 responses and Tregs. IL-10$^{-/-}$ mice have been found to have enhanced Th1 and Th17-like responses[52,53], thereby conversely significant amounts of IL-10 could result in a reduction of Th1 and Th17 responses. Indeed, we observe a significantly decreased induction of splenic Th1 cells and splenic Th17 cells, as well as circulating Th17 cells. The capacity of TLR2-dependent IL-10 production to inhibit IFN-γ-producing T cells has previously been described[16]. Moreover, IL-10 is known to indirectly prevent antigen-specific T cell activation by modulating APCs and directly by inhibiting T cell expansion[10]. In fact, we also observed a reduced proliferative capacity of splenic T cells.

In agreement with earlier observations that IL-10 reduces monocyte adhesion and recruitment[54], we found a significantly reduced expression of adhesion molecules, ICAM-1 and VCAM-1, as well as CCL2 in the aortic arch, resulting in reduced leukocyte recruitment to the lesions. Both macrophage and T cell presence was dramatically reduced in the aortic arch and the aortic root. A decrease of circulating inflammatory Ly-6C^{hi} monocytes, which is in line with reduced lesional macrophages, likely resulted from a decrease in CCL2 and a reduced recruitment of monocytes from bone marrow.

In summary, we demonstrate that HK-SA treatment significantly reduces atherosclerotic lesion development by 34%, likely through inducing IL-10-producing immunoregulatory M2b-like macrophages upon *intraperitoneal* treatment. Our results indicate that TLR2/PI3K activation in macrophages via HK-SA could be beneficial for atherosclerosis. Therefore, it will be important to determine which specific components of HK-SA are responsible for the observed protective effect. Eventually, it will be interesting to specifically target lesional macrophages, ideally with purified *Staphylococcus aureus* components. We believe our study will help to reassess the role of the TLR2/PI3K pathway in atherosclerosis and will support the discovery and further development of specific molecules to modulate macrophages via TLR2/PI3K pathways for atherosclerosis therapy.

References

1. Watanabe, T., Hirata, M., Yoshikawa, Y., Nagafuchi, Y. & Toyoshima, H. Role of macrophages in atherosclerosis. Sequential observations of cholesterol-induced rabbit aortic lesion by the immunoperoxidase technique using monoclonal antimacrophage antibody. *Lab. Invest.* 53, 80–90 (1985).
2. Gown, A. M., Tsukada, T. & Ross, R. Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am. J. Pathol.* 125, 191–207 (1986).
3. Murray, P. J. *et al.* Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* 41, 14–20 (2014).
4. Taghavie-Moghadam, P. L., Butcher, M. J. & Galkina, E. V. The dynamic lives of macrophage and dendritic cell subsets in atherosclerosis. *Ann. N. Y. Acad. Sci.* 1319, 19–37 (2014).
5. Chinetti-Gbaguidi, G. *et al.* Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARγ and LXRα pathways. *Circ. Res.* 108, 985–95 (2011).
6. Mallat, Z. *et al.* Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* 85, e17–24 (1999).
7. Von Der Thüsen, J. H. *et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J.* 15, 2730–2 (2001).
8. Yoshioka, T. *et al.* Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice. *Gene Ther.* 11, 1772–1779 (2004).
9. Liu, Y. *et al.* Inhibition of atherogenesis in LDLR knockout mice by systemic delivery of adeno-associated virus type 2-hIL-10. *Atherosclerosis* 188, 19–27 (2006).
10. De Vries, J. E. Immunosuppressive and anti-inflammatory properties of interleukin 10. *Ann. Med.* 27, 537–41 (1995).
11. Miller, Y. I. *et al.* Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. *J. Biol. Chem.* 278, 1561–8 (2003).
12. Xu, X. H. *et al.* Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation* 104, 3103–8 (2001).
13. O'Neill, L. A. J., Golenbock, D. & Bowie, A. G. The history of Toll-like receptors - redefining innate immunity. *Nat. Rev. Immunol.* 13, 453–60 (2013).
14. De Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. & de Vries, J. E. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174, 1209–20 (1991).
15. Netea, M. G., Van der Meer, J. W. M. & Kullberg, B.-J. Toll-like receptors as an escape mechanism from the host defense. *Trends Microbiol.* 12, 484–8 (2004).
16. Gaddis, D. E., Maynard, C. L., Weaver, C. T., Michalek, S. M. & Katz, J. Role of TLR2-dependent IL-10 production in the inhibition of the initial IFN-γ T cell response to Porphyromonas gingivalis. *J. Leukoc. Biol.* 93, 21–31 (2013).
17. Sing, A. *et al.* Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J. Exp. Med.* 196, 1017–24 (2002).
18. Chau, T. A. *et al.* Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. *Nat. Med.* 15, 641–8 (2009).
19. Frodermann, V. *et al.* A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to Staphylococcus aureus. *J. Infect. Dis.* 204, 253–62 (2011).
20. Swirski, F. K. *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J. Clin. Invest.* 117, 195–205 (2007).
21. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6, 13 (2014).
22. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25, 677–86 (2004).
23. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–69 (2008).
24. Wolfs, I. M. J., Donners, M. M. P. C. & de Winther, M. P. J. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. *Thromb. Haemost.* 106, 763–71 (2011).
25. Puccetti, P. & Grohmann, U. IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-kappaB activation. *Nat. Rev. Immunol.* 7, 817–23 (2007).
26. Francisco, L. M. *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* 206, 3015–29 (2009).
27. Niedbala, W., Wei, X. Q., Piedrafita, D., Xu, D. & Liew, F. Y. Effects of nitric oxide on the induction and differentiation of Th1 cells. *Eur. J. Immunol.* 29, 2498–505 (1999).

28. Niedbala, W. *et al.* Nitric oxide induces CD4+CD25+ Foxp3 regulatory T cells from CD4+CD25 T cells via p53, IL-2, and OX40. *Proc. Natl. Acad. Sci. U. S. A.* 104, 15478–83 (2007).
29. Bingisser, R. M., Tilbrook, P. A., Holt, P. G. & Kees, U. R. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J. Immunol.* 160, 5729–34 (1998).
30. Albina, J. E., Abate, J. A. & Henry, W. L. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. *J. Immunol.* 147, 144–8 (1991).
31. Chen, X. & Oppenheim, J. J. Contrasting effects of TNF and anti-TNF on the activation of effector T cells and regulatory T cells in autoimmunity. *FEBS Lett.* 585, 3611–8 (2011).
32. Moreira, L. O. *et al.* The TLR2-MyD88-NOD2-RIPK2 signalling axis regulates a balanced pro-inflammatory and IL-10-mediated anti-inflammatory cytokine response to Gram-positive cell walls. *Cell. Microbiol.* 10, 2067–77 (2008).
33. Johansson, M. E. *et al.* Innate immune receptor NOD2 promotes vascular inflammation and formation of lipid-rich necrotic cores in hypercholesterolemic mice. *Eur. J. Immunol.* 44, 3081–92 (2014).
34. Martin, M. *et al.* Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. *J. Immunol.* 171, 717–25 (2003).
35. Polumuri, S. K., Toshchakov, V. Y. & Vogel, S. N. Role of phosphatidylinositol-3 kinase in transcriptional regulation of TLR-induced IL-12 and IL-10 by Fc gamma receptor ligation in murine macrophages. *J. Immunol.* 179, 236–46 (2007).
36. Chamberlain, N. D. et al. Ligation of TLR7 by rheumatoid arthritis synovial fluid single strand RNA induces transcription of TNFα in monocytes. *Ann. Rheum. Dis.* 72, 418–26 (2013).
37. Fukao, T. *et al*. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat. Immunol.* 3, 875–81 (2002).
38. Guha, M. & Mackman, N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem.* 277, 32124–32 (2002).
39. Fukao, T. & Koyasu, S. PI3K and negative regulation of TLR signaling. *Trends Immunol.* 24, 358–363 (2003).
40. Briken, V. & Mosser, D. M. Editorial: switching on arginase in M2 macrophages. *J. Leukoc. Biol.* 90, 839–41 (2011).
41. Chang, J. D. *et al.* Deletion of the phosphoinositide 3-kinase p110gamma gene attenuates murine atherosclerosis. *Proc. Natl. Acad. Sci. U. S. A.* 104, 8077–82 (2007).
42. Zotes, T. M. *et al.* PI3K p110γ deletion attenuates murine atherosclerosis by reducing macrophage proliferation but not polarization or apoptosis in lesions. *PLoS One* 8, e72674 (2013).
43. Farrar, C. A. *et al.* Inhibition of TLR2 promotes graft function in a murine model of renal transplant ischemia-reperfusion injury. *FASEB J.* 26, 799–807 (2012).
44. Arslan, F. *et al.* Treatment with OPN-305, a humanized anti-Toll-Like receptor-2 antibody, reduces myocardial ischemia/reperfusion injury in pigs. *Circ. Cardiovasc. Interv.* 5, 279–87 (2012).
45. Schoneveld, A. H. *et al.* Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development. *Cardiovasc. Res.* 66, 162–9 (2005).
46. Mullick, A. E., Tobias, P. S. & Curtiss, L. K. Modulation of atherosclerosis in mice by Toll-like receptor 2. *J. Clin. Invest.* 115, 3149–56 (2005).
47. Curtiss, L. K., Black, A. S., Bonnet, D. J. & Tobias, P. S. Atherosclerosis induced by endogenous and exogenous toll-like receptor (TLR)1 or TLR6 agonists. *J. Lipid Res.* 53, 2126–32 (2012).
48. Liu, X. *et al.* Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids. *Atherosclerosis* 196, 146–54 (2008).
49. Ha, T. *et al.* TLR2 ligands induce cardioprotection against ischaemia/reperfusion injury through a PI3K/Akt-dependent mechanism. *Cardiovasc. Res.* 87, 694–703 (2010).
50. Nawijn, M. C. *et al.* TLR-2 activation induces regulatory T cells and long-term suppression of asthma manifestations in mice. *PLoS One* 8, e55307 (2013).
51. Filippi, C. M. *et al.* TLR2 signaling improves immunoregulation to prevent type 1 diabetes. *Eur. J. Immunol.* 41, 1399–409 (2011).
52. Berg, D. J. *et al.* Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J. Clin. Invest.* 98, 1010–20 (1996).
53. Yen, D. *et al.* IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J. Clin. Invest. 116, 1310–6 (2006).
54. Pinderski Oslund, L. J. *et al.* Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler. Thromb. Vasc. Biol.* 19, 2847–53 (1999).

Amanda C. Foks[1]
Vanessa Frodermann[1]
Mariette N.D. ter Borg[1]
Kim L.L. Habets[1]
Ilze Bot[1]
Ying Zhao[1]
Theo J.C. van Berkel[1]
Johan Kuiper[1]
Gijs H.M. van Puijvelde[1]

6

[1] Division of Biopharmaceutics, LACDR, Leiden University, Leiden, The Netherlands

Differential Effects of Regulatory T cells on the Initiation and Regression of Atherosclerosis

Adapted with permission from Atherosclerosis. 2011 Sep;218(1):53-60.

Abstract

Objective Regulatory T cells (Tregs) play an important role in the regulation of T cell-mediated immune responses through suppression of T cell proliferation and cytokine production. In atherosclerosis, a chronic autoimmune-like disease, an imbalance between pro-inflammatory cells (Th1/Th2) and anti-inflammatory cells (Tregs) exists. Therefore, increased Treg numbers may be beneficial for patients suffering from atherosclerosis. In the present study, we determined the effect of a vast expansion of Tregs on the initiation and regression of well-established lesions.

Methods and Results For in vivo Treg expansion, LDL receptor deficient ($LDLr^{-/-}$) mice received repeated *intraperitoneal* injections of a complex of IL-2 and anti-IL-2 mAb. This resulted in a 10-fold increase in $CD4^{+}CD25^{hi}Foxp3^{+}$ T cells, which potently suppressed effector T cells ex vivo. During initial atherosclerosis, IL-2 complex treatment of $LDLr^{-/-}$ mice fed a Western-type diet reduced atherosclerotic lesion formation by 39%. The effect on pre-existing lesions was assessed by combining IL-2 complex treatment with a vigorous lowering of blood lipid levels in $LDLr^{-/-}$ mice. This did not induce regression of atherosclerosis, but significantly enhanced lesion stability.

Conclusion Our data show differential roles for Tregs during atherosclerosis: Tregs suppress inflammatory responses and attenuate initial atherosclerosis development, while during regression Tregs can improve stabilization of atherosclerotic lesions.

Introduction

Atherosclerosis is considered to be a chronic autoimmune-like disease with an underlying imbalance between pro-inflammatory and anti-inflammatory processes[1,2]. Restoration of this delicate balance by induction of Tregs has proven to be of therapeutic potential in the treatment of several autoimmune diseases such as diabetes and rheumatoid arthritis[3,4]. As key regulators of T cell-mediated immune responses, Tregs exert suppressive effects on effector T cells. Suppression mainly occurs through secretion of IL-10 and TGF-β, and cell-cell contact, mediated by membrane-bound TGF-β, CTLA-4 or GITR[5,6]. In mice, Tregs are characterized by the expression of the surface molecules CD4 and CD25, and expression of the transcription factor Forkhead box protein P3 (Foxp3)[7].

The role of Tregs in atherosclerosis has been the subject of intense investigation. Adoptive transfer of $CD4^+CD25^+$ T cells causes a reduction in atherosclerotic lesion development[8] while a depletion of $CD4^+CD25^+$ T cells or more specifically Foxp3 expressing Tregs aggravates lesion development[8,9]. Our group has demonstrated that induction of antigen-specific Tregs via oral tolerance induction against oxLDL shows the beneficial effect of Tregs on the initiation and progression of atherosclerosis[10].

Tregs have been shown to depend on IL-2 for optimal growth and survival[11-14]. Recently, it was shown that repeated injections of an IL-2 complex consisting of recombinant IL-2 and a specific anti-IL-2 monoclonal antibody (JES6-1A12) results in a specific expansion of Tregs[15], which very potently induce resistance to experimental autoimmune encephalomyelitis and suppressed graft rejections[16], type I diabetes[17], murine asthma[18] and myasthenia gravis[19].

In order to obtain a clinically relevant therapy for atherosclerosis, an experimental therapy inducing regression of atherosclerosis is a prerequisite, as most cardiovascular patients will already have well-established lesions. The effect of Tregs on the stabilization and regression of established atherosclerosis, however, remains to be elucidated. In the present study, we therefore not only determined the beneficial effect of IL-2 complex induced Tregs on the initiation of atherosclerosis, but more importantly, determined their therapeutic potential in a model for regression of atherosclerotic lesions.

Material and methods

Animals

Male LDLr deficient ($LDLr^{-/-}$) mice, 10-12 weeks old, were obtained from Jackson Laboratories. The animals 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). Diet and water were provided *ad libitum*. All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines.

Preparation of IL-2 complexes
IL-2 complexes were prepared by mixing 1 µg recombinant IL-2 (Peprotech) with 5 µg anti-IL-2 mAb (clone JES6-1A12, R&D) in sterile PBS and incubated at 37°C for 30 minutes before injecting *intraperitoneally*.

Initiation and regression of atherosclerosis
Atherosclerosis was induced in $LDLr^{-/-}$ mice by feeding a Western-type diet for 8 weeks. Two weeks after start of Western-type diet mice were treated *intraperitoneally* with the IL-2 complex (n=11) or with sterile PBS as a control (n=11). Initially, mice were treated *intraperitoneally* with IL-2 complexes for three consecutive days to boost the expansion of Tregs, thereafter mice were injected every 10 days to maintain high levels of Tregs. To study regression of atherosclerosis, mice were put on a Western-type diet for 10 weeks. At week 10, a baseline group (n=11) was sacrificed to determine disease extent at the beginning of the treatment. Subsequently, mice were put on a chow diet and simultaneously treated *intraperitoneally* with the IL-2 complex as mentioned above (n=13). As a control, mice were treated with sterile PBS (n=14). At week 20, mice were sacrificed and tissues were harvested after in situ perfusion using PBS and subsequent perfusion using Zinc Formal-Fixx (Shandon Inc.). Tissues were frozen in nitrogen and stored at -80°C until further use.

Serum cholesterol levels
During the experiments, mice were weighed and blood samples were obtained by tail vein bleeding. The total cholesterol levels in serum were determined at week 0, 2 and 8 after start of the initial atherosclerosis experiment and at week 0, 5, 10, 14, 18 and 20 after start of the regression experiment. The concentrations of serum cholesterol were determined using enzymatic colorimetric procedures (Roche/Hitachi). Precipath (Roche/Hitachi) was used as an internal standard.

Histological analysis and morphometry
Cryosections of the aortic root (10 µm) were made and stained with Oil-red-O. Lesional collagen content was determined with a Masson's Trichrome staining. Furthermore, corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:1000). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. To determine the number of T cells in the lesions, a CD3 staining was performed using anti-mouse CD3 (1:50, BD Biosciences Pharmingen, San Diego. CA). In addition, the aortic arch and its main branch points were excised (4 µm), fixed, and embedded in paraffin. Longitudinal sections of the aortic arch were analyzed for lesion extent with a hematoxylin and eosin staining. Morphology was studied using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems).

Flow cytometry

During the experiments, levels of Tregs were monitored in the blood at several time points. Red blood cells were lysed using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). For the detection of $CD4^+CD25^{hi}Foxp3^+$ T cells, the blood cells were stained with the surface markers CD4 and CD25 (0.25 µg Ab/2 × 10^5 cells). For intracellular staining of Foxp3, cells were fixated and permeabilized overnight and subsequently stained against Foxp3 according to manufacturer's protocol (eBioscience). At sacrifice, blood, spleen, mediastinal lymph nodes near the heart (HLN) and liver were isolated (n=5 per group). Single cell suspensions were obtained by squeezing the organs through a 70µm cell strainer. Red blood cells were removed from blood and splenocytes using erythrocyte lysis buffer. For the detection of $CD4^+CD25^{hi}Foxp3^+$ T cells, the spleen, blood, HLN and liver cells were stained with CD4, CD25 and Foxp3. In addition, cells were stained for the transcription factors T-bet, RORγt and GATA-3 and the cytokines IFN-γ, IL-17A and IL-4. All antibodies were purchased from eBioscience. FACS analysis was performed on a FACSCantoII (BD Biosciences). Data were analyzed using FACSDiva software (BD Biosciences).

Spleen cell proliferation

Splenocytes (n=5 per group) were cultured for 48 hours in triplicate in a 96-wells round-bottom plate (3 × 10^5 cells/well) in RPMI 1640 supplemented with L-Glutamine, 100 U/ml streptomycin/penicillin and 10% FCS. As a positive control cells were stimulated with anti-CD3 and anti-CD28 (2 µg/ml). Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences) for the last 16 hours. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as stimulation index (SI): ratio of mean counts per minute of triplicate cultures with anti-CD3/CD28 stimulation to triplicate cultures without stimulation.

Suppression assay

Tregs were isolated with greater than 95% purity from splenocytes using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit from Miltenyi Biotec. 7.5 × 10^4 splenocytes were plated out per well of a 96-well plate with or without titrated amounts of isolated Tregs from IL-2 complex and control treated mice. Cells were activated with anti-CD3 and anti-CD28 (2 µg/ml) and pulsed with ^{3}H-thymidine (0.5 µCi/well) on day 3. Proliferation was assessed 16 hours later using a liquid scintillation counter. All results are expressed as the mean disintegration per minute (DPM) of triplicate cultures.

Cytokine determination in supernatant of the suppression assay

IL-10 and TGF-β concentrations in the supernatant of effector T cells cultured in a 1:1 ratio with Tregs for 72 hours were determined by ELISA according to manufacturer's protocol (eBioscience).

Real-time PCR

Spleens from baseline mice (n=11), control mice (n=14) and IL-2 complex mice

(n=13) were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (RevertAid M-MuLV reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using SYBR green technology. The following primer pairs were used:

IL-10:	5′-TCTTACTGACTGGCATGAGGATCA-3′	and	5‘-GTCCGCAGCTCTAGGAGCAT-3′
TGF-β:	5′-AGGGCTACCATGCCAACTTCT-3′	and	5‘-GCAAGGACCTTGCTGTACTGTGT-3′

The following primers were used as endogenous references:

36B4:	5′-GGACCCGAGAAGACCTCCTT-3′	and	5′ GCACATCACTCAGAATTTCAATGG-3′
HPRT:	5′-TTGCTCGAGATGTCATGAAGGA-3′	and	5′ AGCAGGTCAGCAAAGAACTTATAG-3′

Statistical analysis

All data are expressed as mean±SEM. A paired two-tailed student T-test was used to compare normally distributed data between two groups of animals. Probability values of $P<0.05$ were considered significant.

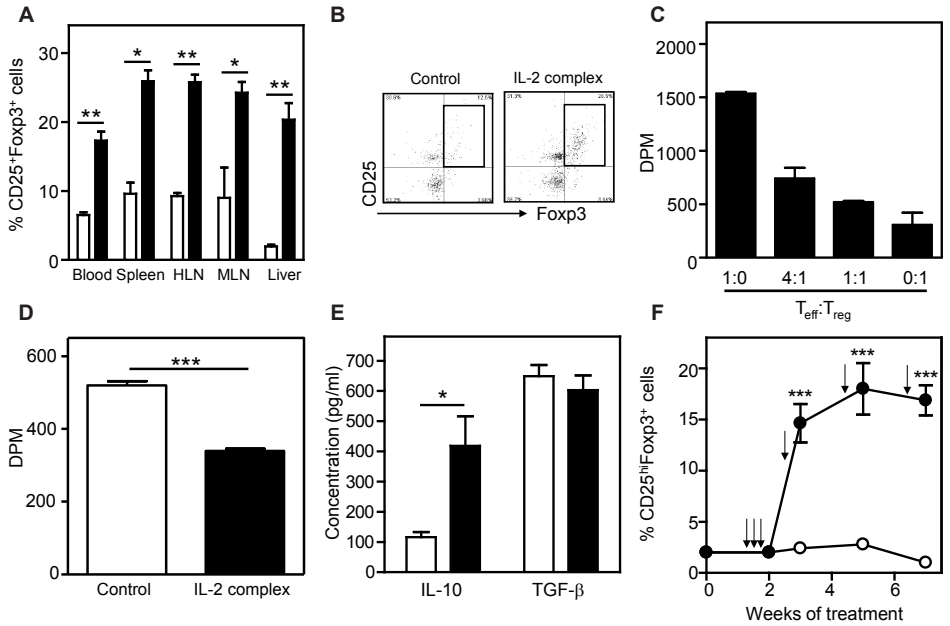


Figure 1. IL-2 complex induces persistent high levels of Tregs in LDLr$^{-/-}$ mice. LDLr$^{-/-}$mice were injected 3 times with an IL-2 complex (n = 3, black bars) or PBS as a control (n = 3, open bars). Mice were sacrificed 5 days after initiation of the experiment. **A.** Blood, spleen, mediastinal lymph nodes near the heart (HLN), mesenteric lymph nodes (MLN) and liver cells were isolated and the percentage of CD25$^+$Foxp3$^+$ cells within CD4$^+$ cells was determined by flow cytometry. **B.** Representative dotplots are shown. **C.** A suppression assay was performed to determine the suppressive capacity of the expanded Tregs by measuring the proliferation of splenocytes (n=3). Data are shown as the mean disintegration per minute (dpm) of triplicate cultures. **D.** In a 1:1 ratio, effector T cells were more potently suppressed by Tregs from IL-2 complex treated mice compared to Tregs from control mice. **E.** Secretion of IL-10 and TGF-β in the supernatant of Tregs cultured with effector T cells in a 1:1 ratio was determined with ELISA. **F.** To induce atherosclerosis, LDLr$^{-/-}$ mice were fed a Western-type diet for 8 weeks. Two weeks after initiation of the experiment, mice received *i.p.* injections with the IL-2 complex (black arrows). Levels of CD4$^+$CD25^{hi}Foxp3$^+$ T cells were monitored in the blood at weeks 2, 3, 5 and 7 using flow cytometry (n=5 per group). *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Persisting high levels of Tregs in LDLr$^{-/-}$ mice due to continuous treatment with the IL-2 complex

To determine whether IL-2 complexes induced Treg expansion in LDLr$^{-/-}$ mice, we injected the IL-2 complex or PBS *intraperitoneally* on 3 consecutive days. Mice were sacrificed 5 days after initiation of the experiment. The administration of IL-2 complexes resulted in a significant 3-fold increase of Tregs in lymphoid organs and an 11-fold increase in the liver, compared to control mice (Figure 1A and B). The expanded Tregs in the IL-2 complex-treated group were functional as they potently suppressed effector T cell proliferation ex vivo (Figure 1C). In addition, Tregs expanded by the IL-2 complex were more suppressive than Tregs from control treated mice ($P<0.001$, Figure 1D), whereas no significant differences between solely effector T cell proliferation and Treg proliferation of both groups were observed (data not shown). Cytokine determination in the supernatant of this suppression assay showed that IL-2 complex expanded Tregs mainly function via IL-10 secretion, whereas no difference in TGF-β secretion was observed ($P<0.05$, Figure 1E).

To determine whether IL-2 complex-induced Treg expansion is still feasible under hyperlipidemic, pro-inflammatory conditions, LDLr$^{-/-}$ mice were fed a Western-type diet for 8 weeks. Two weeks after initiation of the experiment, mice were *intraperitoneally* injected with IL-2 complexes for 3 consecutive days to boost the expansion of Tregs. Thereafter, mice were injected every 10 days to maintain persistently high Treg levels essential for investigating the effect of high Treg levels on atherosclerosis development and regression. As shown in Figure 1F, the IL-2 complex is still able to enhance Tregs in blood under hyperlipidemic conditions. In addition, the level of Tregs in the blood persistently remained at a significantly 10-fold higher level than in control treated mice ($P<0.001$).

IL-2 complex administration reduces the development of atherosclerosis

Since a limited increase in Tregs already affects initiation of atherosclerosis, we postulated that a 10-fold expansion of Tregs in blood observed after IL-2 complex administration may significantly potentiate this effect. During the experiment enhanced levels of Tregs did not affect body weight and total plasma cholesterol levels (data not shown). Eight weeks after the start of the high fat diet mice were sacrificed and atherosclerotic lesion size was determined. We observed a significant 39% reduction in aortic root lesion size of IL-2 complex treated mice ($1.73\times10^5 \pm 0.13\times10^5$ μm^2) in comparison with control mice ($2.84\times10^5 \pm 0.30\times10^5$ μm^2, $P<0.01$, Figure 2A). No difference in lesion stability, as determined by Masson's Trichrome staining, was observed between IL-2 complex treated mice (16.0 ± 1.5%) and control treated mice (18.8 ± 0.9%, Figure 2B). Furthermore, no difference in macrophage content was observed (control: 61.4 ± 3.3% and IL-2 complex: 53.0 ± 4.4%, Figure 2C). At sacrifice, we determined whether the high amounts of CD4$^+$CD25^{hi}Foxp3$^+$ T cells measured in blood corresponded to increased Treg levels in spleen, mediastinal lymph nodes located near the heart (HLN), and liver. In agreement with increased

Treg levels in the blood of IL-2 complex treated mice, we observed a significant 3-fold ($P<0.001$), 1.5-fold ($P<0.05$), and 9.2-fold ($P<0.05$) increase in Tregs in the spleen, HLN, and liver, respectively, as compared with control treated mice (Figure 3A). To determine the suppressive capacity of the IL-2 complex expanded Tregs, splenocytes isolated from both groups were cultured for 48 hours in the presence of αCD3/αCD28 stimulation. A significant 43% decrease in T cell proliferation was observed in mice treated with the IL-2 complex (stimulation index of 9.7 ± 1.5) compared to control mice (stimulation index of 17.1 ± 3.3), showing that the induced Tregs are functional (Figure 3B, $P<0.05$). Since Tregs function in part via IL-10 and TGF-β secretion, we determined the gene expression of these cytokines in the spleen. IL-2 complex treated mice showed a 4.7-fold increase in IL-10 expression compared with control mice ($P<0.05$), whereas TGF-β expression remained unchanged (Figure 3C). This suggests that Tregs induced by the IL-2 complex may exert their suppressive function predominantly via the secretion of IL-10.

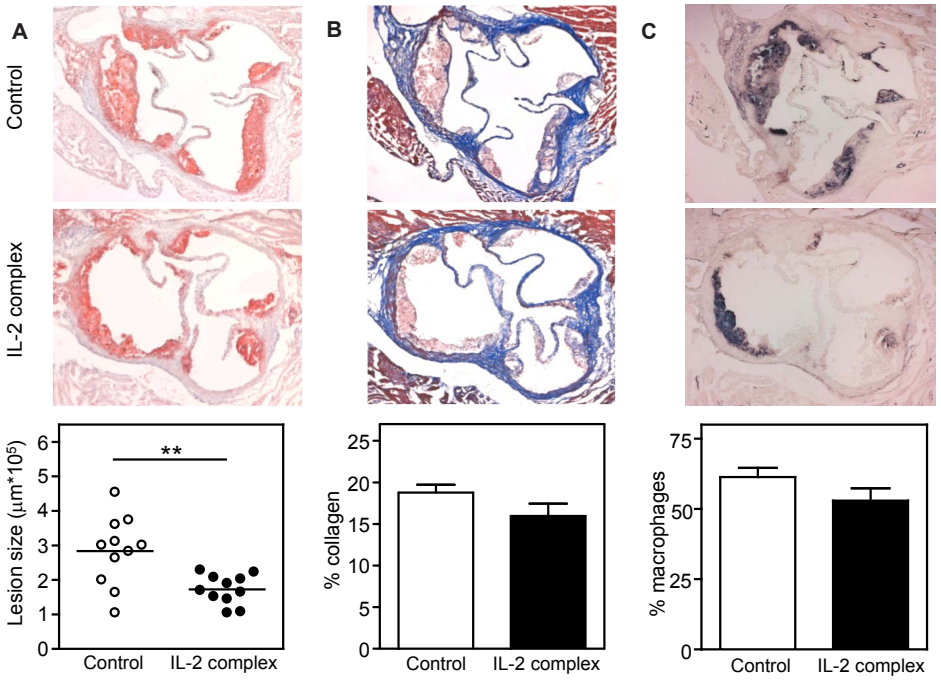


Figure 2. Expansion of Tregs reduces lesion formation. A. Representative cross-sections of lesion formation in the three valves area of the aortic root stained with Oil-red-O and hematoxylin are shown and lesion size was determined. **B.** Corresponding sections on separate slides were stained for collagen using Masson's Trichrome staining. The percentage of collagen relative to the lesion size was determined. **C.** Relative macrophage content was determined with a MOMA-2 staining and quantified. **P < 0.01.

Effect of Treg expansion on other T cell subsets

It has been suggested that Tregs have the capacity to specifically target and suppress effector T cells, such as Th1, Th2 and Th17 cells[19,20]. To evaluate whether the IL-2 complex expanded Tregs inhibit a specific T cell subset in vivo during initiation of atherosclerosis, splenocytes were stained for the transcription factors T-bet, GATA-3,

and RORγt, which control the differentiation of Th0 cells into Th1, Th2 and Th17 cells, respectively. Flow cytometry analysis showed that IL-2 complex treated mice have significant reduced T-bet expression (1.7 ± 0.2% vs. 3.6 ± 0.4%, P<0.01, Figure 3D) and reduced GATA-3 expression (16.1 ± 0.6% vs. 19.8 ± 1.3%, P<0.05, Figure 3D) in the $CD4^+$ T cell population of the spleen, compared to the control group. Accordingly, reduced $CD4^+IFN\text{-}\gamma^+$ T cells were observed (6.1 ± 0.5% vs. 9.4 ± 1.0%, P<0.05, Figure 3E). Interestingly, the percentage of IL-4^+ cells did not change in the spleen, but was decreased in the blood (13.5 ± 2.1% vs. 23.8 ± 1.8%, P<0.01, Figure 3F). Th17 responses, on the other hand, remained unchanged following Treg expansion (Figure 3D-F).

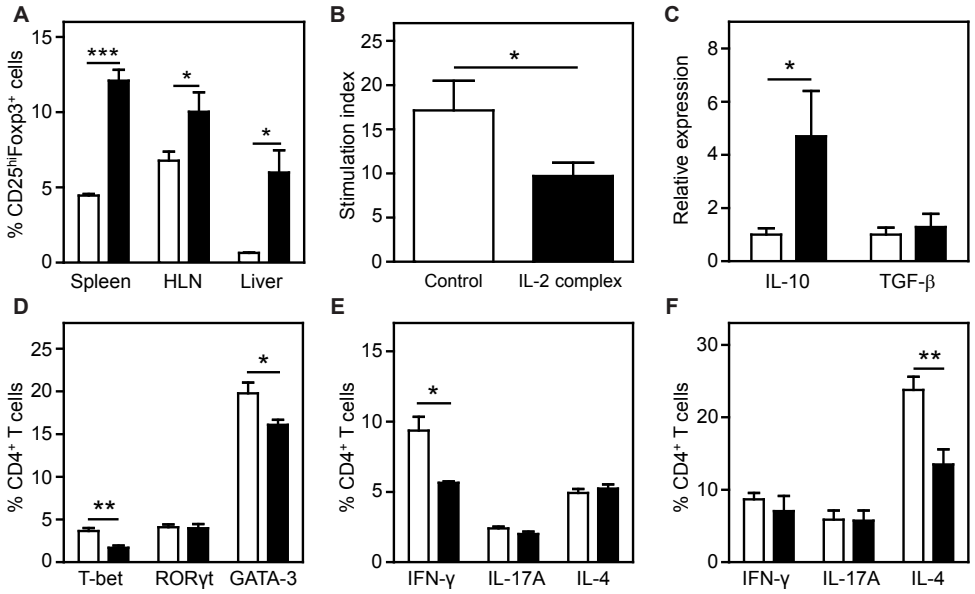


Figure 3. Effect of the IL-2 complex on the percentage and functionality of the Tregs. A. After eight weeks, spleen, HLN and liver cells were isolated and stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry (n=5). **B.** The effect of boosting Tregs with the IL-2 complex on spleen cell proliferation was determined by culturing splenocytes (n=5) in the presence or absence of CD3/CD28 stimulation. Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells. The proliferation is expressed as stimulation index. **C.** mRNA levels of IL-10 and TGF-β in the spleen were determined with RT-PCR. **D.** Spleen cells were stained for CD4 and the transcription factors T-bet (Th1), RORγt (Th17) and GATA-3 (Th2). **E.** Cytokine production in the spleen and **F.** blood was evaluated by flow cytometry. Cells were stained for CD4, IFN-γ, IL-17A and IL-4. *P < 0.05, **P < 0.01, ***P < 0.001.

IL-2 complex-expanded Tregs stabilize lesions in a regression model

Since it is clinically more relevant to determine the effect of Tregs on pre-existing lesions, we combined lipid lowering with IL-2 complex treatment. To this end, we put $LDLr^{-/-}$ mice, which were fed a Western-type diet for 10 weeks, on a chow diet for another 10 weeks, combined with simultaneous administration of IL-2 complexes. In addition, a baseline group was sacrificed after 10 weeks of Western-type diet to determine the effect of treatment on atherosclerotic lesion size. No differences in weight and cholesterol levels were found between baseline, control and IL-2 complex

treated mice during Western-type diet feeding (Figure 4A and B). In addition, no differences in weight were found between control and IL-2 complex-treated mice after switching to chow diet and Treg induction (Figure 4A). Only a 10-20% reduction ($P<0.05$) in plasma cholesterol levels could be observed in IL-2 complex-treated mice compared to control mice at 4, 8 and 10 weeks after switching to a low cholesterol diet (Figure 4B). Throughout the experiment, Treg levels in blood remained significantly higher in the IL-2 complex group as compared to control treated mice (Figure 4C). In addition, highly elevated Treg levels were observed in blood, spleen and HLN at sacrifice (Figure 4D). At sacrifice ex vivo effector T cell proliferation was suppressed (Figure 4D).

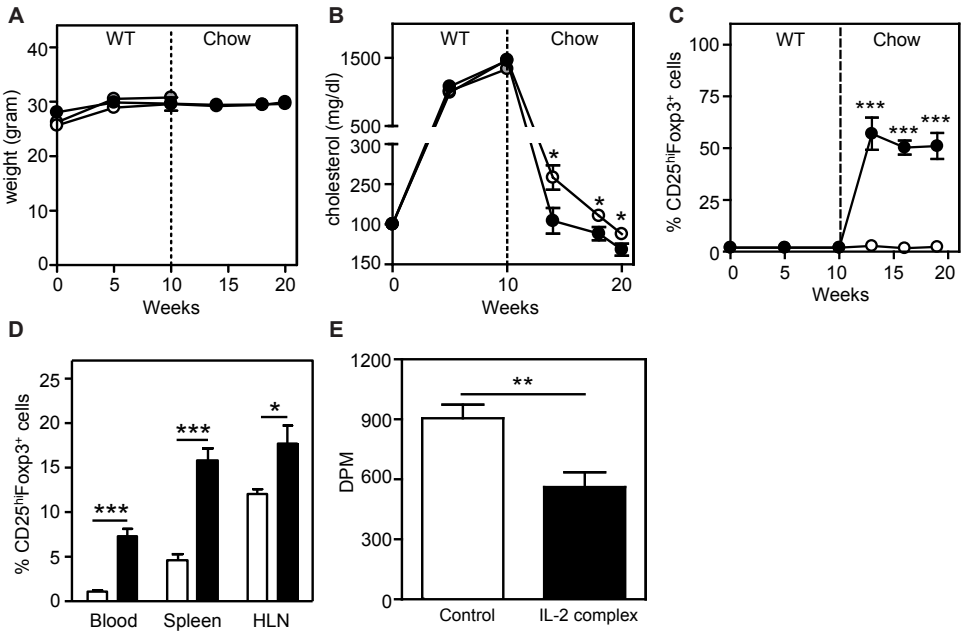


Figure 4. Effect of IL-2 complex on weight, cholesterol levels and Tregs during regression of atherosclerosis. LDLr$^{-/-}$ mice received 10 weeks Western-type diet and were subsequently put on chow diet for 10 weeks and treated with the IL-2 complex (n=13, black circles) or PBS (n=14, open circles). A baseline group was sacrificed after 10 weeks of Western-type diet (n=11, grey circles). **A.** During the experiment, mice were weighed. **B.** Blood was taken by tail vein bleeding and total cholesterol concentration was determined within the serum. **C.** To check whether IL-2 complexes expanded Tregs, we monitored these in the circulation. **D.** At sacrifice, blood, spleen and HLN cells were isolated and stained for CD4, CD25, and Foxp3, and analyzed with flow cytometry (n=5 per group). **E.** The effect of boosting Tregs with the IL-2 complex on spleen cell proliferation was determined by culturing splenocytes (n=5) in the presence or absence of CD3/CD28 stimulation. In a 1:1 ratio, effector T cells were more potently suppressed by Tregs from IL-2 complex treated mice compared to Tregs from control mice. Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells and is expressed as disintegrations per minute (DPM). *P < 0.05, ***P < 0.001.

Both the control and the IL-2 complex-treated mice displayed no reduction in lesion size (control: $3.06\times10^5 \pm 0.25\times10^5$ μm^2 and IL-2 complex: $3.10\times10^5 \pm 0.25\times10^5$ μm^2) when compared with the baseline group ($2.74\times10^5 \pm 0.10\times10^5$ μm^2, Figure 5A and C). The same effect was observed in the aortic arch, where both IL-2 complex treated

and control mice showed no reduction in lesion size (data not shown). Furthermore, the collagen content of the lesions was determined (Figure 5B and D). Lesions from IL-2 complex-treated mice showed a substantially increased stability (38.3 ± 2.3% collagen) compared to control mice (31.6 ± 1.8% collagen, P<0.05). Additionally, both groups showed very significantly increased collagen content compared to baseline mice (12.2±1.2%, P<0.001). No difference in relative macrophage content was observed between the IL-2 complex treated group (12.4 ± 1.9%) and the control group (14.3 ± 1.6%). Both groups, however, showed a significant 60% reduction in the relative macrophage content compared to the baseline group (36.7 ± 2.6%, P<0.001, Figure 5E), indicative for regressed lesions in both groups. Together these results suggest that increasing the number of Tregs during well-established atherosclerosis results in more stable lesions, with increased collagen content, but does not affect lesion size.
In addition, we analyzed the aortic root for $CD3^+$ T cells within lesions and found almost no T cells in lesions of both IL-2 complex-treated mice and control mice. However, we found a 60% increase of $CD3^+$ T cells within the adventitia of IL-2 complex

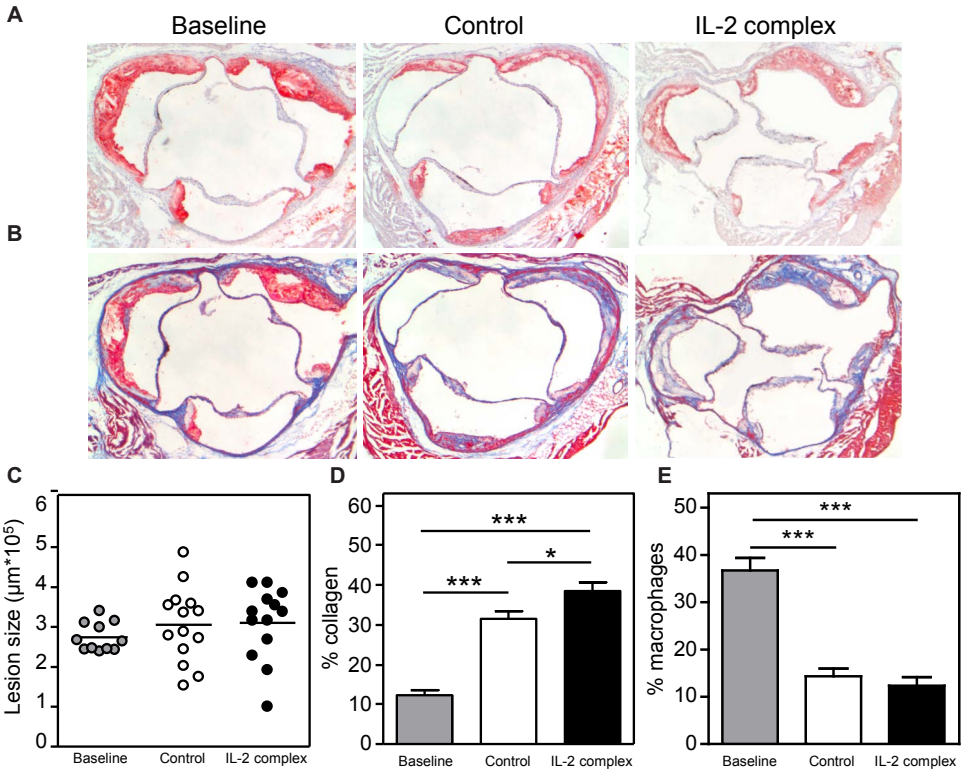


Figure 5. IL-2 complex treatment stabilizes atherosclerotic lesions during regression. **A.** Sections of the aortic root were stained with Oil-red-O and hematoxylin. **B.** Corresponding sections on separate slides were also stained for collagen using Masson's Trichrome staining. **C.** Lesion size was determined by Oil-red-O staining. **D.** The percentage of collagen relative to the lesion size was quantified by Masson's Trichrome staining. **E.** Relative macrophage content was determined by MOMA-2 staining and quantified. *P < 0.05, ***P < 0.001.

treated mice (86.4 ± 10.6 cells/section) compared to control treated mice (40.3 ± 6.3 cells/section, P<0.01, Figure 6).

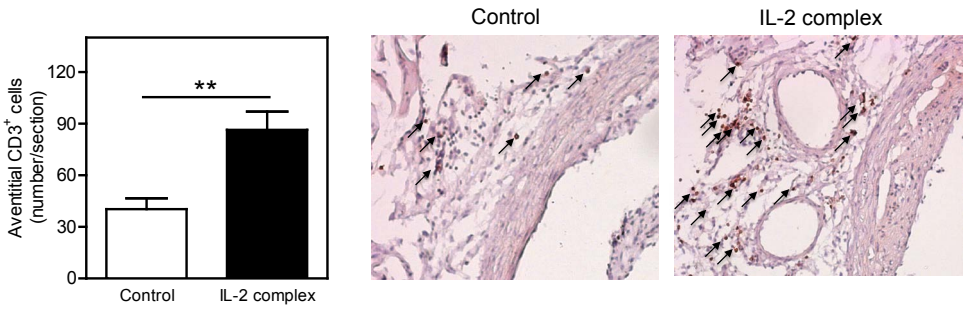


Figure 6. Increased adventitial CD3⁺ T cell infiltration in IL-2 complex treated mice. Sections of the aortic root were stained for CD3 to determine infiltrating T cells. Arrows indicate T cells. ** P <0.01

Discussion

Regulatory $CD4^+CD25^{hi}Foxp3^+$ T cells are important regulators of immune responses and have been shown to play a major role in autoimmune diseases. Since autoimmune diseases result from an imbalance between effector and regulatory cells, with reduced numbers of the latter, Tregs show great potential to be used as a therapeutic regime. Their beneficial role in atherosclerosis has been particularly elucidated in the initiation of atherosclerosis using adoptive transfer, induction and depletion of Tregs[8,9,11,21-23]. However, in these studies, only a modest increase in Treg numbers was achieved in the order of 1.5- to 2-fold, mostly for two to three weeks. We therefore determined whether significantly higher Treg levels for longer periods of time could even more drastically attenuate atherosclerosis development. We used the recently published technique using the IL-2 complex consisting of IL-2 and a neutralizing anti-IL-2 mAb, a treatment that beneficially affected the outcome of a number of autoimmune-like diseases[16-19]. In the present study, $LDLr^{-/-}$ mice were fed a Western-type diet for 8 weeks. The treatment with the IL-2 complex was started after two weeks of feeding the Western-type diet in order to counteract the pro-inflammatory effects of the diet. We now show that stimulation of Tregs with the IL-2 complex resulted in a highly significant 10-fold increase of $CD4^+CD25^{hi}Foxp3^+$ T cells in blood of $LDLr^{-/-}$ mice, which was maintained for six weeks. The expansion of Tregs during diet was comparable to the level we obtained in chow fed animals indicating that the pro-inflammatory effect of the high-cholesterol diet did not affect this expansion. The extent of expansion is in line with a previous study by Webster *et al.*, in which IL-2 complexes with a similar molar ratio of IL-2 and anti-IL-2 mAb induced a 10-fold increase of Tregs[16].

The significantly strong expansion of $CD4^+CD25^{hi}Foxp3^+$ T cells resulted in a 39% decrease in initial atherosclerosis in the aortic root. It was previously shown that oral tolerance induction to oxLDL, an atherosclerosis-specific antigen, induced a maximal 2-fold increase in Tregs, with a 30% reduction in lesion size in the aortic

root[10]. These Tregs however, were antigen-specific Tregs, whereas the IL-2 complex has been shown to expand all present peripheral Tregs[16,19]. In addition, the number of antigen-specific Tregs dropped two weeks after treatment, while the present technique enabled us to maintain the enhanced numbers of Tregs for more than six weeks. Interestingly, only an additional reduction of approximately 10% in lesion size was observed. These results may indicate that Tregs only reduce lesion development to a maximal extent and it may be suggested that only a vast, prolonged expansion of antigen-specific Tregs by combining oral tolerance with IL-2 complex treatment may lead to a greater reduction in lesion size.

The IL-2 complex expanded Tregs in the LDLr$^{-/-}$ mice reduced Th1 and Th2 responses and potently suppressed proliferation of splenocytes by 43%. We observed an increase in gene expression of IL-10 in the spleen and increased IL-10 secretion by Tregs, which suggests that the IL-2 complex expanded Tregs exert their suppressive capacity via IL-10. In agreement with this finding, Webster *et al.* show enhanced expression of IL-10 mRNA but little change in TGF-β by the IL-2 complex expanded Tregs[16]. In addition, the suppression of airway inflammation via the IL-2 complex is dependent on IL-10[18]. The increase of IL-10 in the IL-2 complex treated mice may at least partially be responsible for the decrease in lesion size since several studies showed the protective role of IL-10 in atherosclerotic lesion development[24-26].

The development of experimental therapies for the treatment of atherosclerosis mainly focus on preventing the initiation and to a minor extent the progression of atherosclerosis. A clinically more relevant therapy for atherosclerosis would be a therapy which induces regression of atherosclerosis as most of the cardiovascular patients already have well-established lesions. In the present study, we therefore aimed to simulate the treatment of cardiovascular patients by changing the diet (as a mimic of statin-induced lipid lowering) in combination with a reduction of the inflammatory status by inducing Tregs. This approach is comparable to Verschuren *et al.* who induced regression of atherosclerosis in apoE*3Leiden mice by switching high-fat diet to chow diet and treatment with the atheroprotective Liver-X-receptor (LXR)-agonist[27]. In our current study, we were able to induce high levels of Tregs in mice that previously were fed a cholesterol rich diet for 10 weeks comparable to the levels obtained in chow fed mice. We observed no lesion regression in the control group (only lipid lowering) and despite extensive Treg induction also no lesion regression was found in the IL-2 complex-treated group. However, we observed that Treg induction increased lesion stability as indicated by increased collagen content in the lesions. This effect cannot be ascribed to the significant 10-20% reduction in cholesterol observed in IL-2 complex treated mice, since we did not find a correlation between collagen content and cholesterol levels. Possibly, the reduction in cholesterol can be ascribed to an increase of IL-10 produced by IL-2 complex expanded Tregs. Previously it was shown that IL-10 influences parenchymal liver cells, thereby lowering cholesterol levels in LDLr$^{-/-}$mice[25].

Interestingly, a 60% increase of adventitial CD3^{+} T cells within lesions of IL-2 complex treated mice was observed. This strong increase correlates with a highly significant 85% increase of circulating Tregs, which likely migrate towards the site of

inflammation via the adventitia. Moreover, we see a significant suppression of effector T cell proliferation in the IL-2 complex-treated group by expanded Tregs. This proves that these Tregs are functional in suppressing effector T cells and in limiting the possibility of effector T cell expansion and migration towards inflammatory sites. In addition to the apparent phenotype of T cells in the IL-2 treated-group, the observed increase in IL-10 additionally suggests an anti-inflammatory environment in these mice. This again suggests an inhibition of effector T cell proliferation but also implies that T cells found in this environment will be of an anti-inflammatory phenotype.

In conclusion, our data clearly illustrate the potential of IL-2 complexes to selectively expand Tregs capable of attenuating initial atherosclerotic lesion development, and further prove their capability to stabilize well-established lesions in a regression model. In the future, it may be of great interest to induce antigen specific Tregs with the IL-2 complex.

References

1. Hansson, G.K., Robertson, A.K., Soderberg-Naucler, C. Inflammation and atherosclerosis. *Annu Rev Pathol.* 1, 297-329 (2006).
2. Mallat, Z., Ait-Oufella, H., Tedgui, A. Regulatory T cell responses: potential role in the control of atherosclerosis. *Curr Opin Lipidol.* 16, 518-524 (2005).
3. Peng, Y., Laouar, Y., Li, M.O., Green, E.A., Flavell, R.A. TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A.* 101, 4572-4577 (2004).
4. Morgan, M.E. *et al.* Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells. Arthritis Rheum. 52, 2212-2221 (2005).
5. von Boehmer, H. Mechanisms of suppression by suppressor T cells. *Nat Immunol.* 6, 338-344 (2005).
6. Nakamura, K., Kitani, A., Strober, W. Cell contact-dependent immunosuppression by CD4(+) CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med.* 194, 629-644 (2001).
7. Hori, S., Nomura, T., Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 299, 1057-1061 (2003).
8. Ait-Oufella, H. *et al.* Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med.* 12, 178-180 (2006).
9. van Es, T. *et al.* Vaccination against Foxp3(+) regulatory T cells aggravates atherosclerosis. *Atherosclerosis*. 209, 74-80 (2009).
10. van Puijvelde, G.H. *et al.* Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis. *Circulation*. 114, 1968-1976 (2006).
11. Murakami, M., Sakamoto, A., Bender, J., Kappler, J., Marrack, P. CD25+CD4+ T cells contribute to the control of memory CD8+ T cells. *Proc Natl Acad Sci U S A.* 99, 8832-8837 (2002).
12. Setoguchi, R., Hori, S., Takahashi, T., Sakaguchi, S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med.* 201, 723-735 (2005).
13. D'Cruz, L.M., Klein, L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol.* 6, 1152-1159 (2005).
14. Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., Rudensky, A.Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol.* 6, 1142-1151 (2005).
15. Boyman, O., Kovar, M., Rubinstein, M.P., Surh, C.D., Sprent, J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science*. 311, 1924-1927 (2006).
16. Webster, K.E. *et al.* In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J Exp Med.* 206, 751-760 (2009).
17. Tang, Q. *et al.* Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity*. 28, 687-697 (2008).
18. Wilson, M.S. *et al.* Suppression of murine allergic airway disease by IL-2:anti-IL-2 monoclonal antibody-induced regulatory T cells. *J Immunol.* 2008;181:6942-6954.

19. Liu, R. *et al.* Expansion of regulatory T cells via IL-2/anti-IL-2 mAb complexes suppresses experimental myasthenia. *Eur J Immunol*;40:1577-1589.
20. Dooms, H., Abbas, A.K. Revisiting the role of IL-2 in autoimmunity. *Eur J Immunol*. 40, 1538-1540 (2010).
21. Harats, D., Yacov, N., Gilburd, B., Shoenfeld, Y., George, J. Oral tolerance with heat shock protein 65 attenuates Mycobacterium tuberculosis-induced and high-fat-diet-driven atherosclerotic lesions. *J Am Coll Cardiol*. 40, 1333-1338 (2002).
22. Maron, R. *et al.* Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. *Circulation*. 106, 1708-1715 (2002).
23. George, J. *et al.* Suppression of early atherosclerosis in LDL-receptor deficient mice by oral tolerance with beta 2-glycoprotein I. *Cardiovasc Res.* 62, 603-609 (2004).
24. Mallat, Z. *et al.* Protective role of interleukin-10 in atherosclerosis. *Circ Res.* 85, e17-24 (1999).
25. Von Der Thusen, J.H. *et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J.* 15, 2730-2732 (2001).
26. Pinderski, L.J. *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.* 90, 1064-1071 (2002).
27. Verschuren, L., de Vries-van der Weij, J., Zadelaar, S., Kleemann, R., Kooistra, T. LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in apoE*3Leiden mice: time course and mechanisms. *J Lipid Res.* 50, 301-311 (2009).

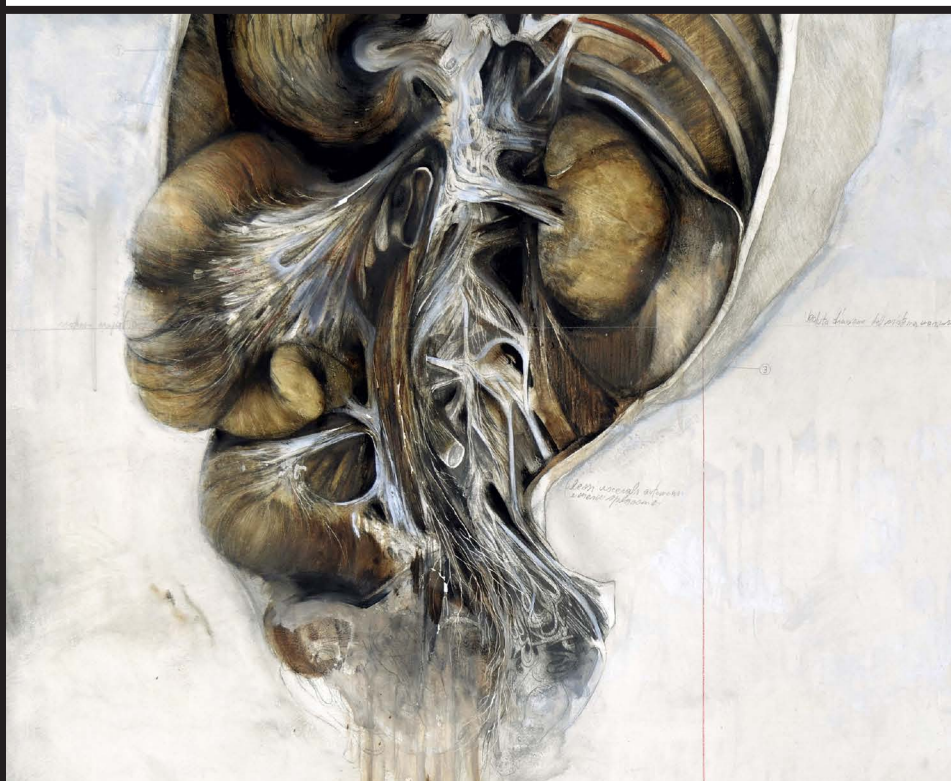
Vanessa Frodermann[1]
Gijs H.M. van Puijvelde[1]
Menno Hoekstra[1]
Gerjan de Bruin[2]
Janine van Duijn[1]
Thomas van der Heijden[1]
Mara J. Kröner[1]
Peter J. van Santbrink[1]
Herman S. Overkleeft[2]
Ilze Bot[1]
Bogdan I. Florea[2]
Saskia C.A. de Jager[1,3]
Johan Kuiper[1]

7

[1] Division of Biopharmaceutics, LACDR, Leiden University, Leiden, The Netherlands
2 Leiden Institute of Chemistry and Netherlands Proteomics Centre, Leiden, The Netherlands
[3] Present address: Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

Bortezomib: A Novel Lipid-Lowering Drug to Prevent Atherosclerosis

Manuscript in preparation.



Abstract

Objective The ubiquitin-proteasome system plays a key role in cellular protein homeostasis. It degrades the majority of proteins, e.g. cell cycle proteins, apoptotis-regulating proteins, and transcription factors. We assessed whether proteasome inhibition in addition to its effect on inflammation could affect lipid metabolism in atherosclerosis-prone low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice.

Methods and Results We found that proteasome inhibition by Bortezomib treatment during eight weeks of Western-type diet resulted in a robust 59% reduction of aortic root lesion sizes in $LDLr^{-/-}$ mice. These lesions show a 1.9-fold reduction in macrophage numbers. Overall, proteasome inhibition significantly reduced inflammation and resulted in reduced aortic VCAM-1 expression, reduced plasma CCL2 levels, and reduced monocyte and macrophage responses. Moreover, pro-atherogenic Th1 responses were decreased and T cells were skewed towards Th2 responses.

Bortezomib also significantly reduced plasma cholesterol levels by 50% due to a significant 62% decrease in plasma very low-density lipoprotein (VLDL) levels. VLDL secretion rates were decreased by 50% in Bortezomib-treated mice, resulting from reduced *de novo* hepatic synthesis of lipids, as determined by a significant 58% reduction of fatty acid synthase. Moreover, increased cholesterol efflux to the bile was observed, as determined by a 2-fold upregulation of ABCG5.

Conclusion Our results show a previously unknown effect of proteasomal inhibition by Bortezomib on dyslipidemia, making proteasome inhibition a very potent target for cardiovascular diseases as dual reduction of inflammation and dyslipidemia can be achieved. Targeting the proteasome may thus be especially beneficial for the treatment of atherosclerosis in statin-unresponsive patients.

Introduction

The 26S proteasome is the primary enzyme complex responsible for protein degradation in mammalian cells[1]. Multiple proteins are targeted for proteasomal degradation by ubiquitination: cell cycle- and apoptosis-regulating proteins, damaged or misfolded proteins, and antigens for major histocompatibility complex (MHC) I presentation[1,2]. As such, the proteasome plays a central role in cellular homeostasis and controls cell cycle progression, differentiation, apoptosis, cellular stress responses and immune responses[3]. Dysregulation of the ubiquitin proteasome system is associated with multiple diseases, such as cancer, neurodegenerative diseases, viral infections, cardiovascular disease and autoimmune diseases[2,4,5].

The mammalian 26S proteasome consists of a barrel-shaped 20S core protein subunit and one or two regulatory cap proteins, which recognize polyubiquitinated proteins and move them to the 20S core[6]. The 20S core degrades proteins in its hollow center and consists of seven α and seven β subunits, of which β1, β2 and β5 have proteolytic activity. The β1 subunit has caspase-like activity, the β2 subunit has trypsin-like activity and the β5 subunit has chymotrypsin-like activity[7]. During inflammation or cellular stress these catalytic subunits are constitutively replaced by the inducible β subunits β1i (LMP2), β2i (MECL-1) and β5i (LMP7). This switch to the "immunoproteasome" is induced by IFN-γ and/or TNF-α and shows enhanced protein processing capacity, resulting in increased MHC I peptide presentation[8,9].

Proteasomal inhibition has been thoroughly investigated in cancer. Initial interest was driven by studies showing that proteasomal inhibitors could block anti-apoptotic nuclear factor (NF)-κB signaling, thereby inducing apoptosis in cancer cells. The inhibition of NF-κB signaling by proteasome inhibitors was attributed to the accumulation of IκBα, an inhibitor of NF-κB, and the lack of proteolytic processing of the p100 precursor of p52 needed for non-canonical NF-κB signaling[10]. However, recent studies suggest that proteasomal inhibition can also induce NF-κB signaling by upregulation of RIP2 and IKKβ, which decrease levels of inhibitory IκBα[11,12]. Besides this proposed effect on NF-κB signaling, proteasome inhibitors have been found to induce cell cycle arrest and degradation of pro-apoptotic factors, e.g. p53, NOXA and Bim[10], in cancer cells. Moreover, proteasome inhibitors result in reduced angiogenesis, by decreasing vascular endothelial growth factor (VEGF) and suppressing proliferation of vascular endothelial cells[13].

Due to its effect on NF-κB, proteasome inhibition has also been investigated in inflammation, as the NF-kB pathway is induced via ligation of Toll-like receptors, CD40, TNF receptors, IL-1 receptor, and the T cell receptor[14]. Indeed, treatment of macrophages with proteasome inhibitors reduced TLR-induced responses, such as iNOS, TNF-α, and IL-6 induction[15]. Proteasome inhibition has been shown to be protective in experimental models of (collagen-induced) arthritis[16,17], acute graft-versus-host disease[18,19], and experimental autoimmune encephalomyelitis[20].

The most well-known and best studied proteasome inhibitor is the reversible dipeptidyl boronic acid inhibitor Bortezomib (PS-341, Velcade®) which primarily blocks β5 and β5i subunits, but also β1 and β1i[3]. It has been approved by the

United States Food and Drug Administration (FDA) for the treatment of multiple myeloma and mantle cell lymphoma[21]. Proteasomal inhibition has been previously addressed in atherosclerosis. Herrmann *et al.* have found increased atherosclerosis in a pig model of diet-induced atherosclerosis due to increased oxidative stress[22]. In ApoE$^{-/-}$ mice no effect on early atherosclerotic lesions was found, but in advanced stages of atherosclerosis Bortezomib induced rupture-prone lesions, likely due to increased smooth muscle cell and macrophage apoptosis[23]. Wilck *et al.*, however, describe reduced atherosclerosis in LDLr$^{-/-}$ mice due to anti-inflammatory and anti-oxidative effects of Bortezomib[24].

Interestingly, mutations in the β5i gene have been shown to correlate with reduced adipose tissue in humans[25]. Recently, a study by Oliva *et al.* has found that Bortezomib reduces ethanol-induced steatosis by downregulation of several lipogenic genes[26]. We therefore hypothesized that Bortezomib, in its actual therapeutic dose, could not only reduce inflammatory responses but also beneficially affect dyslipidemia. Indeed, in this study we show that Bortezomib reduces atherosclerosis in LDLr$^{-/-}$ mice in part via a reduction in inflammation but also in part via a novel, previously unrecognized, effect of Bortezomib on lipid metabolism and VLDL secretion.

Material and Methods

Animals

C57BL/6 and LDLr$^{-/-}$ mice were originally obtained from the Jacksons Laboratory, kept under standard laboratory conditions, and administered food and water *ad libitum*. All animal work was approved by the Ethics Committee for Animal Experiments of Leiden University and conforms to Dutch government guidelines.

Atherosclerosis

Atherosclerosis was induced in 10-12 weeks old female LDLr$^{-/-}$ mice by feeding a Western-type diet (WTD; 0.25% cholesterol and 15% cocoa butter; Special Diet Services) for eight weeks. Mice were treated twice weekly *intraperitoneally* with 0.5 mg/kg Bortezomib or PBS containing DMSO as a vehicle control. Treatment was started one week prior to WTD and continued during the entire experiment. A 20mM stock solution of Bortezomib was made in DMSO und adjusted to the final concentration, which was on average 0.0125 mg/mouse, in PBS. All solutions were prepared prior to injections after determination of the weight of the mice.

Proteasomal Activity-Based Protein profiling

Proteasomal activity was determined as previously described[27]. Briefly, samples were treated with lysis buffer (50 mM Tris, pH 7.5, 10% glycerol, 5 mM $MgCl_2$, 2 mM ATP and 2 mM DTT, 20-50 µL) for 1 hour on ice, followed by centrifugation at 14 000 rpm for 10 min. Samples containing 10 µg/9 µL protein (protein concentration determined using Qubit® protein assay kit) were then incubated for 1 hour with BODIPY(FL)-epoxomicin (1 µL, 0.5 µM end concentration; developed at the Leiden Institute of Chemistry) at 37°C. Next, Laemmli sample buffer (4x, 4 µL) was added and samples

were boiled for 3 minutes. Gel electrophoresis was performed on a 12.5% SDS-PAGE (15 min 80 V, 120 min 120 V). In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on a ChemiDoc MP system using Cy3 settings.

In vivo hepatic VLDL production rate

Mice were fasted for 16 hours prior to the experiment. At t=0 min, venous blood was drawn from the tail vein and mice were subsequently injected *intravenous* with 500 mg/kg Triton WR-1339 (Sigma-Aldrich) to inhibit lipoprotein lipase and prevent lipolysis of newly secreted hepatic VLDL. Additional tail vein blood samples were taken after 60, 120, and 180 min to determine VLDL-triglyceride (TG) levels. The VLDL-TG secretion rate was determined by the slope of the TG rise over time by linear regression analysis.

Tissue lipid analysis

Lipids were extracted from liver using the Folch method as described previously[28]. Briefly, 100 mg of tissue was homogenized with chloroform/methanol (1:2). The homogenate was centrifuged to recover the upper phase, which was further washed with chloroform–0.9% NaCl (1:1, pH 1.0). After centrifugation, the lower chloroform phase containing lipids was evaporated and the retained lipids were solubilized in 2% Triton X-100 by sonication. Protein content of the tissue homogenates was analyzed by BCA assay (Pierce Biotechnology, Thermo Fisher Scientific). Total cholesterol and triglyceride contents of the lipid extract were determined using enzymatic colorimetric assays (Roche Diagnostics). Data were expressed relative to the protein content.

Plasma cholesterol levels and FPLC seperation

Plasma concentrations of total cholesterol were determined by enzymatic colorimetric assays (Roche Diagnostics). Absorbance was read at 490 nm. Precipath (standardized serum; Roche Diagnostics) was used as internal standard. The distribution of cholesterol over the different lipoproteins in plasma was determined by fractionation of 30 µl of plasma of each mouse using a Superose 6 column (3.2 x 300 mm, Smart-System; Pharmacia). Total cholesterol content of the effluent was determined as described above.

$CD4^+$ T cell isolation and proliferation

Single cell suspensions of spleens from $LDLr^{-/-}$ mice were obtained by using a 70 µm cell strainer (VWR International). Red blood cells were lysed with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). $CD4^+$ T cells (>95% purity) were isolated from splenocytes by using the $CD4^+$ T cell isolation kit according to manufacturer's protocol (Miltenyi Biotec). 1 x 10^5 $CD4^+$ T cells were cultured in 96-well plates (Greiner Bio-One) in the presence or absence of αCD3 and αCD28 (1 µg/mL, eBioscience) for 72 hours in complete RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2mM L-glutamine (all obtained from PAA) and 20 µm β-mercaptoethanol (Sigma Aldrich). Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences) for the last 16 hours of culture.

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). T cell subsets were determined by flow cytometry.

Real-time PCR

mRNA was isolated from the aortic arch and liver using the guanidium isothiocyanate method and reverse transcribed (RevertAid Moloney murine leukemia virus reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast real-time PCR system (Applied Biosystems) using SYBR Green technology. The expression was determined relative to the average expression of three household genes: Succinate dehydrogenase complex, subunit A, flavoprotein (Sdha), hypoxanthine phosphoribosyltransferase (HPRT), and 60S ribosomal protein L27 (Rpl27). For used primer pairs refer to Table 1.

Gene	*Forward*	*Reverse*
36B4	CTGAGTACACCTTCCCACTTACTGA	CGACTCTTCCTTTGCTTCAGCTTT
ABCG5	TGGCCCTGCTCAGCATCT	ATTTTTAAAGGAATGGGCATCTCTT
ABCG8	CCGTCGTCAGATTTCCAATGA	GGCTTCCGACCCATGAATG
CD36	ATGGTAGAGATGGCCTTACTTGGG	AGATGTAGCCAGTGTATATGTAGGCTC
CD68	TGCCTGACAAGGGACACTTCGGG	GCGGGTGATGCAGAAGGCGATG
DGAT	GGTGCCCTGACAGAGCAGAT	CAGTAAGGCCACAGCTGCTG
FAS	GGCGGCACCTATGGCGAGG	CTCCAGCAGTGTGCGGTGGTC
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
GAPDH	ATCCTGCACCACCAACTGCTTA	CATCACGCCACAGCTTTCCAG
LPL	CCCCTAGACAACGTCCACCTC	TGGGGGCTTCTGCATACTCAAA
LRP1	CTTCTGGTGGCTGGCGTGGTG	CATCCGCTGGTGCTGGAAGCC
MCP-1	CTGAAGCCAGCTCTCTCTTCCTC	GGTGAATGAGTAGCAGCAGGTGA
MTTP	TCTCACAGTACCCGTTCTTGGT	GAGAGACATATCCCCTGCCTGT
RPL27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
PPARγ	GACGCGGGCTGAGAAGTC	CACCGCTTCTTTCAAATCTTGTC
Scd1	TACTACAAGCCCGGCCTCC	CAGCAGTACCAGGGCACCA
SREBP-1C	TCTGAGGAGGAGGGCAGGTTCCA	GGAAGGCAGGGGGCAGATAGCA
VCAM-1	AGACTGAAGTTGGCTCACAATTAAGAAG	AGTAGAGTGCAAGGAGTTCGGG

Table 1. Primer Pairs used for qPCR analysis. The relative expression of genes was determined relative to the average expression of four housekeeping genes: ribosomal protein 36B4, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT), and ribosomal protein L27 (Rpl27). Abbreviations: ABCG5/8, ATP-binding cassette sub-family G member 5/8; DGAT, diglyceride acyltransferase; FAS, fatty acid synthase; LPL, lipoprotein lipase; LRP1, Low density lipoprotein receptor-related protein 1; MTTP, microsomal triglyceride transfer protein; PPARγ, peroxisome proliferator-activated receptor γ; Scd1; stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element-binding protein 1; VCAM-1, Vascular cell adhesion protein 1.

Flow Cytometry

At sacrifice retro-orbital blood was collected and erythrocytes were lysed as described. Per sample, 3 x 10^5 white blood cells or 1 x 10^5 $CD4^+$ T cells from proliferation assays were stained with the appropriate antibodies. The following antibodies were used: CD11b-eFluor450 (clone M1/70), CD4-PerCP (clone RM4-5; BD Biosciences), CD25-FITC (clone eBio3C7), FoxP3-APC (clone FJK-16s), Gata-3-PE (clone TWAJ), Ly-6C-PerCP (clone HK1.4), Ly-6G-FITC (clone 1A8; BD Biosciences), RORγt-PE (clone AFKJS-9), and T-bet-APC (clone eBio4B10). All antibodies were purchased from eBioscience, unless stated otherwise. For intracellular staining, cells were fixed and permeabilized according to the manufacturer's protocol (eBioscience). Flow cytometry analysis was performed on the FACSCantoII and data were analyzed using FACSDiva software (BD Biosciences).

Cytokine and analysis

IL-2 and CCL2 were determined by ELISA, according to manufacturer's protocol (BD Biosciences).

Histological analysis

10 μm cryosections of the liver were stained with Oil-Red-O and hematoxylin to determine lipid content. To determine aortic root plaque size, 10 μm cryosections were stained with Oil-Red-O and haematoxylin (Sigma Aldrich). Corresponding sections were stained for collagen fibers using Sirius Red (Sigma Aldrich) and analyzed under polarized light. Macrophage content was analyzed by immunohistochemical staining against a macrophage-specific antigen (MOMA-2, polyclonal rat IgG2b, 1:1000, Serotec Ltd), respectively. Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100; Sigma Aldrich) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. To determine the number of adventitial T cells, CD3 staining was performed using anti-mouse CD3 (clone SP7, 1:150, ThermoScientific). BrightVision anti-rabbit-HRP was used as secondary antibody (Immunologic). The section with the largest lesion and four flanking sections were analyzed for lesion size and collagen content, two flanking sections were analyzed for macrophage and T cell content. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). The percentage of collagen and macrophages in the lesions was determined by dividing the collagen- or MOMA-2-positive area by the total lesion surface area.

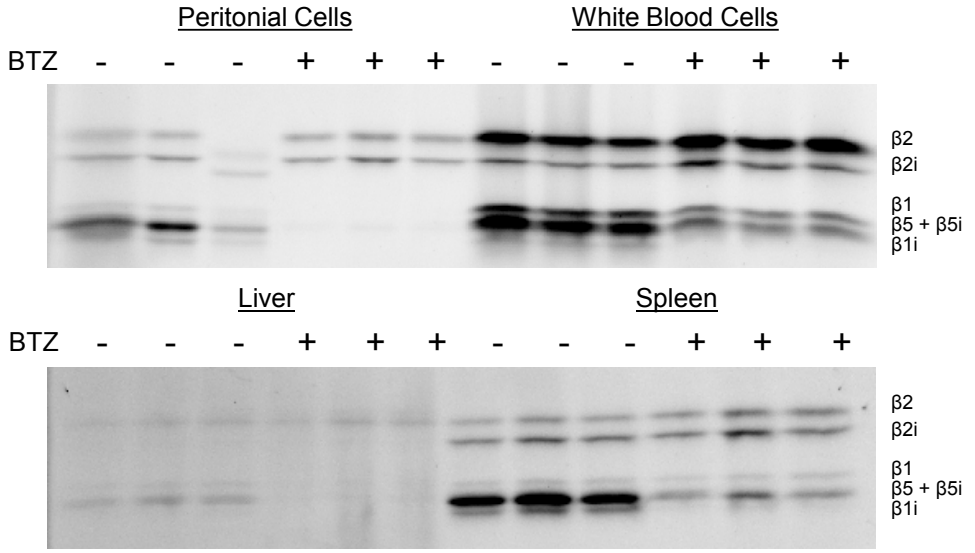
Statistical analysis

Values are expressed as mean±SEM. Data of two normally distributed groups were analyzed by Student's T-test, data of three groups were analyzed by one-way ANOVA and data of two or more groups with more than one variable were analyzed by two-way ANOVA, followed by Bonferroni post-testing. Statistical analysis was performed using Prism (GraphPad). Probability values of $P<0.05$ were considered significant.

Results

Bortezomib Reduces Proteasomal Activity in LDLr$^{-/-}$ Mice

Bortezomib is approved by the FDA for treatment of multiple myeloma and mantle cell lymphoma[21]. The recommended *intravenous* dose of Bortezomib in humans is 1.3 mg/m^2 (administered in cycles of twice weekly for 2 weeks followed by a 10-day rest period)[29]. Since this equals an *intravenous* dose of Bortezomib of about 0.42 mg/kg in mice[30], we chose to treat mice with an *intraperitoneal* dose of 0.5 mg/kg twice weekly, which is below the maximum tolerated dose in mice of 1.0 mg/kg twice weekly and this dose does not induce liver or kidney toxicity[17].



% inhibition	Peritoneal	Blood	Spleen	Liver
β1c	100	63	30	n.d.
β1i	100	n.d.	79	n.d.
β5	100	46	79	85

Figure 1. Bortezomib reduces proteasomal activity. Female LDLr$^{-/-}$ mice received two weekly injections of vehicle control (PBS/DMSO) or bortezomib (BTZ). One hour after the last injection, peritoneal cells, blood, liver cells, and splenocytes were isolated. Proteasome activity was determined by proteasomal activity-based profiling. Samples of three control mice and three mice treated with Bortezomib are shown. Table indicates average inhibition over three mice. Bands have been quantified and corrected for gel loading using coomassie quantification. n.d.: band could not be quantified due to overlap or too low intensity in samples.

To determine the extent of proteasome inhibition, we injected LDLr$^{-/-}$ mice with two weekly injections of Bortezomib after which we determined proteasome activity in peritoneal cells, white blood cells, liver and spleen. Peritoneal cell showed a complete inhibition of proteasome activity by the subunits β1, β1i, and β5/β5i. White blood cells in the circulation showed a 63% reduction of β1 and a 46% reduction of β5/β5i

subunits. Splenocytes showed a strong 79% inhibition of β1i and β5/β5i, while β1 was only inhibited by 30%. In the liver we found an 85% inhibition of β5/β5i subunits. No inhibition of β2 or β2i was observed. β5 and β5i subunits cannot be separated in murine samples by this analysis and their activity is therefore expressed together (Figure 1).

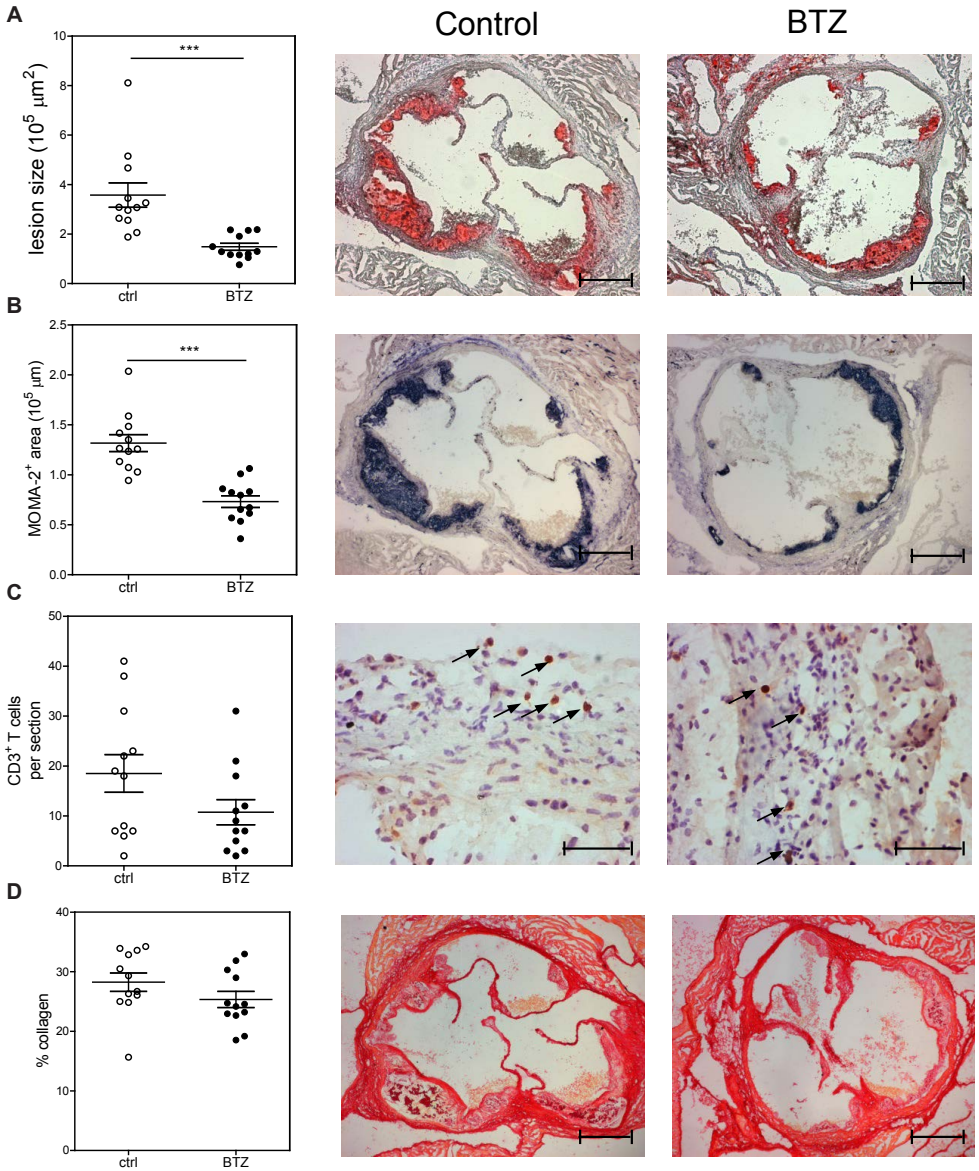


Figure 2. Bortezomib reduces atherosclerotic lesion development. A. At sacrifice, lesion size in the three valve area of the aortic root was determined; representative cross-sections stained with Oil-Red-O and hematoxylin are shown. Scale bar, 300 µm. **B.** Macrophage positive area was determined by MOMA-2 staining. Scale bar, 300 µm. **C.** $CD3^+$ T cells were determined by αCD3 staining. Arrows indicate T cells. Scale bar, 200 µm. **D.** Collagen positive area was determined by Sirius Red staining. Scale bar, 300 µm. All values are expressed as mean±SEM and are representative of six mice. *** $P<0.001$.

Proteasomal Inhibition Reduces Atherosclerotic Lesion Development

To assess the effect of proteasome inhibition on atherosclerosis, we treated female LDLr$^{-/-}$ mice twice weekly with *intraperitoneal* injections of Bortezomib. After one week, mice were put on a Western-type diet (WTD) for eight weeks to induce atherosclerotic lesion development. Bortezomib treatment was continued throughout the experiment. We observed a significant 58.6% reduction in lesion development (control: 3.6 x 10^5 ± 0.5 x 10^5 µm versus Bortezomib: 1.5 x 10^5 ± 0.1 x 10^5 µm; Figure 2A) and a 44.4% reduction in lesional macrophages (control: 1.3 x 10^5 ± 0.1 x 10^5 µm versus Bortezomib: 0.7 x 10^5 ± 0.1 x 10^5 µm; Figure 2B). The number of recruited CD3$^+$ T cells did not significantly differ (control: 18.5 ± 3.8 T cells per lesion versus Bortezomib: 10.7 ± 2.5 T cells per lesion; Figure 2C) and also no differences in collagen content of the lesions, as determined by Sirius red, were observed (control: 28.3 ± 1.5% versus Bortezomib: 25.3 ± 1.4%; Figure 2D).

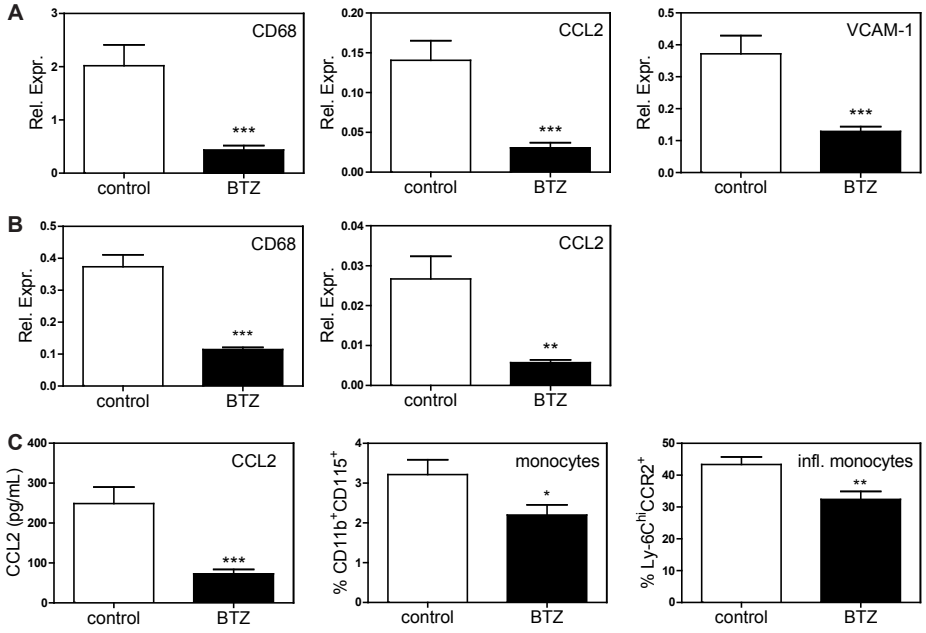


Figure 3. Bortezomib reduces monocytes and macrophages. A. The expression of CD68, CCL2 and VCAM-1 were determined in the aortic arch by qPCR. **B.** The expression of CD68 and CCL2 were determined in the liver by qPCR. Expression was determined relative to four housekeeping genes (36B4, GAPDH, HPRT, Rpl27). **C.** Serum CCL2 levels were determined by ELISA. Circulating monocytes and inflammatory monocytes as a percentage of total monocytes were determined by flow cytometry. All values are expressed as mean±SEM and are representative of at least six mice.* P<0.05, ** P<0.01, *** P<0.001.

Proteasomal Inhibition Significantly Reduces Monocyte Recruitment and Macrophages

In line with a reduction in lesional macrophages in the aortic root, we also observed a significant 78.4% reduced CD68 expression in the aortic arch of Bortezomib-treated mice indicative of reduced macrophages. This likely resulted from a reduced

recruitment of monocytes into the lesions as we observed a 77.9% reduced CCL2 and a 65.3% reduced VCAM-1 expression (Figure 3A). In the liver of Bortezomib-treated mice we also found a 69.4% reduction of CD68 expression and a 77.8% reduction of CCL2 expression (Figure 3B). Overall we established a 70.7% decrease in plasma CCL2 in the Bortezomib group: This corresponded with a 31.6% reduction in circulating monocytes and a 25.3% reduction in inflammatory Ly-6C^{hi}CCR2^{+} monocytes (Figure 3C).

Proteasomal Inhibition Skews T cells towards Th2 Responses and Reduces Th1 Responses

We argued that Bortezomib-treatment should affect T cell subset induction as proteasome inhibition has been suggested to preferentially induce Tregs and reduce Th1 and Th17 responses[31], although we did not observe significant effects on CD3^{+} T cells in the aortic root. We isolated splenic CD4^{+} T cells from Bortezomib-treated mice and control mice and induced general T cell proliferation by stimulation with αCD3 and αCD28 for 72 hours. We found a significant 43.4% and 72.8% reduced proliferative capacity, as determined by ^{3}H-thymidine incorporation and IL-2 production, respectively (Figure 4A). The relative percentages of T cell responses were significantly skewed towards Th2 responses, which were increased by 43.1%, while Th1 responses were decreased by 21.3% upon Bortezomib-treatment. Th17 responses were not affected and Treg percentages were slightly but non-significantly increased (Figure 4B), indicating that overall pro-atherogenic T cell responses were reduced after Bortezomib-treatment.

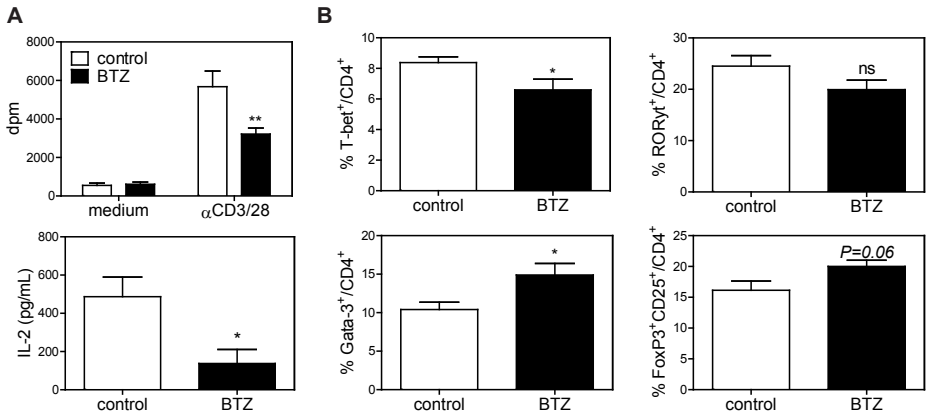


Figure 4. Bortezomib reduces T cell responses. A. Splenocytes were cultured in the presence of 1 μg/mL αCD3/αCD28 for 72 hours. Proliferation was assessed by the amount of ^{3}H-thymidine incorporation during the last 16 hours of culture and by IL-2 production determined by ELISA. **B.** Th1 (T-bet^{+}), Th2 (Gata-3^{+}), Th17 (RORγt^{+}) cells and Treg (FoxP3^{+}CD25^{+}) within CD4^{+} T cells were determined by flow cytometry. All values are expressed as mean±SEM and are representative of six mice.* P<0.05, ** P<0.01.

Proteasomal Inhibition Significantly Reduces Plasma Cholesterol and VLDL Secretion

During the entire experiment we monitored weight, food intake, plasma glucose and cholesterol levels to determine possible effects on metabolism. While we found no significant effects on food intake, weight development and plasma glucose levels (Figure 5A), we intriguingly observed that after one week WTD cholesterol levels plateaued in mice treated with Bortezomib. After eight weeks diet, proteasome inhibition resulted in a significant 50.2% decrease in total plasma cholesterol levels compared to control mice (Figure 5B). The large reduction in cholesterol levels was due to a substantial 62% decrease in plasma VLDL levels as determined by fast-performance liquid chromatography (FPLC) separation, while LDL and HDL levels were not affected (Figure 5B).

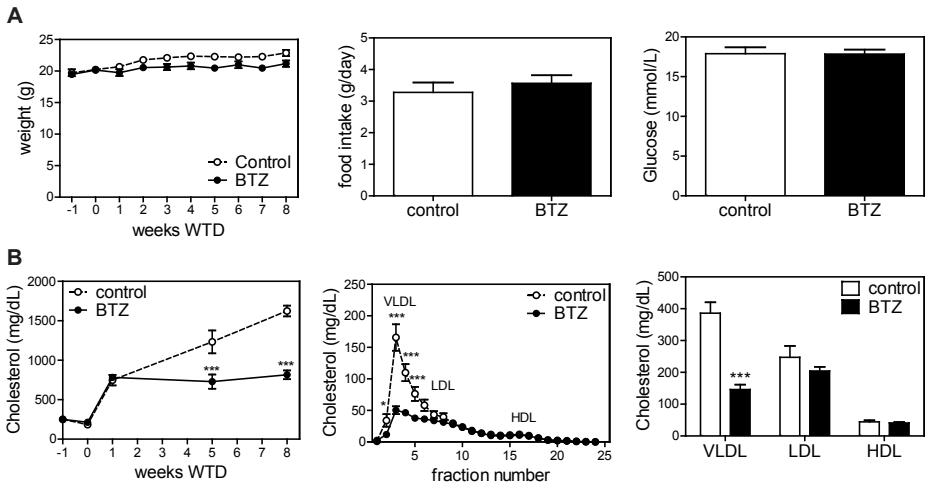


Figure 5. Bortezomib treatment reduces serum cholesterol levels. A. Mice were weighed weekly, food intake was determined during the last two weeks, and glucose was measured at the end of the experiment. **B.** Cholesterol levels were monitored throughout the entire experiment. For FPLC analysis 3 mice per group were pooled. Fractions 2–5 represent VLDL, fractions 6–14 represent LDL, and fractions 15–20 represent HDL. All values are expressed as mean±SEM and are representative of at least eight mice. * P<0.05, *** P<0.001.

Additionally, we observed a reduction in Oil-Red O positive staining in livers of Bortezomib-treated mice (Figure 6A). Oil-red O stains neutral lipids (e.g. triglycerides and cholesterol esters) and it is the most accurate method for determining the level of hepatic steatosis[32]. Steatosis, an abnormal lipid accumulation within cells, has been shown to be associated with an increased risk for atherosclerosis[33]. This finding indicates that Bortezomib-treated mice had significantly reduced WTD-induced liver steatosis and thereby decreasing a risk factor for atherosclerosis. Direct measurement of the cholesterol levels in the liver confirmed that cholesterol levels were decreased by 1.4-fold upon Bortezomib-treatment, while triglyceride content was not affected (Figure 6B).

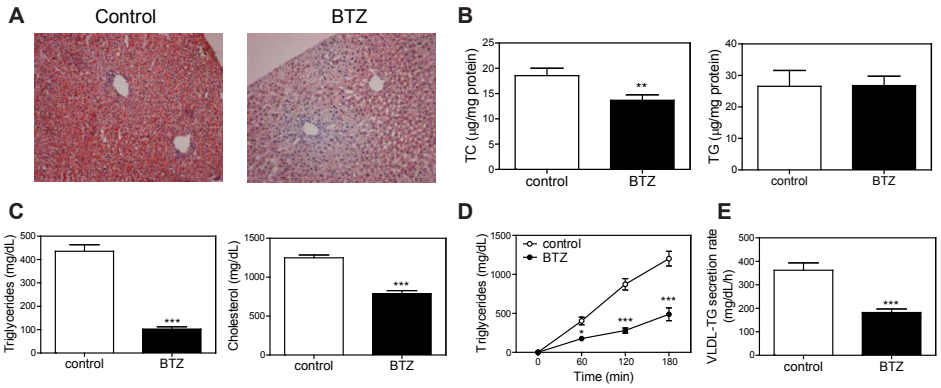


Figure 6. Bortezomib reduces VLDL secretion. A. Liver sections were stained with Oil-Red O, representative sections are shown. **B.** Cholesterol and triglyceride amounts per mg protein in the liver were determined. **C.** Female LDLr$^{-/-}$ were fasted for 16 hours after two weeks of WTD. Plasma Triglycerides and cholesterol levels were determined. **D.** Increases in triglyceride levels in circulation after administration of Triton WR-1339. **E.** VLDL-Triglyceride (TG) secretion determined over 180 min. All values are expressed as mean±SEM and are representative of six mice. * P<0.05, ** P<0.01, *** P<0.001.

To establish whether the reduction in plasma VLDL was associated with a reduction in VLDL secretion, we performed a Triton WR-1339 study. After two weeks WTD, when plasma cholesterol levels had plateaued in Bortezomib-treated mice, we fasted mice for 16 hours and found a significant 76% and 37% reduction in plasma triglycerides and cholesterol, respectively (Figure 6C). Subsequently, we injected Triton WR-1339 and found a significant reduction in the secretion of VLDL-triglycerides in Bortezomib-treated mice, when corrected for initial differences (Figure 6D). Overall, Bortezomib-treated mice showed a 49.6% reduction in their VLDL-triglyceride secretion rate (Figure 6E). Reduced VLDL secretion has often been seen to increase triglyceride content of the liver, but we did not observe any effect on liver triglyceride content.

Proteasomal Inhibition Significantly Reduces Lipogenesis and Increases Bile Acid Excretion

To determine if reduced VLDL secretion was due to reduced hepatic lipid uptake we assessed the expression of CD36 and LDL receptor-related protein 1 (LRP1), both receptors for VLDL and chylomicrons. CD36 and LRP1 were not affected by Bortezomib-treatment (Figure 7A), indicating that hepatic VLDL clearance from the circulation was not affected.

We further assessed whether expression of genes involved in hepatic *de novo* lipogenesis was modified. Fatty acid synthase (FAS) is the main biosynthetic enzyme in lipogenesis, which synthesizes palmitate from acetyl-CoA and malonyl-CoA. Interestingly, we observed a striking downregulation by 57.8%. Stearoyl-CoA desaturase-1 (Scd1) is the rate-limiting enzyme in the synthesis of unsaturated fatty acids further downstream of FAS, resulting in production of oleic acid. We found that Scd1 was also reduced by 44%, but this was not significant (Figure 7 B). In contrast, diglyceride acyltransferase 1 (DGAT1), which is involved in the last steps of triglyceride synthesis, and microsomal triglyceride transfer protein (MTTP), which plays a central

role in lipoprotein assembly, were not affected (Figure 7C).

Sterol regulatory element binding protein-1c (SREBP-1c), a transcription factor regulation *de novo* lipogenesis genes, was also not affected (Figure 7D). However, peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor known to be involved in high fat diet-induced liver steatosis[34], was significantly downregulated by 28.6% in line with the observed reduction in steatosis (Figure 7D). Additionally, we found a significant increase of ABCG5 by 101.3% indicating increased efflux of cholesterol to the bile for excretion. However, ABCG8, which has the same role in the liver, was not affected (Figure 7E). Interestingly, expression of lipoprotein lipase (LPL), which hydrolyzes triglycerides from VLDL and chylomicrons into free fatty acids, was significantly decreased by 70.5% (Figure 7F). LPL is not expressed in adult liver but mainly expressed by macrophages[35]. Therefore reduced LPL levels likely simply reflect reduced hepatic macrophage content.

Overall Bortezomib-treated mice show profound effects on hepatic liver metabolism: cholesterol efflux to the bile is likely increased and *de novo* lipogenesis is significantly reduced, which ultimately results in reduced VLDL secretion.

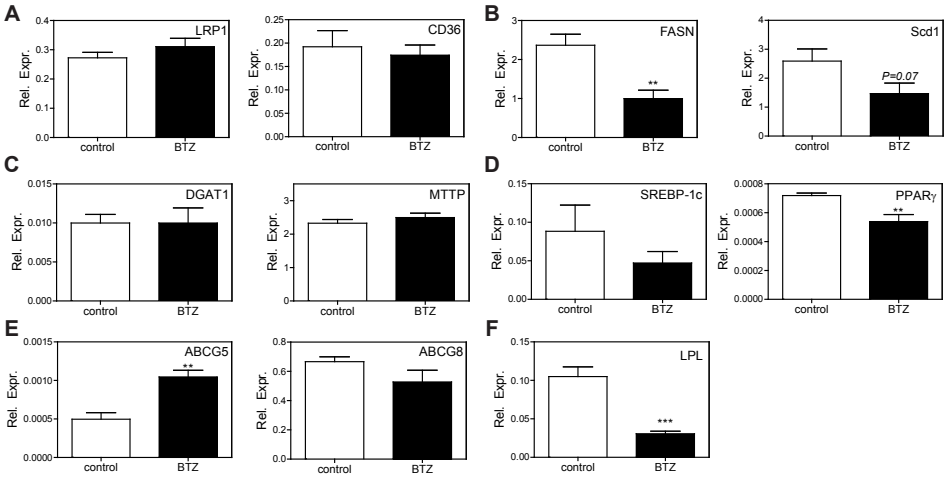


Figure 7. Bortezomib inhibits *de novo* lipogenesis. Genes involved in lipoprotein uptake (**A**), genes of key enzymes involved in initial steps of lipogenesis (**B**) and other further downstream enzymes of lipogenesis (**C**) , transcription factors (**D**), genes involved in cholesterol efflux (**E**) and lipoprotein lipase (**F**) were determined by qPCR. The expression is expressed relative to four housekeeping genes (36B4, GAPDH, HPRT, Rpl27). All values are expressed as mean±SEM and are representative of six mice. ** P<0.01, *** P<0.001.

Discussion

To our knowledge we show here for the first time that Bortezomib can have a potent lipid-lowering effect, a quality that in addition to its anti-inflammatory effects strengthens the beneficial effect of Bortezomib on atherosclerosis. Bortezomib is therefore the perfect dual drug to inhibit both underlying causes of atherosclerosis: inflammation and dyslipidemia. We find that as a result Bortezomib significantly

reduces atherosclerosis development by 58.6%.

We see a strong reduction of inflammatory responses. In line with an earlier atherosclerosis study by Wilck *et al.* we observe a significant reduction in VCAM-1, CCL2 levels and monocyte responses[24]. Moreover, we observe reduced Th1 cell induction and a skewing towards Th2 cells, which is in line with earlier described effects of proteasome inhibition on $CD4^+$ T cell subsets[31]. Anti-inflammatory effects of Bortezomib have also been described in experimental models for rheumatoid arthritis and graft-versus-host disease[17,18]. Interestingly, we see a large reduction of macrophage content in the liver. Uptake of oxLDL or cholesterol crystals has been found to increase expression of inflammatory genes in macrophages and may contribute to the development of hepatosteatosis[36,37]. Non-alcoholic steatohepatitis (NASH) is dramatically reduced when inflammatory responses of liver macrophages (Kupffer cells) are inhibited or reduced[36,38]. Therefore, an inhibition of pro-inflammatory responses in macrophages by Bortezomib could contribute to reduced steatosis.

In addition to reduced inflammation, we observed a robust decrease in plasma cholesterol and triglyceride levels, which was mainly the result of a robust reduction in circulating VLDL levels. We further determined that this reduction in VLDL was most likely related to multiple effects of Bortezomib. Bortezomib resulted in reduced *de novo* lipid synthesis due to a significant reduction of FAS expression and reduced hepatic cholesterol levels, which likely result from increased expression of liver ABCG5 and thus increased bile acid excretion (Figure 8). We additionally observed a very strong decrease of LPL. As LPL in the liver is mainly expressed by macrophages[35], it likely reflects decreases we observe in macrophage inflammation and numbers. However, LPL has been shown to promote foam cell formation[39] and reduced LPL expression in macrophages could thus contribute to the reduced cholesterol levels observed in the liver. Because LPL is secreted by macrophages, reduced hydrolysis of triglycerides from VLDL could result in less free fatty acid availability for VLDL production.

Information on the role of the ubiquitin-proteasome pathway in lipid metabolism is limited. A mutation in the PSMB8 gene, encoding β5i, results in reduced expression and reduced proteasome activity. This was found to induce loss of adipose tissue in the upper part of the human body by blocking adipocyte differentiation[25]. Interestingly, it was found that corticosteroid therapy reduces inflammation in these patients, but does not affect lipodystrophy, suggesting that effects on adipose tissue are likely not due to secondary effects of inflammation[25]. In the earlier atherosclerosis study by Wilck *et al.* changes in HMG-CoA reductase and HMG-CoA synthase in the aorta were observed[24], but they did not report on the expression of these genes in the liver. In a study by Oliva *et al.* Bortezomib was investigated in rats to determine possible beneficial effects on ethanol-induced steatosis. A reduction in several lipogenic genes, including SREBP-1c, FAS, Scd1, DGAT, HMG-CoA synthase, was observed, however, LPL in this study was significantly increased[26]. It has to be noted that in this study rats were on a normal chow diet. Interestingly, statins have been proposed to, besides their well-known effect on HMG-CoA reductase inhibition, also inhibit the proteasome[40,41]. However, whether this has any contribution to cholesterol-lowering capacities of statin treatment is unknown. Proteasome activator 28-null mice, which

show a 30-40% reduction of overall proteasomal activity, have increased SREBP-1c activity, hepatic steatosis and hepatic insulin resistance[42], indicating that the β2 and β2i subunits might have a beneficial effects on lipid metabolism as those are the only subunits not inhibited by Bortezomib. It will be interesting to determine if specific effects on one proteasomal subunit or combined inhibition are needed for the beneficial effect that Bortezomib exerts.

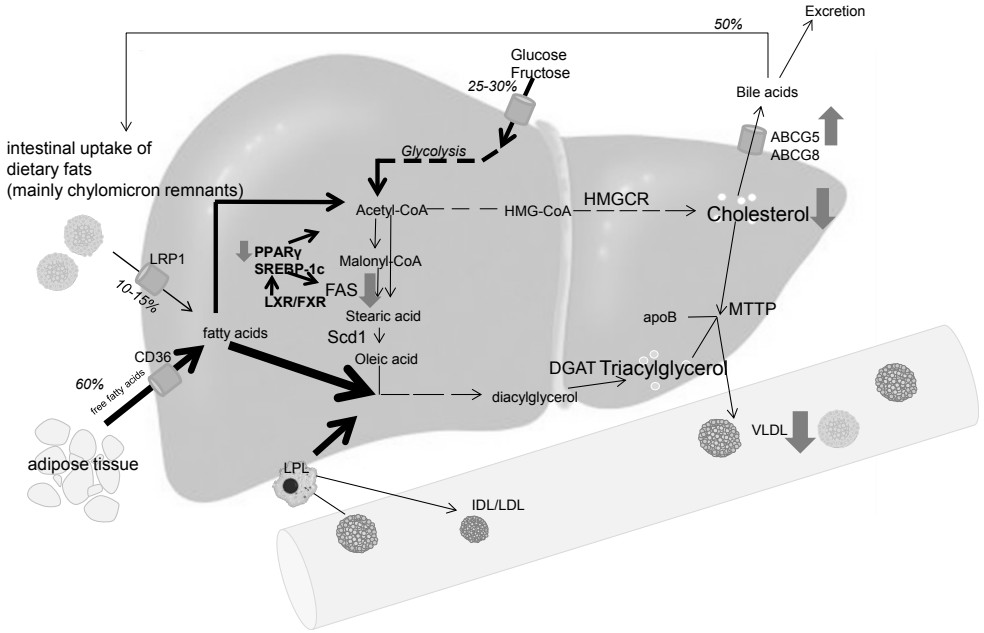


Figure 8. Scheme of Bortezomib effects on hepatic lipid metabolism. Bortezomib treatment did not affect lipoprotein uptake. However *de novo* lipogenesis is reduced, as FAS is significantly reduced, as well as PPARγ. Additionally ABCG5 is significantly increased resulting in increased cholesterol efflux. Overall this reduces hepatic cholesterol levels and reduced VLDL assembly, resulting in a decreased VLDL secretion rate. Grey arrows indicate positive effects of Bortezomib-treatment. Percentages indicate contribution to VLDL-triglycerides under conditions of non-alcoholic steatosis. ABCG5/8, ATP-binding cassette sub-family G member 5/8; DGAT, diglyceride acyltransferase; FAS, fatty acid synthase; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LRP1, LDL receptor-related protein 1; PPARγ, peroxisome proliferator-activated receptor ; Scd1, Stearoyl-CoA desaturase-1; SREBP-1c, Sterol regulatory element binding protein-1c.

Bortezomib is already used in the clinic for multiple myeloma and it would be very interesting to determine whether patients receiving treatment show initial decreases in their plasma cholesterol levels after treatment. However, the cholesterol lowering effect of Bortezomib might have been overlooked as multiple myeloma patients have low basal cholesterol levels likely due to increased cholesterol utilization by myeloma cells[43]. After Bortezomib therapy patients did not show significant changes in their plasma cholesterol compared to pre-treatment values[43], indicating that a lower cholesterol level was maintained despite cancer remission.

The dramatic effect of Bortezomib-treatment on dyslipidemia is especially important as a substantial proportion of patients will not respond to statin treatment, either due to statin resistance or intolerance. Of patients receiving statin therapy only

73% will actually achieve target plasma LDL levels[44] and a large number of these patients will still have cardiovascular events[45]. Alternatives for patients unresponsive to statins include ezetimibe and bile acid sequestrants, but these treatments lack potency when compared to statin therapy[46]. For example, the use of ezetimibe in addition to statins was recently assessed in the IMPROVE-IT trial and found to reduce primary endpoint events, such as myocardial infarction and stroke, by only an additional 6.4%[47]. Nonetheless, a proportion of patients remain where none of the above mentioned therapies help to reduce plasma cholesterol, leaving these patients at a high risk for future cardiovascular complications. Currently, clinical trials establishing the effect of PCSK9 inhibition are ongoing and seem promising to reduce LDL levels. We show here that Bortezomib could be another potent alternative treatment, especially as it additionally significantly reduces inflammation. We envision that Bortezomib therefore will be a potent drug for the treatment of cardiovascular diseases due to its dual effects on inflammation and dyslipidemia.

References

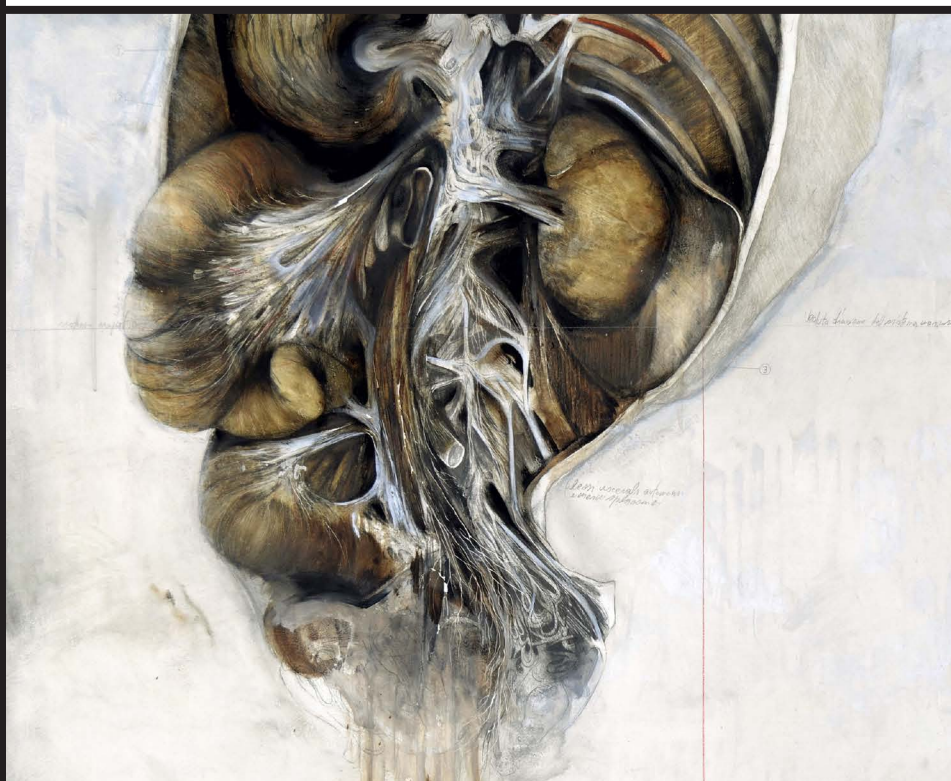
1. Rock, K. L. *et al.* Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78, 761–71 (1994).
2. Wang, J. & Maldonado, M. A. The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell. Mol. Immunol.* 3, 255–61 (2006).
3. Paniagua Soriano, G., De Bruin, G., Overkleeft, H. S. & Florea, B. I. Toward understanding induction of oxidative stress and apoptosis by proteasome inhibitors. *Antioxid. Redox Signal.* 21, 2419–43 (2014).
4. Pagan, J., Seto, T., Pagano, M. & Cittadini, A. Role of the ubiquitin proteasome system in the heart. *Circ. Res.* 112, 1046–58 (2013).
5. Ciechanover, A. & Brundin, P. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron* 40, 427–46 (2003).
6. Bedford, L., Paine, S., Sheppard, P. W., Mayer, R. J. & Roelofs, J. Assembly, structure, and function of the 26S proteasome. *Trends Cell Biol.* 20, 391–401 (2010).
7. Murata, S., Yashiroda, H. & Tanaka, K. Molecular mechanisms of proteasome assembly. *Nat. Rev. Mol. Cell Biol.* 10, 104–15 (2009).
8. Hisamatsu, H. *et al.* Newly identified pair of proteasomal subunits regulated reciprocally by interferon gamma. *J. Exp. Med.* 183, 1807–16 (1996).
9. Seifert, U. *et al.* Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. *Cell* 142, 613–24 (2010).
10. McConkey, D. J. & Zhu, K. Mechanisms of proteasome inhibitor action and resistance in cancer. *Drug Resist. Updat.* 11, 164–79 (2008).
11. Hideshima, T. *et al.* Bortezomib induces canonical nuclear factor-kappaB activation in multiple myeloma cells. *Blood* 114, 1046–52 (2009).
12. McConkey, D. J. Bortezomib paradigm shift in myeloma. *Blood* 114, 931–2 (2009).
13. Crawford, L. J., Walker, B. & Irvine, A. E. Proteasome inhibitors in cancer therapy. *J. Cell Commun. Signal.* 5, 101–10 (2011).
14. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb. Perspect. Biol.* 1, a001651 (2009).
15. Qureshi, N., Morrison, D. C. & Reis, J. Proteasome protease mediated regulation of cytokine induction and inflammation. *Biochim. Biophys. Acta* 1823, 2087–93 (2012).
16. Muchamuel, T. *et al.* A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat. Med.* 15, 781–7 (2009).
17. Lee, S.-W., Kim, J.-H., Park, Y.-B. & Lee, S.-K. Bortezomib attenuates murine collagen-induced arthritis. *Ann. Rheum. Dis.* 68, 1761–7 (2009).
18. Pai, C.-C. S. *et al.* Treatment of chronic graft-versus-host disease with bortezomib. *Blood* 124, 1677–88 (2014).
19. Sun, K. *et al.* Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. *Proc. Natl. Acad. Sci. U. S. A.* 101, 8120–5 (2004).

20. Vanderlugt, C. L., Rahbe, S. M., Elliott, P. J., Dal Canto, M. C. & Miller, S. D. Treatment of established relapsing experimental autoimmune encephalomyelitis with the proteasome inhibitor PS-519. *J. Autoimmun.* 14, 205–11 (2000).
21. Adams, J. The proteasome: a suitable antineoplastic target. *Nat. Rev. Cancer* 4, 349–60 (2004).
22. Herrmann, J. *et al.* Chronic proteasome inhibition contributes to coronary atherosclerosis. *Circ. Res.* 101, 865–74 (2007).
23. Van Herck, J. L. *et al.* Proteasome inhibitor bortezomib promotes a rupture-prone plaque phenotype in ApoE-deficient mice. *Basic Res. Cardiol.* 105, 39–50 (2010).
24. Wilck, N. *et al.* Attenuation of early atherogenesis in low-density lipoprotein receptor-deficient mice by proteasome inhibition. *Arterioscler. Thromb. Vasc. Biol.* 32, 1418–26 (2012).
25. Kitamura, A. *et al.* A mutation in the immunoproteasome subunit PSMB8 causes autoinflammation and lipodystrophy in humans. *J. Clin. Invest.* 121, 4150–60 (2011).
26. Oliva, J., French, S. W., Li, J. & Bardag-Gorce, F. Proteasome inhibitor treatment reduced fatty acid, triacylglycerol and cholesterol synthesis. *Exp. Mol. Pathol.* 93, 26–34 (2012).
27. Li, N. *et al.* Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS. *Nat. Protoc.* 8, 1155–68 (2013).
28. Li, Z. *et al.* Niacin reduces plasma CETP levels by diminishing liver macrophage content in CETP transgenic mice. *Biochem. Pharmacol.* 84, 821–9 (2012).
29. Bross, P. F. *et al.* Approval summary for bortezomib for injection in the treatment of multiple myeloma. *Clin. Cancer Res.* 10, 3954–64 (2004).
30. Reagan-Shaw, S., Nihal, M. & Ahmad, N. Dose translation from animal to human studies revisited. *FASEB J.* 22, 659–61 (2008).
31. Kalim, K. W., Basler, M., Kirk, C. J. & Groettrup, M. Immunoproteasome subunit LMP7 deficiency and inhibition suppresses Th1 and Th17 but enhances regulatory T cell differentiation. *J. Immunol.* 189, 4182–93 (2012).
32. Mehlem, A., Hagberg, C. E., Muhl, L., Eriksson, U. & Falkevall, A. Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease. *Nat. Protoc.* 8, 1149–54 (2013).
33. Targher, G. *et al.* Relation of nonalcoholic hepatic steatosis to early carotid atherosclerosis in healthy men: role of visceral fat accumulation. *Diabetes Care* 27, 2498–500 (2004).
34. Morán-Salvador, E. *et al.* Role for PPARγ in obesity-induced hepatic steatosis as determined by hepatocyte- and macrophage-specific conditional knockouts. *FASEB J.* 25, 2538–50 (2011).
35. Semenkovich, C. F. *et al.* Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation, and evolution. *J. Lipid Res.* 30, 423–31 (1989).
36. Hendrikx, T. *et al.* Macrophage specific caspase-1/11 deficiency protects against cholesterol crystallization and hepatic inflammation in hyperlipidemic mice. *PLoS One* 8, e78792 (2013).
37. Bieghs, V. *et al.* Trapping of oxidized LDL in lysosomes of Kupffer cells is a trigger for hepatic inflammation. *Liver Int.* 33, 1056–61 (2013).
38. Bieghs, V. *et al.* Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice. *Gastroenterology* 138, 2477–86, 2486.e1–3 (2010).
39. Babaev, V. R. *et al.* Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J. Clin. Invest.* 103, 1697–705 (1999).
40. Wójcik, C. *et al.* Lovastatin and simvastatin are modulators of the proteasome. Int. J. Biochem. *Cell Biol.* 32, 957–65 (2000).
41. Rao, S. *et al.* Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. *Proc. Natl. Acad. Sci. U. S. A.* 96, 7797–802 (1999).
42. Otoda, T. *et al.* Proteasome dysfunction mediates obesity-induced endoplasmic reticulum stress and insulin resistance in the liver. Diabetes 62, 811–24 (2013).
43. Yavasoglu, I. et al. Cholesterol levels in patients with multiple myeloma. *Ann. Hematol.* 87, 223–8 (2008).
44. Waters, D. D. *et al.* Lipid treatment assessment project 2: a multinational survey to evaluate the proportion of patients achieving low-density lipoprotein cholesterol goals. *Circulation* 120, 28–34 (2009).
45. Superko, H. R. & King, S. Lipid management to reduce cardiovascular risk: a new strategy is required. *Circulation* 117, 560–8; discussion 568 (2008).
46. Reiner, Z. Resistance and intolerance to statins. *Nutr. Metab. Cardiovasc. Dis.* 24, 1057–66 (2014).
47. DiNicolantonio, J. J., Chatterjee, S., Lavie, C. J., Bangalore, S. & O'Keefe, J. H. Ezetimibe Plus Moderate Dose Simvastatin After Acute Coronary Syndrome: What are we IMPROVEing on? *Am. J. Med.* (2015).

7

8

General Discussion and Perspectives



Background

Atherosclerosis is the main underlying cause of cardiovascular disease and mainly affects medium to large arteries. The disease develops due to dyslipidemia and inflammation, resulting in both a massive lipid accumulation and a chronic pro-inflammatory response in the vessel wall. Current treatment is primarily based on lowering blood cholesterol levels, one of the main risk factors for atherosclerosis. Statins, which were first marketed for their lipid lowering properties in the late 1980s, inhibit 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, which is the rate-limiting enzyme in cholesterol synthesis, but have also recently been suggested to have anti-inflammatory properties[1]. Nonetheless, statins can only lower cardiovascular risk by 25-30% and patients with coronary syndromes have a higher than 20% chance of a recurrent event, despite optimal treatment[2]. This emphasizes the urgent need for the development of new therapeutic strategies.

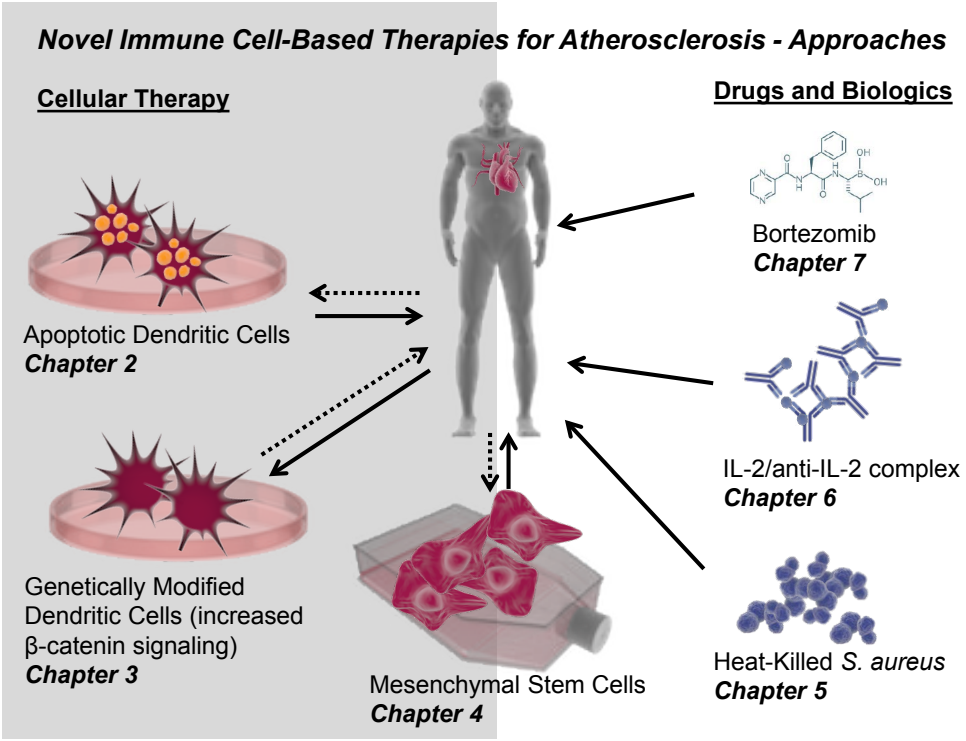


Figure 1. Approaches taken in this thesis to establish a novel immune cell-based therapy for atherosclerosis. Cellular therapy and drugs/biologics to modify immune cells were evaluated. Apoptotic DCs (chapter 2) and DCs with a β-catenin stabilization (chapter 3), as well as MSCs (chapter 4) were used as cellular therapy. MSCs were also used to indirectly modify immune cell function, thus, representing an intermediate approach. For indirect modulation of immune cells, we administered heat-killed *S. aureus* (chapter 5), IL-2/anti-IL-2 complexes (chapter 6), and the proteasome inhibitor Bortezomib (chapter 7).

This Thesis

The goal of this thesis was to establish novel immune cell-based therapies for atherosclerosis. Two approaches were used: **(1)** direct cellular therapy or **(2)** use of drugs and biologics to modify immune cell function in vivo to reduce atherosclerosis in

low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice, which develop atherosclerosis on a high-fat (Western-type, WTD) diet (Figure 1). While our therapeutic strategies were specifically aimed to reduce inflammation, we observed in two studies a beneficial effect on lipid homeostasis.

(1) Cellular Therapy: Direct Delivery of (Modified) Immune Cells to Treat Atherosclerosis

The goal of an anti-inflammatory treatment for atherosclerosis is to modulate ongoing inflammatory responses of both the innate and adaptive arm. Dendritic cells (DCs) are the most potent antigen-presenting cells and bridge innate and adaptive immune responses. We hypothesized that modulation of DCs towards a tolerogenic phenotype, i.e. producing anti-inflammatory mediators and inducing regulatory T cell (Treg) responses can beneficially affect atherosclerosis. We addressed this in two ways: (1) by administration of apoptotic DCs, which results in tolerogenic DC responses, and (2) by increasing β-catenin signaling in DCs to directly induce a tolerogenic phenotype.

In **chapter 2**, we investigated the potential of an adoptive transfer of oxidized LDL (oxLDL)-induced apoptotic DCs in both early and advanced atherosclerosis. It has been established that clearance of apoptotic cells (efferocytosis) in advanced atherosclerotic lesions is impaired[3] and earlier studies by our lab and others have shown that blockade of efferocytosis worsens atherosclerotic disease burden in experimental models for atherosclerosis[4,5]. Here we provide first evidence that clearance of oxLDL-induced apoptotic DCs has the potential to induce atheroprotective responses. We show that administration of oxLDL-induced apoptotic DCs results in induction of tolerogenic DCs, increased numbers of regulatory T cells, and reduced inflammatory monocyte responses.

After adoptive transfer of apoptotic DCs, either before lesion initiation or after established lesions had developed, we found that apoptotic DCs were mainly cleared by DCs and macrophages in the marginal zone of the spleen, inducing a tolerogenic phenotype and enabling an induction of Treg responses. Additionally, we found that overall (inflammatory) monocyte numbers were reduced, a previously unknown effect of apoptotic cell treatment. Moreover, in already established lesions, which resemble a more clinical setting as most patients enter the clinic with evident atherosclerosis, our treatment was able to increase lesion stability. Our study not only indicates the potential of such a therapeutic approach for the clinic, but also emphasizes the importance of research into the emigration potential of DCs from lesions. This is further reinforced by an earlier study from our laboratory showing that the adoptive transfer of oxLDL-pulsed DCs reduces initial atherosclerosis[6]. If DCs, which clear oxLDL or oxLDL-induced apoptotic DCs/macrophages (both containing atherosclerosis-relevant antigens), can be induced to emigrate early on from lesions, they might in fact contribute to inhibition of lesion development and stabilization.

Interestingly, at the same time as we published our results, Hosseini *et al.* found that *intraperitoneal* administration of apoptotic thymocytes also reduced atherosclerotic lesion development in $ApoE^{-/-}$ mice[7]. Similar to our study, mice show reduced numbers of macrophages and T cells in atherosclerotic lesions. However, it will

be interesting to determine why we mainly observed effects on monocyte responses and Hosseini *et al.* mainly found effects on B cell responses, specifically an induction of atheroprotective B1a cells producing IgM[7].

Another interesting aspect of our study was that control apoptotic DCs, which were not loaded with oxLDL, did reduce inflammatory cytokine responses, but did not induce tolerogenic DC phenotypes when taken up by bone marrow-derived or splenic DCs. This is in line with the study by Hosseini *et al.* who did not find any effect on Treg responses, as their apoptotic cells did not contain oxLDL[7]. We found that only DCs exposed to oxLDL-induced apoptotic DCs upregulated the expression of PD-L1, IL-10, ABCA1 and ABCG1, indicating that liver X receptor (LXR) signaling was increased. Indeed exposure to apoptotic cells and cholesterol both induce LXR activation[8–10]. In fact, LXR activation of DCs has been shown to result in a tolerogenic DC phenotype[11]. We therefore speculate that while oxLDL-induced apoptotic DCs might indeed transfer an atherosclerosis-relevant antigen in the context of an anti-inflammatory setting (efferocytosis), excess cholesterol present in apoptotic cells might contribute to the additional benefit we observe using oxLDL-induced apoptotic DCs.

It has also been suggested that phosphatidylserine (PS)-containing liposomes, which are thought to mimic apoptotic cells, can modulate inflammatory responses. PS is needed for apoptotic cell recognition by DCs and macrophages and inhibits DC maturation[12–14]. Indeed, *intravenous* administration of PS-containing liposomes induces anti-inflammatory TGF-β and IL-10 responses by macrophages, which improves infarct repair after myocardial infarction (MI)[15]. This approach would enable an easier translation to the clinic as liposomes could be generally produced. Indeed Hosseini *et al.* found that PS liposomes could mimic apoptotic cells and attenuate atherosclerosis in ApoE$^{-/-}$ mice[7]. However, in a preliminary study we did not find any effect of a lower dose of PS-containing liposomes on atherosclerosis development in LDLr$^{-/-}$ mice.

In another approach to induce tolerogenic DCs, we assessed whether increased β-catenin signaling in DCs could have a beneficial effect on atherosclerotic lesion development. In **chapter 3**, we made use of CD11c-βcat^{EX3} mice, which have constitutively active β-catenin signaling in all CD11c^{+} cells. β-catenin signaling in DCs was previously suggested to be involved in promoting a tolerogenic phenotype in DCs[16–18]. Indeed, upon bone marrow transplantation of CD11c-βcat^{EX3} bone marrow into LDLr$^{-/-}$ mice, we found a significant increase in Tregs, which reduced aortic root lesions by 26%. These lesions had significantly less necrotic areas and showed a trend towards more collagen. Similarly, an adoptive transfer of CD11c-βcat^{EX3} DCs into LDLr$^{-/-}$ mice decreased atherosclerotic lesion development by 21%. However, we used CD11c as a promoter in this study and it has to be acknowledged that CD11c is not an exclusive marker for DCs[19]. Monocytes and macrophages can also express CD11c, especially under hypercholesterolemic conditions[19,20]. Nonetheless, also in macrophages Wnt3a signaling, which induces β-catenin, was found to result in an anti-inflammatory phenotype[21] and this may account for some of the beneficial responses we observed after bone marrow transplantation. In the future it will be interesting to determine the effect of an adoptive transfer of DCs with a β-catenin stabilization in

advanced atherosclerosis, possibly with an increased treatment regimen e.g. three doses about two weeks apart as DCs have a half-life up to a week[22] and a similar regimen is advised for Provenge, a DC-based therapy for prostate cancer[23].

In recent years interest in the potential and benefit of stem cell therapies has increased and stem cells have frequently been described to "cure" multiple diseases. Indeed, tremendous advances have been made in stem cell research and in stem cell use in regenerative medicine. Mesenchymal stem cells (MSCs) specifically have been used for e.g. bone regeneration[24], neuroregeneration[25] and myocardial regeneration[26]. Recently, the immunoregulatory properties of MSCs have been acknowledged, since MSCs have a beneficial effect on inflammatory diseases as established in experimental mouse models for experimental autoimmune encephalomyelitis, collagen-induced arthritis, and type 2 diabetes[27–30] and in clinical trials for graft-versus-host disease and multiple sclerosis[31,32]. In **chapter 4**, we therefore adoptively transferred mouse MSCs into LDLr$^{-/-}$ mice on a WTD and found that MSCs reduced circulating CCL2 levels, monocytes and effector CD4^{+} and CD8^{+} T cells. Additionally, we found a striking effect on lipid metabolism: MSCs reduced total cholesterol levels by significantly decreasing VLDL levels as a result of a decreased *de novo* lipid synthesis. Overall, MSC therapy resulted in a 33% decreased atherosclerotic lesion development.

The effect of MSC therapy on cholesterol metabolism is novel, but since the effect was only seen four to five weeks after injections and atherosclerosis induction, we speculate that the effect is an indirect result of reduced inflammation. For example IL-10 has been found to decrease VLDL synthesis[33], while TNF-α increases VLDL synthesis[34], indicating that modulation of cytokine responses by MSCs could already reduce dyslipidemia. Because MSCs have been investigated for their role in cardiac repair after MI, it would be interesting to also establish effects on cholesterol levels in in these patients. Interestingly, in a small 18 patient cohort, it was shown that intracoronary stem cell infusion following MI reduced plaque burden in the coronary tree four years after treatment[35], indicating MSC treatment can be beneficial for atherosclerotic patients.

Two recent studies have provided evidence that MSCs can ameliorate atherosclerosis. Lin *et al.* demonstrate that MSC therapy improved vasodilation, by increasing eNOS expression in endothelial cells, and thereby reduced atherosclerotic lesions in the aorta of ApoE$^{-/-}$ mice[36]. Another study by Wang *et al.* found that MSC reduced atherosclerotic lesions in the aortic root in ApoE$^{-/-}$ mice, by inducing Tregs[37]. While the first study reported no effect on serum cholesterol levels[36], the second study does not mention total serum cholesterol levels. Lin *et al.* adoptively transferred human MSCs into ApoE$^{-/-}$ mice, harboring the risk of xenograft rejection. Wang *et al.* used MSCs derived from ApoE$^{-/-}$ mice, while we used MSCs from a C57BL/6 background. Increased cholesterol in stem cells of ApoE$^{-/-}$ mice has been found to increase proliferation of hematopoietic stem and progenitor cells[38], suggesting that disturbed cholesterol homeostasis of ApoE$^{-/-}$ MSCs could result in the differences to wild type MSCs. Future studies will have to address which type of MSCs, when and how often they should be administered.

(2) Drugs and biologics to modulate immune cell function in vivo

In addition to cellular therapy, drugs and biologics can be used to modulate immune cell function or to induce a specific immune cell subset in vivo.

In a first approach we made use of the fact that the immune system has an intrinsic control mechanism to reduce inflammatory responses when a pathogen is eliminated to ensure a return to tissue homeostasis. Toll-like receptors (TLRs), which recognize pathogens, have been found to in addition to initial inflammatory responses, result in anti-inflammatory IL-10 production[39]. Several studies have suggested that TLR2 activation specifically induces IL-10 responses[39–41]. A fundamental difference appears to exist between different types of antigen presenting cells in their capacity to produce IL-10: macrophages produce high levels of IL-10 in response to TLR2 signaling, while DCs produce much lower amounts of IL-10[40,42]. In **chapter 5**, we show that *intraperitoneal* injections of heat-killed *Staphylococcus aureus* (HK-SA) induce a potent anti-inflammatory IL-10 response, which is capable of reducing inflammation and thereby reduces atherosclerotic lesion development in the aortic root by 34%. We demonstrate that IL-10 responses are crucially dependent on TLR2/PI3K signaling and that this induces an immunoregulatory M2b phenotype in macrophages. The strong IL-10 production reduces the expression of adhesion molecules (VCAM-1 and ICAM-1) and CCL2 in the aorta. The amount of circulating inflammatory Ly-6C^{hi} monocytes and the amount of lesional macrophages is dramatically reduced. Additionally less Th1 and Th17 responses were observed and also lesional T cell numbers were reduced by HK-SA treatment. Here we demonstrate that TLR2/PI3K-dependent signaling can be exploited to induce anti-inflammatory IL-10 responses, which reduce monocyte/macrophage and T cell responses, as a treatment for atherosclerosis.

Several immunomodulatory strategies induce immunoregulatory/tolerogenic cells to reduce inflammation. One of the most investigated immune cells in the context of atherosclerosis is the Treg. In earlier chapters (chapter 2, 3, and 4) our treatment indirectly increased Tregs and this contributed to beneficial effects on atherosclerotic lesion development. However, in these studies we only achieved a modest increase (1.5-fold to 1.8-fold) and these generally did not last throughout the entire experiment. In **chapter 6**, we therefore assessed whether an immense and prolonged increase in Tregs, by administration of an IL-2/anti-IL2 complex, could reduce atherosclerotic lesion development and induce regression of pre-established lesions in parallel to a switch to a normal chow diet. This treatment resulted in an overall 10-fold induction of Tregs and increased splenic IL-10 responses. Treg expansion prevented atherosclerotic lesion development by 39%, suggesting that Tregs can potently inhibit initial atherosclerosis. However, while Tregs did not affect lesion size in established atherosclerosis, IL-2/anti-IL-2 complex treatment did increase lesional collagen content by 21%, indicating a more stable lesion phenotype. The latter is more clinically relevant as patients in the clinic will already have lesions and it suggests that a vast Treg expansion could stabilize such lesions.

Macrophages and DCs present atherosclerosis-relevant antigens in the context of cytokine production and can thereby modulate T cell responses. For presentation of antigens on major histocompatibility complex (MHC) I, antigen-processing by the

proteasome is required. Additionally the proteasome plays a central role in several inflammatory signaling pathways in atherosclerosis as it degrades key signaling/ inhibitory molecules, e.g. in TLR, TNF receptor and T cell receptor signaling pathways. However, also signaling proteins and transcription factors involved in lipid metabolism are degraded by the proteasome. We therefore in **chapter 7** sought to investigate if proteasomal inhibition by Bortezomib could both reduce inflammation and reduce dyslipidemia. Indeed, we found a significant reduction of monocyte/macrophage responses: reduced absolute (inflammatory) circulating monocytes and reduced macrophages in the aortic arch, aortic root and liver were observed. We also found reduced Th1 responses and increased Th2 responses. But more strikingly, we found dramatic effects on VLDL metabolism. The VLDL secretion rate was more than half reduced in mice treated with Bortezomib, which resulted from a significant decrease in *de novo* lipid synthesis and an increased bile acid secretion by the liver. Bortezomib consequently resulted in decreased inflammation, but also dramatically reduced hepatic steatosis, which culminated in a robust 58% reduction in atherosclerotic lesion size. Effects on monocytes were in line with a study by Wilck *et al.*, but they did not describe effects of Bortezomib on T cell subsets or lipid metabolism[43]. Interestingly, however, they do mention a reduction of the expression of HMG-CoA reductase in the aorta, but measurements in the liver were not performed. Likely the therapeutic dose for lipid lowering was not achieved in their study and it is therefore interesting to determine the minimal dose of Bortezomib to still affect lipid metabolism.

Considerations

In this thesis preclinical mouse models for atherosclerosis were used and it has to be noted that immune cell numbers largely differ from humans. Humans have about 30% lymphocytes and 60% neutrophils, while C57BL/6 mice have about 80% lymphocytes and 10% neutrophils[44]. Monocytes represent up to 15% of circulating leukocytes in humans and represent three subsets, while only up to 4% are found in mice with two subsets[45,46]. However, whether these differences in immune cell numbers translate into functional differences remains to be established. Additionally LDLr$^{-/-}$ and ApoE$^{-/-}$ mice already have increased total cholesterol levels on a regular chow diet: 175-225mg/dL and 400-500mg/dL, respectively. Upon WTD these levels can increase over 2000 mg/dL[47–49]. In contrast cholesterol levels in humans above 190 mg/dL are already considered as very high[50]. Atherosclerosis development in mice and men has one fundamental difference: lesion rupture and thrombosis are not observed in mice[51]. Furthermore, most treatment strategies are currently assessed by their effect on atherosclerotic lesion development in mouse models, while patients in the clinic mostly present with pre-established lesions. These differences have to be kept in mind when translating murine pre-clinical studies to human clinical trials, i.e. from "bench-to-bedside". For example the drug dose translation from animals to humans cannot simply be done by conversion according to body weight and specific formulas have been established and are recommended by the United States Food and Drug Administration (FDA)[52].

Despite these differences between mice and men, initial atherosclerosis development

is similar, making the mouse a relevant model system. Recently a large scale genomic correlation study additionally found that murine inflammatory responses mostly correlate with genomic responses in humans[53], suggesting that mouse models are good models for human inflammatory diseases. Indeed, a vast amount of novel insights into the atherosclerotic disease process stem from pre-clinical murine studies. Recently there have been efforts to "improve" mouse models further by generating more "humanized" mice with a human hematopoietic system to make mouse-human translations easier[54].

Perspectives

Immune cell therapies have been thoroughly investigated in cancer. Already in 1955, it has been described that adoptive transfer of cells from tumor-draining lymph nodes to a secondary host could confer immunity to the tumor[55]. Since then several advancements have been made to develop T cell therapies for cancer, which are currently successfully investigated for chemotherapy-resistant leukemia in clinical trials[56] and the first DC therapy for prostate cancer has been approved by the FDA[23]. In type 1 diabetes and rheumatoid arthritis clinical studies investigating the potential of tolerogenic DCs are still ongoing[57,58]. In renal transplantation 'the ONE study' (A Unified Approach to Evaluating Cellular Immunotherapy in Solid Organ Transplantation) was set up as a cooperative project to find and develop an immunoregulatory cell (product)-based therapy[59]. It will be interesting to follow the outcome of these comparative studies of different cell therapies. Additionally, MSCs have been investigated in clinical trials e.g. to enhance cardiac function after MI[60] and to reduce acute graft-versus-host disease[31]. Overall these initial clinical studies indicate that cell therapies are well-tolerated and lack side-effects. For MSCs it has been established in clinical trials that they can reduce inflammatory responses in some diseases, however, the outcome of clinical trials for DC therapy of inflammatory diseases is still anticipated.

In this thesis, we investigated cell therapies with apoptotic DCs, DCs with a β-catenin stabilization, and MSCs. The treatment with oxLDL-induced apoptotic DCs in **chapter 2** is translatable into the clinic. Both DCs and LDL can be isolated from patients and can therefore generate a patient-tailored therapy. For the treatment of multiple sclerosis a similar approach has been used: coupling of antigenic myelin peptides to apoptotic peripheral blood mononuclear cells derived from patients. Last year a phase I clinical trial showed this approach to be safe and to decrease antigen-specific T cells[61]. This trial provides first evidence in men that antigen-coupled apoptotic cells results in antigen-specific tolerance. In graft-versus-host disease, extracorporeal photopheresis has been approved by the FDA and is employed to reduce immune responses. Here patient-derived leukocytes are irradiated to induce apoptosis and are re-infused, which induces Tregs[62]. These studies provide evidence that our approach is not only promising and feasible, but should be well-tolerated by patients. Nonetheless, in both cases no effects on monocytes have been described and it would be worthwhile to investigate this. Treatment with apoptotic DCs should prove beneficial for atherosclerosis and unstable angina, but could also prove beneficial after MI, which results in a massive recruitment of monocytes and a worsening of

underlying atherosclerosis. Apoptotic cell treatment could reduce these monocyte responses and simultaneously stabilize lesions, preventing a recurrent event. However, treatment would have to be immediately upon MI and could therefore not include oxLDL loading or DC culture, so here the potential of extracorporeal photopheresis should be explored.

In atherosclerosis, vaccination with IL-10-treated ApoB100-pulsed (tolerogenic) DCs has been shown to reduce lesion development in the aorta by 70%, due to an inhibition of T cell responses[63]. In an opposite approach, our laboratory has shown that DC therapy with oxLDL-pulsed LPS-stimulated (mature) DCs also reduced carotid artery lesion development induced by collar placement by 85%. The reduced lesion was found to likely result from the induction of oxLDL-specific IgG antibodies. However, a similar study using malondialdehyde-modified (MDA) LDL-pulsed LPS-stimulated (mature) DCs found an aggravation of atherosclerosis[64]. The differences between these two studies were that one used wild type DCs and the other $ApoE^{-/-}$ DCs. It could therefore be interesting to determine whether the DC phenotype accounted for this difference as this will enable a better targeted modulation of DCs for atherosclerosis therapy. We therefore in **chapter 3** used tolerogenic DC therapy as this seemed a more straightforward approach to reduce inflammation. Indeed, we show that adoptive transfer of tolerogenic DCs can reduce atherosclerotic lesions providing further evidence that a tolerogenic DC-based therapy is a feasible approach to treat atherosclerosis. Moreover, we show that enhancing β-catenin signaling in transferred DCs could increase their therapeutic potential.

In addition to DC therapies, we describe in **chapter 4** that MSCs can significantly inhibit atherosclerotic lesion development. Because MSCs have been found to have enhanced immunoregulatory properties under inflammatory conditions[65,66], we can envision that a MSC therapy during ongoing atherosclerosis could have a more pronounced effect. Interestingly, a recent study found that MSCs can recruit myeloid-derived suppressor cells[67], which have very potent anti-inflammatory properties. It could therefore prove interesting to combine a MSC therapy with a myeloid-derived suppressor cell therapy to increase the amount of myeloid-derived suppressor cells that can be recruited to lesions.

A challenge for cellular therapies in general is that upon injection cells will be exposed to a different environment, which will affect them and unavoidable influence their phenotype. Therefore genetic engineering may be a promising approach for cell therapies in the future. More specifically, the direct genetic modification of immune cells, as e.g. stabilization of β-catenin, will enable the adoptive transfer of potent immunoregulators that execute targeted actions without undergoing differentiation or modulation in vivo. However, translating such a strategy to clinical practice will currently be difficult due to fear of genetic modifications in the general public. As an alternative, to avoid complications when administering cells, immune cell functions could be directly targeted. As the cells are already in their environment, modulation will occur after they have been exposed to other factors. This ensures that induced phenotypes are not reverted.

In **chapter 5**, we found that macrophages exposed to HK-SA produce a vast

amount of IL-10 and express other anti-inflammatory molecules, such as IDO, PD-L1, and CCL22. We found that TLR2/PI3K signaling is needed for this modulation, indicating that this signaling pathway can be exploited to induce anti-inflammatory responses. We thus challenge the current understanding that TLR2 and PI3K signaling are pro-atherogenic. It will be interesting to develop small molecules that can modulate TLR2 and PI3K signaling in a similar way to HK-SA and possibly specifically target these small molecules to lesional macrophages, as a therapy for atherosclerosis.

As previously mentioned a direct approach to induce tolerance would be to induce a significant expansion of Tregs, which we did in **chapter 6** by administration of an IL-2/anti-IL-2 complex. This approach could be translated in two ways to the clinic: either by direct administration of this complex to expand Tregs in vivo or by isolating T cells and expanding Tregs in vitro. In clinical trials, low dose subcutaneous administration of IL-2 (Proleukin®) was found to induce Tregs and resulted in clinical improvement of hepatitis C virus-induced vasculitis[68], graft-versus-host disease[69] and type 1 diabetes[70,71]. The potency of IL-2 was found to be increased in complexes with IL-2 monoclonal antibodies, which was found to significantly expand Tregs in mice and e.g. potently reduce experimental autoimmune encephalomyelitis, islet transplantation and asthma[72,73]. We have shown that these IL-2 complexes can also reduce atherosclerosis development and increase stability of advanced lesions in $LDLr^{-/-}$, which was later confirmed by another group in $ApoE^{-/-}$ mice[74]. Future studies will have to determine whether these IL-2 complexes are superior to regular IL-2 administration in patients and whether its administration is safe. IL-2 in a combination therapy with rapamycin has also been found to increase Tregs in patients with type 1 diabetes but was found to result in β-cell dysfunction[75]. The combination of IL-2 complexes with rapamycin could also be beneficial as has been found in an animal model of graft-versus-host disease[76].

Tregs can also be isolated from patients and then expanded in vitro before administration. This approach has been found to be safe and effective in clinical trials for graft-versus-host disease, transplantation, and autoimmunity[77]. Future studies will have to determine whether direct in vivo expansion or in vitro expansion and subsequent administration is more beneficial. An exciting challenge will also be the generation of antigen-specific Tregs for atherosclerosis therapy. Our laboratory has shown that antigen-specific Tregs can be generated by oral tolerance induction to oxLDL and HSP60[78,79]. A combination treatment to induce antigen-specific Tregs and then expand them seems most favorable.

Atherosclerosis is both determined by dyslipidemia and inflammation and we show in **chapter 7** that Bortezomib can reduce both underlying causes. As Bortezomib targets different enzymes in lipogenesis than statins, it is likely that combination therapies would be able to more dramatically reduce cholesterol levels. Moreover, Bortezomib could be effective in patients unresponsive to statin treatment. In addition, its anti-inflammatory effect could also compliment anti-inflammatory effects of statins, which have been for example found to induce eNOS in endothelium, to inhibit pro-inflammatory cytokine production by VSMCs, to inhibit B cell proliferation, to inhibit platelet function, and to decrease lesional T cells and macrophages[80]. Due to

their differential effects on lipid metabolism and inflammation, combination treatment with statins and Bortezomib should be greatly beneficial.

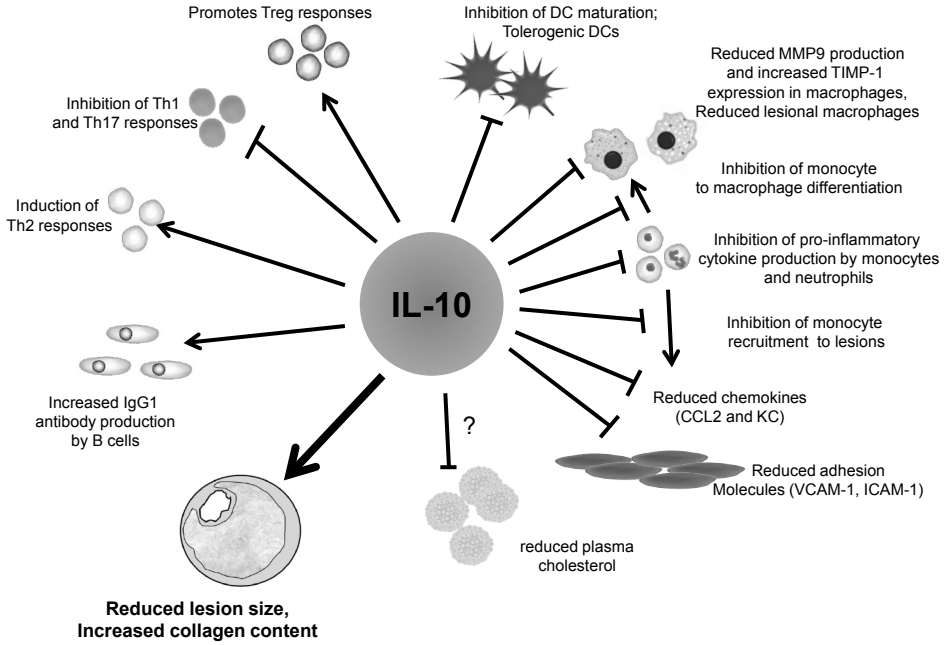


Figure 2. IL-10 affects multiple inflammatory responses in atherosclerosis and has been implicated in affecting serum cholesterol levels. Overall, IL-10 has been shown to contribute to atherosclerotic lesion size reduction and increased collagen content of lesion. Th, T helper; DC, dendritic cell; MMP, matrixmetalloproteinase; TIMP, tissue inhibitor of metalloproteinases; CCL2, chemokine (C-C motif) ligand 2; KC, keratinocyte chemoattractant; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; IgG1, Immunoglobulin G1.

All of our studies induced IL-10 responses (chapters 2, 3, 4, 5, 6, and 7) and some induced Treg responses (chapter 2, 3, 4, 6), which also produce IL-10. SNPs in the IL-10 promoter region have been found to be associated with several risk factor for atherosclerosis and the pathobiology of atherosclerosis[81,82]. Indeed, IL-10 has been found to have extensive effects on atherosclerosis (Figure 2). Numerous studies have assessed the beneficial effects of IL-10 on atherosclerosis, such as adenoviral gene transfer[33,83,84], transgenic mice expressing IL-10 under the IL-2 promoter[85,86] or the CD68 promoter[87], and IL-10 deficient C57BL/6 mice[85,88] and ApoE$^{-/-}$ mice[89]. Interestingly, some studies found that IL-10 reduces cholesterol levels[33,83], while others found enhanced cholesterol[89], or no effects[84,85,88]. In general, IL-10 potently affects many inflammatory processes, for example it reduces expression of adhesion molecules on endothelial cells[85,90], reduces CCL2 levels[83], inhibits DC maturation[90], affects monocyte responses[90], reduces lesional macrophages[84], increases macrophage cholesterol uptake and clearance[87], modulates T cell responses[88,90], affects B cell responses[90] and increases lesional collagen content[88]. This makes IL-10 a potent anti-inflammatory cytokine.

It could well be argued that a large part of the responses we observe in our

studies is mediated by IL-10. However, the clinical development of recombinant human IL-10 (Tenovil) produced by Schering Plough was discontinued due to insufficient beneficial effects in Crohn's disease[91]. Additionally, cellular therapies or specific drugs/biologics offer the advantage of a targeted approach, i.e. defined cells will be modulated, which can migrate to/reside in lesions or draining lymph nodes to modulate specific responses. Cells can be engineered to perform more than one (specific) task and to adapt/respond to their environment. However, the most promising aspect of cellular therapy is the potential to induce antigen-specific tolerance. This would ensure a more targeted therapy and leave other immune responses, such as an ongoing infection, unaffected.

In the past, several other anti-inflammatory strategies for atherosclerosis have been explored. Just to name a few: subcutaneous vaccination with native LDL or antigenic peptides from LDL[92–96], intramuscular vaccination against IL-12[97], immunization against apoB100-specific TCRs[98], CD40L blockade[99,100] and OX40L blockade[101,102] have all been evaluated in preclinical animal models. Anti-inflammatory approaches are now starting to be validated in first clinical trials. Currently two large placebo-controlled clinical trials, the Cardiovascular Inflammation Reduction Trial (CIRT) and the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS), are ongoing to establish whether lowering inflammation will reduce cardiovascular events. The CIRT evaluates the effect of methotrexate[103], which in low doses is anti-inflammatory, and the CANTOS evaluates the effect of inhibiting IL-1β[104]. In phase IIb of the CANTOS it was found that treatment with canakinumab (anti-human IL-1β monoclonal antibody) reduces inflammation in patients, as determined by reduced IL-6 and C-reactive protein, a marker for cardiovascular risk[105], indicating its potential. Both methotrexate and canakinumab are already approved by the FDA for other inflammatory diseases, e.g. juvenile idiopathic arthritis. Additionally other strategies have been validated in first clinical trials, such as a P-selectin antagonist (RO4905417)[106], 5-lipoxygenase inhibitor (Atreleuton) reducing leukotriene production[107], and an anti-CCR2 antibody (MLN1202)[108].

Furthermore, several anti-inflammatory therapies have been tested in other autoimmune diseases and could possibly be translated to atherosclerosis, e.g. abatacept and anti-TNF-α therapies. Abatacept (a fusion protein of IgG and CTLA-4) binds and blocks CD80/CD86 and thereby reduces T cell responses. It has been approved by the FDA for the treatment of rheumatoid arthritis and juvenile idiopathic arthritis; and has been indicated to reduce experimental atherosclerosis[109]. Additionally, anti-TNF-α therapies have been approved by the FDA for the treatment of rheumatoid arthritis and Crohn's disease, among others. In mice, TNF-α inhibition or deficiency has been described to either have no effect[110] or to reduce atherosclerosis[111,112]. In patients, a clinical trial of infliximab (monoclonal antibody against TNF-α), however, found that at high doses of 10 mg/kg TNF-α patients had a much higher risk of heart failure[113]. This clearly indicates that careful evaluation of therapies and their treatment regimens is needed when translating from mice to men

It will be interesting to follow the outcome of large clinical trials such as the CIRT and CANTOS as they will establish on a large scale for the first time whether

interfering with inflammatory pathways will reduce cardiovascular events. As not all patients enrolled in the CIRT and CANTOS trial are taking statins[103,104,114], it could be interesting to determine if combination with statin use is more beneficial. Likely, as atherosclerosis is determined by dyslipidemia and inflammation, the future of cardiovascular treatment strategies will lie in a combination treatment for reducing lipids and inflammation. When comparing all approaches we took in this thesis, it becomes evident that strategies that only affect inflammation result in at most a 40% reduction of lesion sizes, while only when both inflammation and lipid metabolism is dramatically reduced by Bortezomib a more significant lesion reduction of up to 58% is possible (Table 1). Therefore, the future of cardiovascular treatment strategies will lie in reducing both inflammation and dyslipidemia, either by administering drugs potently affecting both (such as bortezomib), or by combination therapies.

In summary, our studies confirm that immune cell therapies show great potential for the treatment of atherosclerosis. However, several challenges in translating these studies into the clinic remain. The journey towards immune cell-based therapies for atherosclerosis, as well as other diseases, will unquestionably be a very exciting one.

Chapter	Therapy	Results	% lesion reduction	% collagen increase
2	**oxLDL-induced apoptotic DCs**	- Reduced monocyte responses - Tolerogenic DCs - Increased Tregs	**28%** (D) **40%** (P)	n/a **45%**
3	**β-catenin stabilization in DCs**	- Tolerogenic DCs - Increased Tregs	**21%** (D) **26%** (BMT)	n/a **25%**
4	**MSCs**	- Reduced T cell responses - Reduced VLDL	**33%** (D)	n/a
5	**HK-SA**	- Reduced monocyte responses - Reduced T cell responses - Increased IL-10 producing M2b-like macrophages	**34%** (D)	n/a
6	**IL-2/anti-IL-2 complex**	- Increased Tregs	**39%** (D) **0%** (R)	n/a **21%**
7	**Bortezomib**	- Reduced monocyte responses - Reduced T cell responses - Reduced VLDL	**58%** (D)	n/a

Table 1. Overview of results of studies presented in this thesis. All studies affected several inflammatory responses; only studies of chapters 4 and 7 (indicated in dark grey) found effects on lipid metabolism. BMT, Bone Marrow Transplantation; D, Development of atherosclerosis; P, Progression of atherosclerosis; R, Regression of atherosclerosis, n/a: not affected likely due to initial stages of atherosclerosis.

References

1. Antonopoulos, A. S., Margaritis, M., Lee, R., Channon, K. & Antoniades, C. Statins as anti-inflammatory agents in atherogenesis: molecular mechanisms and lessons from the recent clinical trials. *Curr. Pharm. Des.* 18, 1519–30 (2012).
2. Cannon, C. P. *et al.* Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N. Engl. J. Med.* 350, 1495–504 (2004).
3. Tabas, I. Macrophage death and defective inflammation resolution in atherosclerosis. Nat. Rev. Immunol. 10, 36–46 (2010).
4. Foks, A. C. *et al.* T-Cell Immunoglobulin and Mucin Domain 3 Acts as a Negative Regulator of Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 33, 2558–65 (2013).

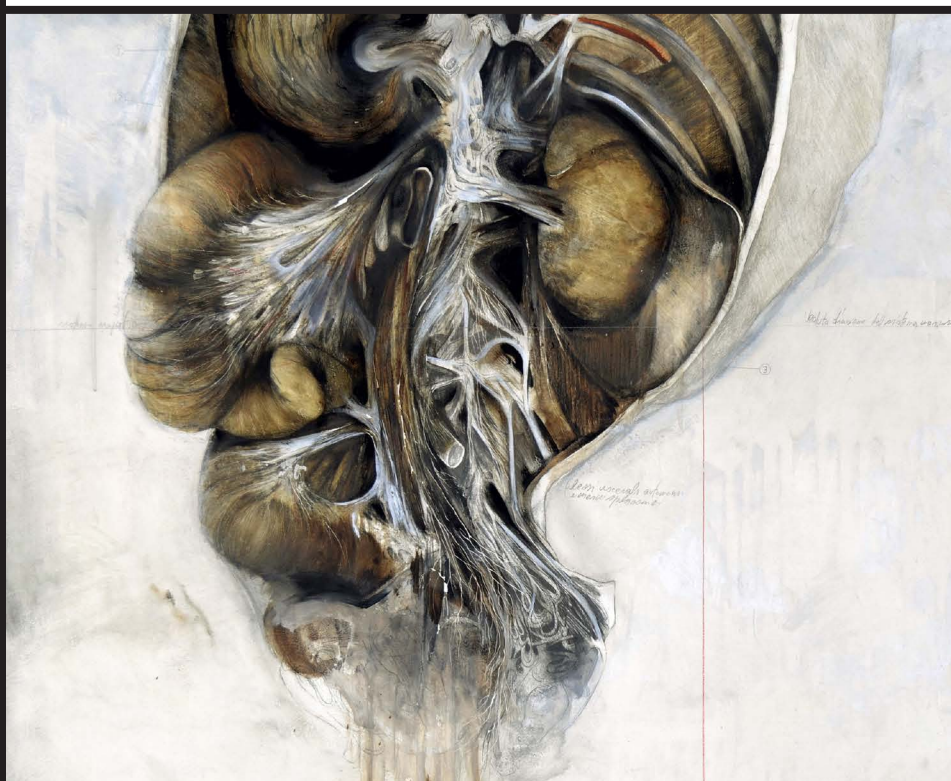
5. Elliott, M. R. & Ravichandran, K. S. Clearance of apoptotic cells: implications in health and disease. *J. Cell Biol.* 189, 1059–70 (2010).
6. Habets, K. L. L. *et al.* Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc. Res.* 85, 622–30 (2010).
7. Hosseini, H. *et al.* Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes. *Cardiovasc. Res.* (2015). doi:10.1093/cvr/cvv037
8. Shiffman, D. *et al.* Large scale gene expression analysis of cholesterol-loaded macrophages. *J. Biol. Chem.* 275, 37324–32 (2000).
9. Kiss, R. S., Elliott, M. R., Ma, Z., Marcel, Y. L. & Ravichandran, K. S. Apoptotic cells induce a phosphatidylserine-dependent homeostatic response from phagocytes. *Curr. Biol.* 16, 2252–8 (2006).
10. A-Gonzalez, N. *et al.* Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity* 31, 245–58 (2009).
11. Geyeregger, R. *et al.* Liver X receptors regulate dendritic cell phenotype and function through blocked induction of the actin-bundling protein fascin. *Blood* 109, 4288–95 (2007).
12. Chen, X., Doffek, K., Sugg, S. L. & Shilyansky, J. Phosphatidylserine regulates the maturation of human dendritic cells. *J. Immunol.* 173, 2985–94 (2004).
13. Fadok, V. A. *et al.* Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148, 2207–16 (1992).
14. Fadok, V. A. *et al.* A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 405, 85–90 (2000).
15. Harel-Adar, T. *et al.* Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. *Proc. Natl. Acad. Sci. U. S. A.* 108, 1827–32 (2011).
16. Jiang, A. *et al.* Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27, 610–24 (2007).
17. Vander Lugt, B. *et al.* TGF-β suppresses β-catenin-dependent tolerogenic activation program in dendritic cells. *PLoS One* 6, e20099 (2011).
18. Manicassamy, S. *et al.* Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* 329, 849–53 (2010).
19. Geissmann, F., Gordon, S., Hume, D. A., Mowat, A. M. & Randolph, G. J. Unravelling mononuclear phagocyte heterogeneity. *Nat. Rev. Immunol.* 10, 453–60 (2010).
20. Wu, H. *et al.* Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 119, 2708–17 (2009).
21. Schaale, K., Neumann, J., Schneider, D., Ehlers, S. & Reiling, N. Wnt signaling in macrophages: augmenting and inhibiting mycobacteria-induced inflammatory responses. *Eur. J. Cell Biol.* 90, 553–9
22. Merad, M. & Manz, M. G. Dendritic cell homeostasis. *Blood* 113, 3418–27 (2009).
23. Ahmed, M. S. & Bae, Y.-S. Dendritic cell-based therapeutic cancer vaccines: past, present and future. *Clin. Exp. Vaccine Res.* 3, 113–6 (2014).
24. Keramaris, N. C. *et al.* Endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) in bone healing. *Curr. Stem Cell Res. Ther.* 7, 293–301 (2012).
25. Glavaski-Joksimovic, A. & Bohn, M. C. Mesenchymal stem cells and neuroregeneration in Parkinson's disease. *Exp. Neurol.* 247, 25–38 (2013).
26. Wen, Z., Zheng, S., Zhou, C., Wang, J. & Wang, T. Repair mechanisms of bone marrow mesenchymal stem cells in myocardial infarction. *J. Cell. Mol. Med.* 15, 1032–43 (2011).
27. Zappia, E. *et al.* Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106, 1755–61 (2005).
28. Augello, A., Tasso, R., Negrini, S. M., Cancedda, R. & Pennesi, G. Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum.* 56, 1175–86 (2007).
29. Chen, M. *et al.* Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation. *Arthritis Rheum*. 65, 1181–93 (2013).
30. Shin, L. & Peterson, D. A. Impaired Therapeutic Capacity of Autologous Stem Cells in a Model of Type 2 Diabetes. *Stem Cells Transl. Med.* 1, 125–135 (2012).
31. Le Blanc, K. *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 371, 1579–86 (2008).
32. Bai, L. *et al*. Hepatocyte growth factor mediates mesenchymal stem cell–induced recovery in multiple sclerosis models. *Nat. Neurosci.* 15, 862–70 (2012).
33. Von Der Thüsen, J. H. *et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J.* 15, 2730–2 (2001).
34. Ruan, H. *et al.* Profiling gene transcription in vivo reveals adipose tissue as an immediate target

of tumor necrosis factor-alpha: implications for insulin resistance. *Diabetes* 51, 3176–88 (2002).
35. Benedek, I., Bucur, O. & Benedek, T. Intracoronary infusion of mononuclear bone marrow-derived stem cells is associated with a lower plaque burden after four years. *J. Atheroscler. Thromb*. 21, 217–29 (2014).
36. Lin, Y.-L., Yet, S.-F., Hsu, Y.-T., Wang, G.-J. & Hung, S.-C. Mesenchymal Stem Cells Ameliorate Atherosclerotic Lesions via Restoring Endothelial Function. *Stem Cells Transl. Med.* 4, 44–55 (2014).
37. Wang, Z. X. *et al.* Mesenchymal stem cells alleviate atherosclerosis by elevating number and function of CD4+CD25+FOXP3+ regulatory T-cells and inhibiting macrophage foam cell formation. *Mol. Cell. Biochem.* 400, 163–172 (2014).
38. Westerterp, M. *et al.* ATP-binding cassette transporters, atherosclerosis, and inflammation. *Circ. Res.* 114, 157–70 (2014).
39. Saraiva, M. & O'Garra, A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10, 170–81 (2010).
40. Frodermann, V. *et al.* A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to Staphylococcus aureus. *J. Infect. Dis.* 204, 253–62 (2011).
41. Netea, M. G. *et al.* Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. *J. Immunol.* 172, 3712–8 (2004).
42. Boonstra, A. *et al.* Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J. Immunol.* 177, 7551–8 (2006).
43. Wilck, N. *et al.* Attenuation of early atherogenesis in low-density lipoprotein receptor-deficient mice by proteasome inhibition. *Arterioscler. Thromb. Vasc. Biol.* 32, 1418–26 (2012).
44. Mestas, J. & Hughes, C. C. W. Of Mice and Not Men: Differences between Mouse and Human Immunology. *J. Immunol.* 172, 2731–2738 (2004).
45. Auffray, C., Sieweke, M. H. & Geissmann, F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu. Rev. Immunol.* 27, 669–92 (2009).
46. Hilgendorf, I. & Swirski, F. K. Making a difference: monocyte heterogeneity in cardiovascular disease. *Curr. Atheroscler. Rep.* 14, 450–9 (2012).
47. Zhang, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258, 468–71 (1992).
48. Plump, A. S. *et al.* Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71, 343–53 (1992).
49. Ishibashi, S. *et al.* Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* 92, 883–93 (1993).
50. Finkel, J. B. & Duffy, D. 2013 ACC/AHA cholesterol treatment guideline: Paradigm shifts in managing atherosclerotic cardiovascular disease risk. *Trends Cardiovasc. Med.* (2014).
51. Whitman, S. C. A practical approach to using mice in atherosclerosis research. *Clin. Biochem. Rev*. 25, 81–93 (2004).
52. Reagan-Shaw, S., Nihal, M. & Ahmad, N. Dose translation from animal to human studies revisited. *FASEB J.* 22, 659–61 (2008).
53. Takao, K. & Miyakawa, T. Genomic responses in mouse models greatly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci.* 112, 1167–72 (2014).
54. Rongvaux, A. *et al.* Development and function of human innate immune cells in a humanized mouse model. *Nat. Biotechnol.* 32, 364–72 (2014).
55. Mitchinson, N. A. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *J. Exp. Med.* 102, 157–77 (1955).
56. Kalos, M. & June, C. H. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity* 39, 49–60 (2013).
57. Giannoukakis, N., Phillips, B., Finegold, D., Harnaha, J. & Trucco, M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. Diabetes Care 34, 2026–32 (2011).
58. Hilkens, C. M. U. & Isaacs, J. D. Tolerogenic dendritic cell therapy for rheumatoid arthritis: where are we now? *Clin. Exp. Immunol.* 172, 148–157 (2013).
59. Geissler, E. K. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplant. Res.* 1, 11 (2012).
60. De Jong, R., Houtgraaf, J. H., Samiei, S., Boersma, E. & Duckers, H. J. Intracoronary stem cell infusion after acute myocardial infarction: a meta-analysis and update on clinical trials. *Circ. Cardiovasc. Interv.* 7, 156–67 (2014).
61. Lutterotti, A. *et al.* Antigen-specific tolerance by autologous myelin peptide-coupled cells: a

phase 1 trial in multiple sclerosis. *Sci. Transl. Med.* 5, 188ra75 (2013).
62. Biagi, E. *et al.* Extracorporeal photochemotherapy is accompanied by increasing levels of circulating CD4+CD25+GITR+Foxp3+CD62L+ functional regulatory T-cells in patients with graft-versus-host disease. *Transplantation* 84, 31–9 (2007).
63. Hermansson, A. *et al.* Immunotherapy with tolerogenic apolipoprotein B-100-loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice. *Circulation* 123, 1083–91 (2011).
64. Hjerpe, C., Johansson, D., Hermansson, A., Hansson, G. K. & Zhou, X. Dendritic cells pulsed with malondialdehyde modified low density lipoprotein aggravate atherosclerosis in Apoe(-/-) mice. *Atherosclerosis* 209, 436–41 (2010).
65. Ma, S. *et al.* Immunobiology of mesenchymal stem cells. Cell Death Differ. 21, 216–25 (2014).
66. English, K., Barry, F. P., Field-Corbett, C. P. & Mahon, B. P. IFN-γ and TNF-α differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol. Lett.* 110, 91–100 (2007).
67. Lee, H. J. *et al.* Mesenchymal Stem/Stromal Cells Protect against Autoimmunity via CCL2-Dependent Recruitment of Myeloid-Derived Suppressor Cells. *J. Immunol.* 1402139 (2015).
68. Saadoun, D. *et al.* Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N. Engl. J. Med.* 365, 2067–77 (2011).
69. Koreth, J. *et al.* Interleukin-2 and regulatory T cells in graft-versus-host disease. *N. Engl. J. Med.* 365, 2055–66 (2011).
70. Rosenzwajg, M. *et al.* Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. *J. Autoimmun*. (2015).
71. Hartemann, A. *et al.* Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial. lancet. *Diabetes Endocrinol.* 1, 295–305 (2013).
72. Webster, K. E. *et al.* In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J. Exp. Med.* 206, 751–60 (2009).
73. Wilson, M. S. *et al.* Suppression of murine allergic airway disease by IL-2:anti-IL-2 monoclonal antibody-induced regulatory T cells. *J. Immunol.* 181, 6942–54 (2008).
74. Dinh, T. N. *et al.* Cytokine therapy with interleukin-2/anti-interleukin-2 monoclonal antibody complexes expands CD4+CD25+Foxp3+ regulatory T cells and attenuates development and progression of atherosclerosis. *Circulation* 126, 1256–66 (2012).
75. Long, S. A. *et al.* Rapamycin/IL-2 combination therapy in patients with type 1 diabetes augments Tregs yet transiently impairs β-cell function. *Diabetes* 61, 2340–8 (2012).
76. Zhang, P. *et al.* Induced regulatory T cells promote tolerance when stabilized by rapamycin and IL-2 in vivo. *J. Immunol.* 191, 5291–303 (2013).
77. Foks, A. C., Lichtman, A. H. & Kuiper, J. Treating Atherosclerosis With Regulatory T Cells. *Arterioscler. Thromb. Vasc. Biol.* (2014).
78. Van Puijvelde, G. H. M. *et al.* Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis. Circulation 114, 1968–76 (2006).
79. Van Puijvelde, G. H. M. *et al.* Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 27, 2677–83 (2007).
80. Antonopoulos, A. S., Margaritis, M., Lee, R., Channon, K. & Antoniades, C. Statins as anti-inflammatory agents in atherogenesis: molecular mechanisms and lessons from the recent clinical trials. *Curr. Pharm. Des.* 18, 1519–30 (2012).
81. Yu, G. I. *et al.* Association of interleukin-10 promoter region polymorphisms with risk factors of Atherosclerosis. *Int. J. Immunogenet.* 42, 31–37 (2015).
82. Heiskanen, M. *et al.* Polymorphism in the IL10 promoter region and early markers of atherosclerosis: the Cardiovascular Risk in Young Finns Study. *Atherosclerosis* 208, 190–6 (2010).
83. Yoshioka, T. *et al.* Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice. *Gene Ther.* 11, 1772–1779 (2004).
84. Liu, Y. *et al.* Inhibition of atherogenesis in LDLR knockout mice by systemic delivery of adeno-associated virus type 2-hIL-10. *Atherosclerosis* 188, 19–27 (2006).
85. Pinderski Oslund, L. J. *et al.* Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler. Thromb. Vasc. Biol.* 19, 2847–53 (1999).
86. Pinderski, L. J. *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.* 90, 1064–71 (2002).
87. Han, X., Kitamoto, S., Wang, H. & Boisvert, W. A. Interleukin-10 overexpression in macrophages suppresses atherosclerosis in hyperlipidemic mice. *FASEB J.* 24, 2869–80 (2010).
88. Mallat, Z. *et al.* Protective role of interleukin-10 in atherosclerosis. Circ. Res. 85, e17–24 (1999).
89. Caligiuri, G. et al. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol. Med.* 9, 10–7 (2003).

90. Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19, 683–765 (2001).
91. Sollid, L. M. & Khosla, C. Future therapeutic options for celiac disease. Nat. Clin. Pract. Gastroenterol. *Hepatol*. 2, 140–7 (2005).
92. Freigang, S., Hörkkö, S., Miller, E., Witztum, J. L. & Palinski, W. 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 neoepitopes. *Arterioscler. Thromb. Vasc. Biol.* 18, 1972–82 (1998).
93. Palinski, W., Miller, E. & Witztum, J. L. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 92, 821–5 (1995).
94. Ameli, S. *et al.* Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. *Arterioscler. Thromb. Vasc. Biol.* 16, 1074–9 (1996).
95. Nilsson, J. *et al.* Immunization with homologous oxidized low density lipoprotein reduces neointimal formation after balloon injury in hypercholesterolemic rabbits. *J. Am. Coll. Cardiol.* 30, 1886–91 (1997).
96. Zhou, X., Caligiuri, G., Hamsten, A., Lefvert, A. K. & Hansson, G. K. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 21, 108–14 (2001).
97. Hauer, A. D. *et al.* Blockade of interleukin-12 function by protein vaccination attenuates atherosclerosis. *Circulation* 112, 1054–62 (2005).
98. Hermansson, A. *et al.* Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis. *J. Exp. Med.* 207, 1081–93 (2010).
99. Mach, F., Schönbeck, U., Sukhova, G. K., Atkinson, E. & Libby, P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature* 394, 200–3 (1998).
100. Lutgens, E. *et al.* Requirement for CD154 in the progression of atherosclerosis. *Nat. Med.* 5, 1313–6 (1999).
101. Van Wanrooij, E. J. A. *et al.* Interruption of the Tnfrsf4/Tnfsf4 (OX40/OX40L) pathway attenuates atherogenesis in low-density lipoprotein receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 27, 204–10 (2007).
102. Foks, A. C. *et al.* Interruption of the OX40-OX40 ligand pathway in LDL receptor-deficient mice causes regression of atherosclerosis. *J. Immunol.* 191, 4573–80 (2013).
103. Ridker, P. M. Testing the inflammatory hypothesis of atherothrombosis: scientific rationale for the cardiovascular inflammation reduction trial (CIRT). *J. Thromb. Haemost.* 7 Suppl 1, 332–9 (2009).
104. Ridker, P. M. Moving beyond JUPITER: will inhibiting inflammation reduce vascular event rates? *Curr. Atheroscler. Rep.* 15, 295 (2013).
105. Ridker, P. M. *et al.* Effects of interleukin-1β inhibition with canakinumab on hemoglobin A1c, lipids, C-reactive protein, interleukin-6, and fibrinogen: a phase IIb randomized, placebo-controlled trial. *Circulation* 126, 2739–48 (2012).
106. Tardif, J.-C. *et al.* Effects of the P-selectin antagonist inclacumab on myocardial damage after percutaneous coronary intervention for non-ST-segment elevation myocardial infarction: results of the SELECT-ACS trial. J. Am. Coll. Cardiol. 61, 2048–55 (2013).
107. Tardif, J.-C. *et al.* Treatment with 5-lipoxygenase inhibitor VIA-2291 (Atreleuton) in patients with recent acute coronary syndrome. *Circ. Cardiovasc. Imaging* 3, 298–307 (2010).
108. Gilbert, J. *et al.* Effect of CC chemokine receptor 2 CCR2 blockade on serum C-reactive protein in individuals at atherosclerotic risk and with a single nucleotide polymorphism of the monocyte chemoattractant protein-1 promoter region. *Am. J. Cardiol.* 107, 906–11 (2011).
109. Ewing, M. M. *et al.* T-cell co-stimulation by CD28-CD80/86 and its negative regulator CTLA-4 strongly influence accelerated atherosclerosis development. *Int. J. Cardiol.* 168, 1965–74 (2013).
110. Hansen, P. R. *et al.* Freunds adjuvant alone is antiatherogenic in apoE-deficient mice and specific immunization against TNFalpha confers no additional benefit. Atherosclerosis 158, 87–94 (2001).
111. Brånén, L. et al. Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 24, 2137–42 (2004).
112. Boesten, L. S. M. *et al.* Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice. *Cardiovasc. Res.* 66, 179–85 (2005).
113. Chung, E. S., Packer, M., Lo, K. H., Fasanmade, A. A. & Willerson, J. T. Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: results of the anti-TNF Therapy Against Congestive Heart Failure (ATTTACH) trial. *Circulation* 107, 3133–40 (2003).
114. Everett, B. M. *et al.* Rationale and design of the Cardiovascular Inflammation Reduction Trial: a test of the inflammatory hypothesis of atherothrombosis. *Am. Heart J.* 166, 199–207.e15 (2013).

Dutch Summary
Nederlandse Samenvatting



Achtergrond

Hart- en vaatziekten (HVZ) is een verzamelnaam voor alle aandoeningen die het hart en de bloedvaten betreffen. De meest voorkomende vormen van HVZ zijn coronair vaatlijden en cerebrovasculaire aandoeningen[1]. Atherosclerose of aderverkalking vormt de belangrijkste oorzaak van het ontstaan van HVZ. De grootste risicofactoren voor het ontwikkelen van hart- en vaatziekten zijn een vetrijk dieet, te weinig lichaamsbeweging, stress en overmatig alcoholgebruik en roken[1,2]. Naast deze factoren kan ook een bacteriële infectie of het lijden aan een auto-immuunziekte, zoals reumatoïde artritis, het risico op HVZ verhogen[3].

HVZ vormen de belangrijkste doodsoorzaak in geïndustrialiseerde landen en zijn elk jaar verantwoordelijk voor 46% van de sterfgevallen in de Europese Unie[4] en 36% van de sterfgevallen in de Verenigde Staten[1]. Toch is er in de afgelopen 10 jaar een aanzienlijke daling (30%) in de sterfte ten gevolge van HVZ waargenomen[1,4]. Deze daling is grotendeels te danken aan een verbeterde preventie en een doeltreffender behandeling van de complicaties van vooral acute vormen van HVZ, zoals hart- en herseninfarcten[5,6]. Helaas neemt als gevolg van deze verbeterde behandeling het aantal gevallen van hartfalen sterk toe[1]. Bovendien stijgen risicofactoren, zoals obesitas[7] en diabetes[8], sterk en dit kan in de nabije toekomst mogelijk een negatieve invloed hebben op het voorkomen van HVZ. Het is ook zorgelijk dat in de Westerse landen de sterftecijfers als gevolg van HVZ weliswaar dalen, terwijl in de rest van de wereld de HVZ sterftecijfers sterk stijgen[9].

Mede vanwege de gevolgen van een hart- of herseninfarct, hebben hart- en vaatziekten een enorme economische impact en de jaarlijkse uitgaven ten gevolge van HVZ belopen in de Europese Unie naar schatting 200 miljard euro[10] en in de Verenigde Staten 400 miljard euro[1]. Deze getallen geven duidelijk aan dat er, naast de klinische behoefte voor een betere behandeling van de patiënten, een grote behoefte is aan nieuwe en betere behandelmethoden van atherosclerose in aanvulling op de bestaande operatieve behandelingen en het gebruik van cholesterol en bloeddrukverlagende middelen.

Atherosclerose is een chronische autoimmuunachtige ziekte van de grote en middelgrote slagaders. Het proces van atherosclerose begint al in de vroege puberjaren[11] en kan een heel leven asymptomatisch blijven of kan leiden tot acute complicaties, zoals een myocardinfarct (MI) of een herseninfarct. De ziekte ontwikkelt zich door een combinatie van dyslipidemie en een ontstekingsproces, waardoor ook de atherosclerotische laesie gekenmerkt: door zowel een grote ophoping van lipiden als een chronische pro-inflammatoire respons.

De huidige behandeling van hart- en vaatziekten is voornamelijk gericht op het verlagen van het niveau van het slechte cholesterol, het LDL cholesterol, in het bloed. Het LDL gehalte is een van de belangrijkste risicofactoren voor atherosclerose en het kan door gebruik van statines effectief worden verlaagd. Statines werden voor het eerst op de markt gebracht aan het eind van de jaren 80 van de vorige eeuw en ze remmen het enzym 3-hydroxy-3-methyl-glutaryl (HMG)-COA reductase, wat het snelheidsbeperkende enzym van de mevalonaat pathway is en verantwoordelijk voor de cholesterolsynthese. Statines blijken het cardiovasculaire risico met 25-30%

te verlagen, maar patiënten die een cardiovasculaire complicatie zoals een hart- of herseninfarct hebben ondervonden, hebben ondanks statine gebruik meer dan twintig procent kans op een tweede cardiovasculaire complicatie[12]. Dit geeft aan dat er een dringende behoefte is aan nieuwe, alternatieve therapeutische strategieën.
Om dit doel te bereiken heeft men zich de laatste jaren ingespannen om een anti-inflammatoire therapie te ontwikkelen teneinde de behandeling van atherosclerose te verbeteren. Sommige van deze methodes zijn al in een tweede en derde fase van een klinische trial, maar de uiteindelijke klinische toepassing is nog niet goedgekeurd.

Dit proefschrift

Het doel van dit proefschrift was om nieuwe, immunomodulerende therapieën te ontwikkelen voor atherosclerose. Grofweg zijn er twee methodes gebruikt: ten eerste een directe cellulaire therapie en ten tweede het gebruik van geneesmiddelen en biologics om de immuunrespons zodanig te beïnvloeden dat atherosclerose vermindert. Het onderzoek is uitgevoerd in transgene muizen die doordat ze de receptor voor het slechte cholesterol, lage dichtheidslipoproteïne (LDLr), missen gevoelig zijn voor het ontwikkelen van atherosclerose. De LDLr deficiënte muizen ($LDLr^{-/-}$) muizen ontwikkelen op een dieet met een hoog vetgehalte (Western-type dieet, WTD) atherosclerose. Hoewel onze therapeutische strategieën primair gericht zijn op het verminderen van de ontstekingsreactie, hebben wij in twee studies een bijkomend gunstig effect op de cholesterol huishouding vastgesteld.

(1) Cellulaire therapie: Directe behandeling met (gemodificeerde) immuuncellen voor de behandeling van atherosclerose

Het doel van een anti-inflammatoire behandeling voor atherosclerose is het moduleren van ontstekingsreacties van zowel het aangeboren als het adaptieve deel van het immuunsysteem. Dendritische cellen (DCs) zijn de krachtigste antigen-presenterende cellen en vormen de verbinding tussen de aangeboren en adaptieve immuunrespons. Onze hypothese was dat de modulatie van DCs naar een tolerogeen fenotype leidt tot de productie van anti-inflammatoire mediatoren en de inductie van regulatoire T cellen (Treg), welke atherosclerose gunstig kunnen beïnvloeden. Wij hebben dit op twee manieren getest: (1) door toediening van apoptotische DCs, met als gevolg de inductie van tolerogene DC reacties en (2) door het verhogen van β-catenin signalering in DCs om direct een tolerogeen fenotype in de DCs te verkrijgen.
In **hoofdstuk 2** onderzochten wij het effect van de behandeling van muizen met DCs, die door behandeling met geoxideerd LDL (oxLDL) apoptotisch waren, op beginnende en bestaande atherosclerose. Het is bekend dat de klaring van apoptotische cellen ("efferocytose") bij vergevorderde atherosclerose verzwakt is[13] en eerdere studies door onder andere ons laboratorium hebben aangetoond dat door de blokkade van efferocytose atherosclerose verergerd wordt[15]. Wij laten nu zien dat de klaring van door oxLDL geïnduceerde apoptotische DCs leidt tot een respons die beschermend is voor atherosclerose. Wij laten zien dat toediening van oxLDL geïnduceerde apoptotische DCs resulteert in de inductie van tolerogene DC's, een verhoogd aantal regulatoire

T cellen, een verminderde inflammatoire respons in de monocyten en verminderde atherosclerose.
Apoptotische DCs, die werden ingespoten in LDLr$^{-/-}$ muizen in een initieel stadium of in een later stadium van atherosclerose, werden voornamelijk opgenomen door DCs en macrofagen in de marginale zone van de milt. Dit leidde tot de inductie van een tolerogeen fenotype wat de ontwikkeling van Tregs faciliteert. Daarnaast vonden wij dat het totaal aantal (inflammatoire) monocyten werd gereduceerd, een tot nu toe onbekend effect van een behandeling met apoptotische cellen. Muizen met bestaande atherosclerotische laesies vormen een meer relevante onderzoekmodel omdat de meeste patiënten in het ziekenhuis komen met bestaande, meestal vergevorderde stadia van atherosclerose. De behandeling van muizen met bestaande atherosclerotische laesies door inspuiting met apoptotische DC's verbeterde de stabiliteit van deze laesies. Ons onderzoek geeft de mogelijke klinische toepassing aan, maar benadrukt ook het belang van het onderzoek naar de emigratie van DCs vanuit de laesies naar de lymfeknopen. Dit sluit aan op eerder onderzoek van ons laboratorium waaruit bleek dat de behandeling van muizen met oxLDL-geladen DCs de start van atherosclerose vermindert[16]. Als DCs, die oxLDL opnemen of oxLDL-geïnduceerde apoptotische DCs dan wel macrofagen (beide met een atherosclerose-relevant antigen) opnemen, kunnen worden geactiveerd om te emigreren uit de atherosclerotische laesies, kunnen ze mogelijk bijdragen aan de remming van laesie ontwikkeling en aan de stabilisatie van bestaande laesies.
Hosseini *et al.* publiceerden tegelijkertijd met ons dat de intraperitoneale toediening van apoptotische thymocyten de ontwikkeling van atherosclerose in muizen verminderde[17]. Vergelijkbaar met onze studie, het aantal macrofagen en T cellen in de atherosclerotische laesies verminderd. Opvallend is dat wij vooral effecten op monocyten hebben waargenomen, terwijl Hosseini *et al.* vooral effecten op de B cel respons vond, die leidde tot een inductie van IgM-producerende B1a cellen die beschermend zijn tegen atherosclerose[17].
Een interessant aspect van onze studie is dat controle apoptotische DCs (niet geladen met oxLDL), weliswaar de expressie van ontstekingsbevorderende cytokines verminderen, maar niet het tolerogene fenotype in DCs induceren. Deze bevinding komt overeen met de studie van Hosseini *et al.* die geen invloed van apoptotische thymocyten op Tregs of IL-10 vond[17]. Wij beschrijven ook dat alleen in DCs die werden blootgesteld aan oxLDL-geïnduceerde apoptotische DCs, de expressie van PD-L1, IL-10, ABCA1 en ABCG1 werd verhoogd, wat erop duidt dat liver X receptor (LXR) signalering verhoogd was en dat is in lijn met het feit dat zowel blootstelling aan apoptotische cellen als aan cholesterol LXR activeert[18-20]. Het is al eerder aangetoond dat de LXR activering in DCs resulteert in een tolerogeen fenotype[21]. Onze hypothese is dat oxLDL-geïnduceerde apoptotische DCs atherosclerose-relevante antigenen kunnen overbrengen in de context van een anti-inflammatoire response (efferocytose) en dat het extra cholesterol aanwezig in de oxLDL-geïnduceerde apoptotische cellen een aanvullend gunstig effect heeft op atherosclerose.
Phosphatidylserine (PS) bevattende liposomen kunnen apoptotische cellen nabootsen en kunnen ook ontstekingsreacties moduleren. PS draagt zorg voor de herkenning

van apoptotische cellen door DCs en macrofagen en het remt de rijping van DCs[22-24]. De intraveneuze toediening van PS-bevattende liposomen induceert de productie van de ontstekingsremmende cytokines TGF-β en IL-10 door macrofagen en in een model voor het herstel na een hartinfarct verbeteren deze liposomen het herstel van de hartspier[25]. Deze benadering zou een eenvoudiger vertaling van onze data naar de kliniek kunnen zijn aangezien liposomen relatief eenvoudig kunnen worden geproduceerd. Hosseini *et al.* beschrijven dat PS liposomen inderdaad apoptotische cellen konden nabootsen en atherosclerose in atherosclerose gevoelige apolipoproteïne E deficiënte (apoE$^{-/-}$) muizen verminderen[17]. In een preliminaire studie met een lagere dosis PS-liposomen konden wij echter geen gunstig effect op de ontwikkeling van atherosclerose in LDLr$^{-/-}$ muizen aantonen.

Een toename in de β-catenine signalering in DCs is een andere benadering om tolerantie in DCs te induceren en wij hebben onderzocht of dit een gunstig effect heeft op de ontwikkeling van atherosclerose. Hiervoor gebruikten wij in **hoofdstuk 3** CD11c-βcat^{EX3} muizen die een door de CD11c-promotor gedreven Cre recombinase bezitten in combinatie met een gefloxt exon 3 van het β-catenine gen, met als resultaat een constitutief actieve β-catenine signalering in CD11c positieve cellen. β-catenine signalering in DCs is een belangrijke factor in het ontstaan van een tolerogeen fenotype in DCs[26-28] en wij vonden na beenmergtransplantatie van CD11c-βcat^{EX3} beenmerg in LDLr$^{-/-}$ muizen een significante toename van het aantal Tregs en een 26% vermindering van de grootte van de atherosclerotische laesies. Deze laesies hadden ook een significant kleinere necrotische kern en vertoonden een verhoogde expressie van collageen. Ook het inspuiten van CD11c-βcat^{EX3} DCs in LDLr$^{-/-}$ muizen verminderde de grootte van de atherosclerose laesie met 21%. In deze experimenten gebruikten wij de expressie van CD11c als de manier om een constitutieve expressie van ß-catenine te bewerkstelligen, CD11c is echter niet een exclusieve marker voor DCs[29], aangezien CD11c expressie ook in monocyten en macrofagen onder hypercholesterolemische condities wordt geïnduceerd[29,30]. Interessant is dat Wnt3a signalering, wat β-catenine induceert, in macrofagen ook resulteert in een anti-inflammatoire fenotype[31] en dit kan bijdragen aan de positieve effecten die wij zien na beenmergtransplantatie. Het zal natuurlijk interessant zijn om het effect van DCs met β-catenine stabilisatie op geavanceerde atherosclerotische laesies te bepalen, eventueel in combinatie met een verhoging van het aantal behandelingen met DCs, aangezien DCs een halfwaardetijd van een week hebben[32]. Deze hogere behandelingsfrequentie wordt ook aanbevolen voor Provenge, een DC-gebaseerde therapie voor prostaatkanker[33].

In de afgelopen jaren is de interesse in de mogelijkheden en voordelen van stamceltherapie sterk toegenomen en stamcellen worden dikwijls genoemd als "genezers" van diverse ziekten. Het onderzoek naar stamcellen en stamcelgebruik in de regeneratieve geneeskunde heeft een sterke ontwikkeling doorgemaakt. Mesenchymale stamcellen (MSCs) zijn specifiek gebruikt voor botvernieuwing[34], neuroregeneratie[35] en het herstel van het hart na een infarct[36]. Meer recent is het duidelijk geworden dat MSCs ook immuun regulerende capaciteiten hebben en een positief effect op ontstekingsziekten kunnen uitoefenen, zoals bij experimentele muismodellen voor multiple sclerose, reumatoïde artritis en type 2 diabetes is vastgesteld[37-40]. In klinische

studies naar Graft-versus-Host ziekte en multiple sclerose is een gunstig effect van de behandeling met MSCs vastgesteld[41,42]. In **hoofdstuk 4** hebben wij daarom MSCs uit muizen ingespoten in LDLr$^{-/-}$ muizen die gevoed werden met een cholesterol-rijk dieet en wij ontdekten dat de toediening van MSCs het plasma niveau van CCL2 en het aantal monocyten en effector CD4^{+} en CD8^{+} T cellen verlaagde. Daarnaast bleken MSCs een opvallend effect op het lipiden metabolisme te hebben; MSCs verlaagden de totale plasma cholesterol niveau door verlaging van het VLDL niveau als gevolg van een verlaagde *de novo* lipidensynthese. Het belangrijkste effect van de MSC therapie was een 33% reductie van de grootte van de atherosclerotische laesie.
Het effect van MSC therapie op het cholesterol metabolisme is nieuw, maar aangezien het effect pas vier tot vijf weken na de MSCs inspuiting ontstond, vermoeden wij dat het effect op het cholesterolmetabolisme een indirect gevolg kan zijn van een afname in de ontsteking. Dit kan afgeleid worden uit het feit dat IL-10 in staat is de VLDL synthese te verlagen[43] en de TNF-α de VLDL synthese[44] kan verhogen, wat aangeeft dat modulatie van cytokine responsen door MSCs dyslipidemie kan verbeteren. Omdat MSCs zijn onderzocht voor hun rol in het herstel van het hartweefsel na een infarct, is het relevant om ook de effecten op het cholesterolgehalte vast te stellen bij deze MSC behandelde patiënten. In een kleine patiëntengroep van 18 personen is aangetoond dat intracoronaire stamcelinfusie volgend op een hartinfarct, de grootte van de atherosclerotische plaque in de coronair vaten vermindert[45], wat erop kan duiden dat MSC behandeling nuttig is voor patiënten met atherosclerose.
Twee recente studies hebben aangetoond dat MSCs de uitkomst van atherosclerose kunnen verbeteren. Lin *et al.* laten zien dat MSC therapie de vaatverwijding verbetert door verhoging van eNOS expressie in endotheelcellen leidend tot een kleinere atherosclerotische laesie in de aorta van ApoE$^{-/-}$ muizen[46]. Een studie van Wang *et al.* beschrijft dat MSC therapie de atherosclerotische laesies in de aortawortel in ApoE$^{-/-}$ muizen verkleint door het induceren van Tregs[47]. Interessant is dat het eerste onderzoek geen effect op plasma cholesterolniveaus vindt[46], terwijl de tweede studie de cholesterol niveaus niet vermeldt. Lin *et al.* gebruikten humane MSCs en Wang *et al.* gebruikten ApoE$^{-/-}$ MSCs, terwijl wij MSCs uit wild type (C57BL/6) muizen gebruikten. Bij humane MSCs bestaat het gevaar van xenotransplantaat afstoting. Verder blijken verhoogde cholesterolwaardes in stamcellen van ApoE$^{-/-}$ knock-out muizen te leiden tot de proliferatie van hematopoïetische stamcellen en progenitor cellen[48], wat kan suggereren dat een verstoorde cholesterol homeostase in ApoE$^{-/-}$ MSCs ten opzichte van de wildtype MSCs tot andere effecten op atherosclerose kan leiden. Toekomstig onderzoek zal meer inzicht moeten verschaffen over het type MSCs met het meest gunstige effect op HVZ.

(2) Geneesmiddelen en biologics om de immuuncel functie in vivo te moduleren

Naast cellulaire therapie kunnen geneesmiddelen (small molecules) en biologics worden gebruikt om immuun cellen te moduleren of een specifieke subset van de immuun cellen te induceren.
In een eerste benadering hebben wij gebruik gemaakt van het feit dat het

immuunsysteem een intrinsiek controlemechanisme heeft om ontstekingsreacties te verminderen tijdens het opruimen van een pathogeen, dit gebeurt om het herstel van de weefselhomeostase te waarborgen. De activatie van Toll-Like Receptoren (TLRs) door pathogenen leidt dus niet alleen tot initiële ontstekingsreacties, maar in latere stadia ook tot anti-inflammatoire responsen zoals de inductie van IL-10[49]. Verschillende studies hebben gesuggereerd dat TLR2 activatie specifiek IL-10 induceert[49-51], waarbij er een fundamenteel verschil is tussen de verschillende types antigeen presenterende cellen en hun vermogen om IL-10 te produceren: macrofagen produceren hoge niveaus van IL-10 in reactie op TLR2 activatie, terwijl TLR2 activatie in DCs tot veel lagere hoeveelheden IL-10 leidt[50,52]. In **hoofdstuk 5** laten wij zien dat een *intraperitoneale* injectie met door verhitting gedode *Staphylococcus aureus* (HK-SA) een krachtige anti-inflammatoire IL-10 respons induceert. Dit leidt tot een verminderde ontsteking en daardoor wordt de ontwikkeling van atherosclerose in de aorta met 34% gereduceerd. Wij laten ook zien dat de inductie van IL-10 door HK-SA afhankelijk is van TLR2/PI3K signalering en dit leidt tot een immunoregulatoir M2b fenotype in macrofagen. De sterke IL-10-productie verminderde de expressie van adhesiemoleculen (VCAM-1 en ICAM-1) en CCL2 in de aorta, terwijl de hoeveelheid circulerende pro-inflammatoire Ly-6C^{hi} monocyten en de hoeveelheid macrofagen in de laesie drastisch verminderde. Daarenboven werden lagere Th1 en Th17 responsen waargenomen en bleken het aantal T cellen in de laesie verminderd na een behandeling met HK-SA. Onze data laten zien dat TLR2/PI3K-afhankelijke signalering een goede target kan zijn om een anti-inflammatoire IL-10 reactie in macrofagen te induceren en daardoor de monocyt/macrofaag en T cel response te verlagen, wat een behandeling voor atherosclerose mogelijk kan maken.

Er zijn verschillende strategieën beschreven die de ontsteking verminderen door immunoregulatoire of tolerogene cellen te induceren. Eén van de meest onderzochte immuuncellen in het kader van atherosclerose is de Treg. In eerdere hoofdstukken (hoofdstukken 2, 3 en 4) verhoogden onze behandelwijzen indirect het niveau van de Tregs en dit droeg bij aan het positieve effect van deze behandelingen op de ontwikkeling van atherosclerose. In deze studies werd slechts een bescheiden toename gevonden van het aantal Tregs (1.5 tot 1.8 maal) en de toename was ook niet erg langdurig. In **hoofdstuk 6** hebben wij daarom onderzocht of een significante en langdurige toename van het aantal Tregs door toediening van een IL-2/anti-IL2 complex de ontwikkeling van atherosclerose zou kunnen verminderen en regressie van bestaande laesies zou kunnen veroorzaken. De behandeling met het IL-2/anti-IL2 complex resulteerde in een sterke, 10-voudige, inductie van Tregs en een verhoogde IL-10 expressie in de milt. De sterke expansie van de Tregs verminderde de ontwikkeling van atherosclerose met 39%, wat erop wijst dat Tregs initiële atherosclerose remmen. De door het IL-2/anti-IL2 complex geïnduceerde Tregs hadden echter geen invloed op de grootte van reeds ontwikkelde laesies, maar het IL-2/anti-IL-2 complex kon wel de stabiliteit van deze laesies bevorderen door een 21% toename van de hoeveelheid collageen. Deze laatste waarneming is klinisch relevant omdat patiënten op het moment dat ze behandeld worden in de meeste gevallen bestaande laesies hebben en mogelijkerwijs kan een sterke expansie van de Tregs de laesies stabiliseren.

Macrofagen en DCs presenteren atherosclerose-relevante antigenen in samenhang met cytokine productie en deze cellen kunnen vervolgens de reactie van T cellen beïnvloeden. Voor de presentatie van antigenen op het "Major Histocompatibility Complex" (MHC) I, is het noodzakelijk dat antigenen door het proteasoom worden verwerkt. Het proteasoom speelt ook een centrale rol in inflammatoire signaleringsroutes tijdens atherosclerose, aangezien het betrokken is bij de afbraak van sleuteleiwitten in de signaleringsroutes, zoals TLR, de TNF receptor en T cel receptor signaalroutes. Maar ook signaaleiwitten en transcriptiefactoren betrokken bij het lipiden metabolisme worden afgebroken door het proteasoom. Wij hebben daarom in **hoofdstuk 7** onderzocht of remming van het proteasoom door Bortezomib zowel ontsteking in de vaatwand als dyslipidemie kan verminderen. Wij vonden dat de behandeling met Bortezomib een significante vermindering van de activiteit van monocyten en macrofagen bewerkstelligde, zoals bleek uit een lager aantal circulerende pro-inflammatoire monocyten en een lager aantal macrofagen in de aorta en in de lever. Bortezomib verminderde ook de Th1 respons en verhoogde Th2 responsen. Het opvallendste effect van Bortezomib was echter het sterke effect op het VLDL metabolisme aangezien de VLDL secretie met meer dan de helft gereduceerd werd in Bortezomib behandelde muizen, ten gevolge van een significante daling van de triglyceride opname, een daling van de *de novo* lipiden synthese en een verhoogde galzuur uitscheiding door de lever. De Bortezomib behandeling verminderde de ontsteking en de leververvetting, wat gezamenlijk bijdroeg aan een robuuste 58% reductie in laesie grootte. De effecten op monocyten waren vergelijkbaar met de effecten die beschreven zijn door Wilck *et al.*, maar zij vonden geen effecten van Bortezomib op T cellen of lipiden metabolisme[53]. Zij vonden echter wel een lagere expressie van HMG-CoA reductase in de aorta, maar geven geen data over de expressie in de lever. Waarschijnlijk is de dosis van Bortezomib die nodig is voor lipiden verlaging in het onderzoek van Wilck *et al.* niet bereikt en daarom is het interessant om te onderzoeken wat de minimale dosis Bortezomib is die nog invloed heeft op het lipiden metabolisme.

Perspectieven

Het zal interessant zijn om de resultaten van grote klinische trials zoals de CIRT en CANTOS te volgen aangezien zij voor de eerste keer op grote schaal zullen bepalen of een remming van ontstekingsreacties cardiovasculaire gebeurtenissen kan reduceren. Bovendien is het interessant om te bepalen of een combinatie van statine behandeling met nieuwe anti-inflammatoire therapieën een gunstiger uitkomst geeft voor de patiënt. Het lijkt ook waarschijnlijk dat de toekomstige cardiovasculaire behandelingsstrategie juist een gecombineerde behandeling voor het verminderen van lipiden en ontsteking zal zijn, aangezien atherosclerose wordt bepaald door zowel dyslipidemie als ontsteking. Bij het vergelijken van alle benaderingen die wij in dit proefschrift hebben gebruikt, is het duidelijk dat de strategieën die alleen ontsteking beïnvloeden de laesie grootte maximaal 40% verminderen, terwijl alleen een remming van zowel de ontsteking als het lipiden metabolisme door Bortezomib, een grotere laesie vermindering tot 58% mogelijk maakt. Daarom zullen toekomstige cardiovasculaire behandelingsstrategieën gericht zijn op het reduceren van zowel

ontsteking en dyslipidemie, hetzij door toediening van geneesmiddelen, zoals bijvoorbeeld Bortezomib, die beide processen sterk remmen of door een combinatie van therapieën.

In dit proefschrift bevestigen wij dat immuuncel-gebaseerde therapieën een sterke potentie hebben voor de behandeling van atherosclerose. Echter, een aantal problemen om experimentele behandelingen naar de kliniek te vertalen blijven bestaan en de ontwikkeling van immuuncel-gebaseerde therapieën voor atherosclerose zal, evenals voor andere ziekten, een uitdaging zijn die de moeite waard is.

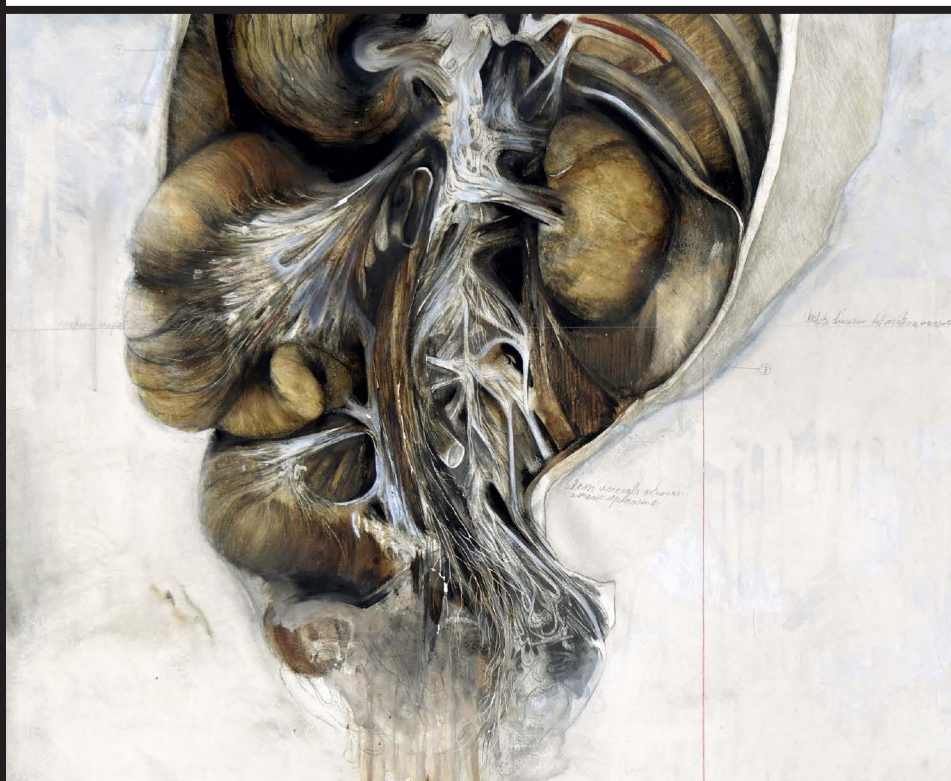
References

1. Go, A. S. *et al.* Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* 129, e28–e292 (2014).
2. Conen, D. Alcohol consumption and incident cardiovascular disease: not just one unifying hypothesis. *Eur. Heart J.* (2015).
3. Legein, B., Temmerman, L., Biessen, E. A. L. & Lutgens, E. Inflammation and immune system interactions in atherosclerosis. *Cell. Mol. Life Sci.* 70, 3847–69 (2013).
4. Nicholas, M., Townsend, N., Scarborough, P. & Rayner, M. Cardiovascular disease in Europe 2014: epidemiological update. *Eur. Heart J.* (2014).
5. Go, A. S. *et al.* Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation* 127, e6–e245 (2013).
6. Smolina, K., Wright, F. L., Rayner, M. & Goldacre, M. J. Determinants of the decline in mortality from acute myocardial infarction in England between 2002 and 2010: linked national database study. *BMJ* 344, d8059 (2012).
7. Finucane, M. M. *et al.* National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9·1 million participants. Lancet 377, 557–67 (2011).
8. Danaei, G. *et al.* National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *Lancet* 378, 31–40 (2011).
9. Finegold, J. A., Asaria, P. & Francis, D. P. Mortality from ischaemic heart disease by country, region, and age: statistics from World Health Organisation and United Nations. *Int. J. Cardiol.* 168, 934–45 (2013).
10. Leal, J., Luengo-Fernández, R., Gray, A., Petersen, S. & Rayner, M. Economic burden of cardiovascular diseases in the enlarged European Union. Eur. Heart J. 27, 1610–9 (2006).
11. Stary, H. C. Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. *Am. J. Clin. Nutr.* 72, 1297S–1306S (2000).
12. Cannon, C. P. *et al.* Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N. Engl. J. Med.* 350, 1495–504 (2004).
13. Tabas, I. Macrophage death and defective inflammation resolution in atherosclerosis. Nat. Rev. Immunol. 10, 36–46 (2010).
14. Foks, A. C. *et al.* T-Cell Immunoglobulin and Mucin Domain 3 Acts as a Negative Regulator of Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol*. 33, 2558–65 (2013).
15. Elliott, M. R. & Ravichandran, K. S. Clearance of apoptotic cells: implications in health and disease. *J. Cell Biol.* 189, 1059–70 (2010).
16. Habets, K. L. L. *et al.* Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc. Res.* 85, 622–30 (2010).
17. Hosseini, H. *et al.* Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes. *Cardiovasc. Res.* (2015). doi:10.1093/cvr/cvv037
18. Shiffman, D. *et al.* Large scale gene expression analysis of cholesterol-loaded macrophages. *J. Biol. Chem.* 275, 37324–32 (2000).
19. Kiss, R. S., Elliott, M. R., Ma, Z., Marcel, Y. L. & Ravichandran, K. S. Apoptotic cells induce a phosphatidylserine-dependent homeostatic response from phagocytes. *Curr. Biol.* 16, 2252–8 (2006).
20. A-Gonzalez, N. *et al.* Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity* 31, 245–58 (2009).
21. Geyeregger, R. *et al.* Liver X receptors regulate dendritic cell phenotype and function through blocked induction of the actin-bundling protein fascin. *Blood* 109, 4288–95 (2007).

22. Chen, X., Doffek, K., Sugg, S. L. & Shilyansky, J. Phosphatidylserine regulates the maturation of human dendritic cells. *J. Immunol.* 173, 2985–94 (2004).
23. Fadok, V. A. *et al.* Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. 148, 2207–16 (1992).
24. Fadok, V. A. *et al.* A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 405, 85–90 (2000).
25. Harel-Adar, T. *et al.* Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. *Proc. Natl. Acad. Sci. U. S. A.* 108, 1827–32 (2011).
26. Jiang, A. et al. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 27, 610–24 (2007).
27. Vander Lugt, B. *et al.* TGF-β suppresses β-catenin-dependent tolerogenic activation program in dendritic cells. *PLoS One* 6, e20099 (2011).
28. Manicassamy, S. *et al.* Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* 329, 849–53 (2010).
29. Geissmann, F., Gordon, S., Hume, D. A., Mowat, A. M. & Randolph, G. J. Unravelling mononuclear phagocyte heterogeneity. *Nat. Rev. Immunol.* 10, 453–60 (2010).
30. Wu, H. *et al.* Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 119, 2708–17 (2009).
31. Schaale, K., Neumann, J., Schneider, D., Ehlers, S. & Reiling, N. Wnt signaling in macrophages: augmenting and inhibiting mycobacteria-induced inflammatory responses. *Eur. J. Cell Biol.* 90, 553–9 (2011).
32. Merad, M. & Manz, M. G. Dendritic cell homeostasis. *Blood* 113, 3418–27 (2009).
33. Ahmed, M. S. & Bae, Y.-S. Dendritic cell-based therapeutic cancer vaccines: past, present and future. *Clin. Exp. Vaccine Res.* 3, 113–6 (2014).
34. Keramaris, N. C. *et al.* Endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) in bone healing. *Curr. Stem Cell Res.* Ther. 7, 293–301 (2012).
35. Glavaski-Joksimovic, A. & Bohn, M. C. Mesenchymal stem cells and neuroregeneration in Parkinson's disease. *Exp. Neurol.* 247, 25–38 (2013).
36. Wen, Z., Zheng, S., Zhou, C., Wang, J. & Wang, T. Repair mechanisms of bone marrow mesenchymal stem cells in myocardial infarction. *J. Cell. Mol. Med.* 15, 1032–43 (2011).
37. Zappia, E. et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106, 1755–61 (2005).
38. Augello, A., Tasso, R., Negrini, S. M., Cancedda, R. & Pennesi, G. Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum.* 56, 1175–86 (2007).
39. Chen, M. *et al.* Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation. *Arthritis Rheum.* 65, 1181–93 (2013).
40. Shin, L. & Peterson, D. A. Impaired Therapeutic Capacity of Autologous Stem Cells in a Model of Type 2 Diabetes. *Stem Cells Transl. Med.* 1, 125–135 (2012).
41. Le Blanc, K. *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 371, 1579–86 (2008).
42. Bai, L. *et al.* Hepatocyte growth factor mediates mesenchymal stem cell–induced recovery in multiple sclerosis models. *Nat. Neurosci.* 15, 862–70 (2012).
43. Von Der Thüsen, J. H. *et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB* J. 15, 2730–2 (2001).
44. Ruan, H. *et al.* Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. *Diabetes* 51, 3176–88 (2002).
45. Benedek, I., Bucur, O. & Benedek, T. Intracoronary infusion of mononuclear bone marrow-derived stem cells is associated with a lower plaque burden after four years. *J. Atheroscler. Thromb.* 21, 217–29 (2014).
46. Lin, Y.-L., Yet, S.-F., Hsu, Y.-T., Wang, G.-J. & Hung, S.-C. Mesenchymal Stem Cells Ameliorate Atherosclerotic Lesions via Restoring Endothelial Function. *Stem Cells Transl. Med.* 4, 44–55 (2014).
47. Wang, Z. X. *et al.* Mesenchymal stem cells alleviate atherosclerosis by elevating number and function of CD4+CD25+FOXP3+ regulatory T-cells and inhibiting macrophage foam cell formation. *Mol. Cell. Biochem.* 400, 163–172 (2014).
48. Westerterp, M. *et al.* ATP-binding cassette transporters, atherosclerosis, and inflammation. *Circ. Res.* 114, 157–70 (2014).
49. Saraiva, M. & O'Garra, A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10, 170–81 (2010).
50. Frodermann, V. *et al.* A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to Staphylococcus aureus. *J. Infect. Dis.* 204, 253–62

(2011).
51. Netea, M. G. *et al.* Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. *J. Immunol.* 172, 3712–8 (2004).
52. Boonstra, A. *et al.* Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J. Immunol.* 177, 7551–8 (2006).
53. Wilck, N. *et al.* Attenuation of early atherogenesis in low-density lipoprotein receptor-deficient mice by proteasome inhibition. *Arterioscler. Thromb. Vasc. Biol.* 32, 1418–26 (2012).

Curriculum Vitae & PhD Portfolio



Curriculum Vitae

Vanessa Frodermann was born on April 6, 1984 in Al Jubail, Saudi Arabia. In 2004 she received her Abitur at the German School Tokyo Yokohama, Japan. In the same year she moved to Germany and started her studies of Molecular Medicine at the University Ulm, where she received her Bachelor in 2007. At the end of her Bachelor, Vanessa decided to pursue a medical degree and started studying Medicine at the University Ulm. However, soon after she realized that she prefered medical research and immediately started her Master in Bio-Pharmaceutical Sciences at the University of Leiden, The Netherlands, in 2008. During the Master she did two major research internships. The first one was at the Division of Biopharmaceutics at the LACDR, Leiden University, The Netherlands, and involved optimizing a protocol for lentiviral transduction of antigen-presenting cells to induce overexpression of suppressors of cytokine signaling. For her Master thesis on this subject Vanessa received the KNMP Student Prize for best Master thesis in 2009. The second internship was at the Madrenas Laboratory, Robarts Research Institute, University of Western Ontario, London, Ontario, Canada, and was financed by the Leiden University Fund Travel Grant, an Outbound Study Grant of the Leiden University and the KNMP Stipendiafonds. During this internship she studied the effect of *Staphylococcus aureus* cell wall components on human innate immune cells and effects on ensuing adaptive immune responses, which resulted in a publication in the Journal of Infectious Diseases. In the summer break of her Master studies Vanessa worked as a student assistant at the Division of Biopharmaceutics. In 2010 Vanessa graduated *cum laude* and returned to the Division of Biopharmaceutics to start her PhD.

The PhD studies were performed under supervision of Prof. Dr. Johan Kuiper and Dr. Saskia C.A. de Jager and were financed by a grant of the Dutch Heart Foundation. During her PhD studies, Vanessa received a travel award from the Federation of Clinical Immunology Societies in 2010 and the Young Investigator Fellowship of the European Atherosclerosis Society in 2015. Moreover, her congress attendances were made possible by the 'dr. W. Stiggelbout-programma' of the Dutch Heart Foundation. In 2012 she received a poster award at the LACDR Spring Symposium and in 2015 she received a Best Young Investigator Oral Presentation Award on the 83rd European Atherosclerosis Society Congress.

After the PhD, Vanessa will start as a postdoctoral fellow in the laboratory of Matthias Nahrendorf at the Massachusetts General Hospital, Harvard Medical School, Boston, USA.

PhD Portfolio

Courses and Workshops

2014 Writing an Excellent Research Grant Proposal
2014 Radiation Protection Course 5B
2012 APCs revisited: The function of APCs in health and disease
2011 Communication in Science
2011 Time for high T: Features and functions of T cells in health and disease
2010 Time Managment Course
2010 LIFI Advanced Course in Immunology
2010 Introductory Course on Drug Research
2009 Flow Cytometry Training
2008 Proefdierkunde (Laboratory Animal Science Course)
2008 Genetically Modified Oragnisms/ Virus Training

Presentations at (Inter)National Conferences and Invited Lectures

2015 83rd European Atherosclerosis Society, Glasgow, UK
2014 LACDR Spring Symposium, Leiden, The Netherlands
2014 Vascular Research Division Seminar Series, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
2014 Leiden Vascular Medicine Meeting, Leiden, The Netherlands
2013 3rd Cardio Vascular Conference, Noordwijkerhout, The Netherlands
2013 Leiden Vascular Medicine Meeting, Leiden, The Netherlands
2013 NVVI Meeting, Noordwijkerhout, The Netherlands
2012 Leiden Vascular Medicine Meeting, Leiden, The Netherlands
2010 Federation of Clinical Immunology Societies Meeting, Boston, MA, USA

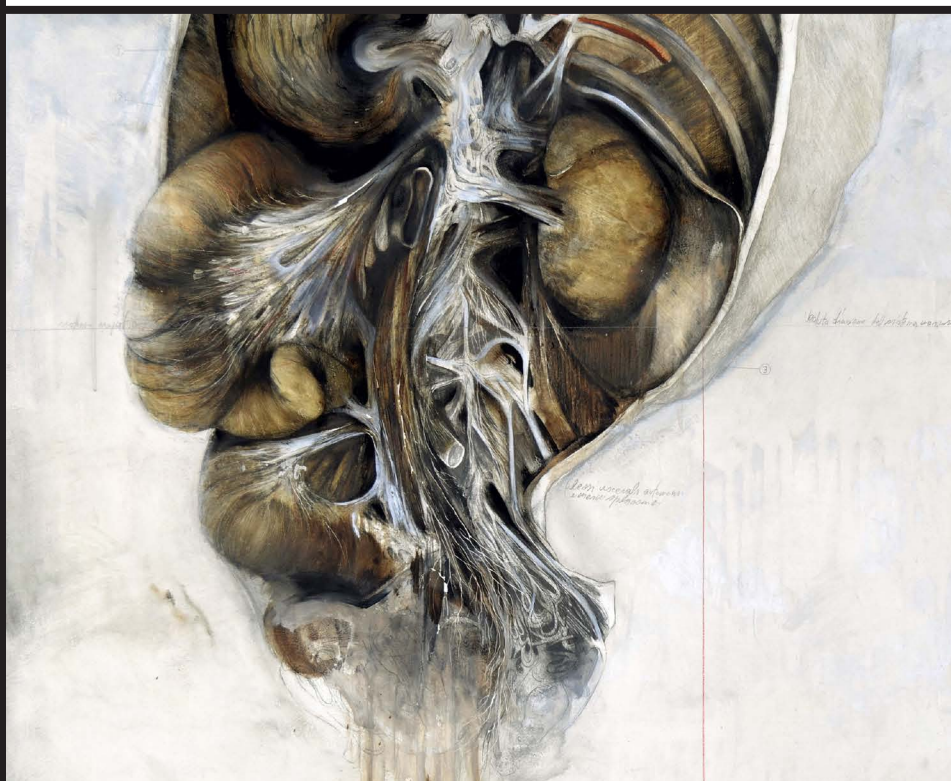
(Inter)National Poster Presentations

2015 83rd European Atherosclerosis Society, Glasgow, Scotland
2014 The 5th Rembrandt Symposium, Noordwijkerhout, The Netherlands
2014 ATVB Conference, Toronto, Canada
2013 The 4th Rembrandt Symposium, Noordwijkerhout, The Netherlands
2013 Gordon Research Conference Atherosclerosis, Stowe, VT, USA
2013 27th Annual European Macrophage Dendritic Cell Society Meeting, Erlangen, Germany
2013 LACDR Spring Symposium, Leiden, The Netherlands
2012 The 3rd Rembrandt Symposium, Noordwijkerhout, The Netherlands
2012 NVVI Meeting, Noordwijkerhout, The Netherlands
2012 3rd European Congress of Immunology, Glasgow, Scotland
2012 Inflammation and Atherosclerosis, Munich, Germany
2012 LACDR Spring Symposium, Amsterdam, The Netherlands
2011 2nd Cardio Vascular Conference, Noordwijkerhout, The Netherlands
2011 NVVI Meeting, Noordwijkerhout, The Netherlands
2011 Gordon Research Conference Atherosclerosis, Newport, RI, USA
2011 The 2nd Rembrandt Symposium, Amsterdam, The Netherlands
2011 LACDR Spring Symposium, Amsterdam, The Netherlands
2010 NVVI Meeting, Noordwijkerhout, The Netherlands
2010 The 1st Rembrandt Symposium, Leiden, The Netherlands
2010 British Atherosclerosis Society Autumn Meeting, Oxford, UK

Awards

2015 Best Young Investigator Oral Presentation Award, 83rd European Atherosclerosis Society Congress
2015 Young Investigator Fellowship European Atherosclerosis Society
2012 Poster Award, LACDR Spring Symposium
2010 Travel Award Federation of Clinical Immunology Societies Meeting
2010 Dutch Heart Foundation PhD Research Grant
2009 KNMP Stipendiafonds
2009 Leiden University Fund Travel Grant
2009 Outbound Study Grant of the Leiden University
2009 KNMP Student Prize

Publications



Publications

Full Papers

Frodermann V, van Duijn J, van Puijvelde GHM, Lagraauw HM, van Santbrink PJ, de Vries M, Quax PHA, Bot I, Foks AC, de Jager SCA, Kuiper J. Modulation of Macrophages in Atherosclerosis by Heat-Killed *S. aureus*– Identification of a Novel Anti-Atherogenic Function for TLR2/PI3K-Signaling. *Submitted for publication.*

Frodermann V, van Duijn J, van Pel M, van Santbrink PJ, Bot I, Kuiper J, de Jager SCA. Mesenchymal Stem Cells Reduce Murine Atherosclerosis Development. *Submitted for publication.*

Frodermann V, van Puijvelde GHM, Hoekstra M, de Bruin G, van Duijn J, van der Heijden T, Kröner MJ, van Santbrink PJ, Bot I, Florea BI, de Jager SCA, Kuiper J. Bortezomib – a Novel Lipid-lowering Drug to Prevent Atherosclerosis. *Manuscript in preparation.*

Frodermann V, Ober-Blöbaum J, Foks A, van Puijvelde GHM, Wierts L, van Santbrink PJ, ter Borg MND, Clausen B, Kuiper J, de Jager SCA. β-Catenin Signaling in Dendritic Cells Reduces Atherosclerosis. *Manuscript in preparation.*

Frodermann V, van Puijvelde GH, Wierts L, Lagraauw HM, Foks AC, van Santbrink PJ, Bot I, Kuiper J, de Jager SC. Oxidized Low-Density Lipoprotein-Induced Apoptotic Dendritic Cells as a Novel Therapy for Atherosclerosis. *J Immunol.* 2015 Mar 1;194(5):2208-18.

Foks AC, Ran IA, Frodermann V, Bot I, van Santbrink PJ, Kuiper J, van Puijvelde GH. Agonistic Anti-TIGIT Treatment Inhibits T Cell Responses in LDLr Deficient Mice without Affecting Atherosclerotic Lesion Development. *PLoS One.* 2013 Dec 20;8(12):e83134.

Foks AC, Ran IA, Wasserman L, Frodermann V, Ter Borg MN, de Jager SC, van Santbrink PJ, Yagita H, Akiba H, Bot I, Kuiper J, van Puijvelde GH. T-cell immunoglobulin and mucin domain 3 acts as a negative regulator of atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2013 Nov;33(11):2558-65.

Hoekstra M, Frodermann V, van den Aardweg T, van der Sluis RJ, Kuiper J. Leukocytosis and enhanced susceptibility to endotoxemia but not atherosclerosis in adrenalectomized APOE knockout mice. *PLoS One.* 2013 Nov 12;8(11):e80441.

Foks AC, Bot I, Frodermann V, de Jager SC, Ter Borg M, van Santbrink PJ, Yagita H, Kuiper J, van Puijvelde GH. Interference of the CD30-CD30L pathway reduces atherosclerosis development. *Arterioscler Thromb Vasc Biol.* 2012 Dec;32(12):2862-8.

Foks AC, Frodermann V, ter Borg M, Habets KLL, Bot I, Zhao Y, van Eck M, van Berkel TJ, Kuiper J, van Puijvelde GH. Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis.* 2011 Sep;218(1):53-60.

Frodermann V, Chau TA, Sayedyahossein S, Toth JM, Heinrichs DE, Madrenas J. A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to Staphylococcus aureus. *J Infect Dis.* 2011 Jul 15;204(2):253-62.

Published Abstracts

Frodermann V, van Puijvelde GHM, Wierts L, Lagraauw HM, van Santbrink PJ, Bot I, Kuiper J, de Jager SCA. OxLDL-induced apoptotic dendritic cells as a novel therapy for atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2014;34:A372.

Foks AC, van Puijvelde GHM, Frodermann V, van der Heijden T, Bot I, Kuiper J. Regulation of atherosclerosis by myeloid-derived suppressor cells. *Arterioscler Thromb Vasc Biol.* 2014;34:A543.

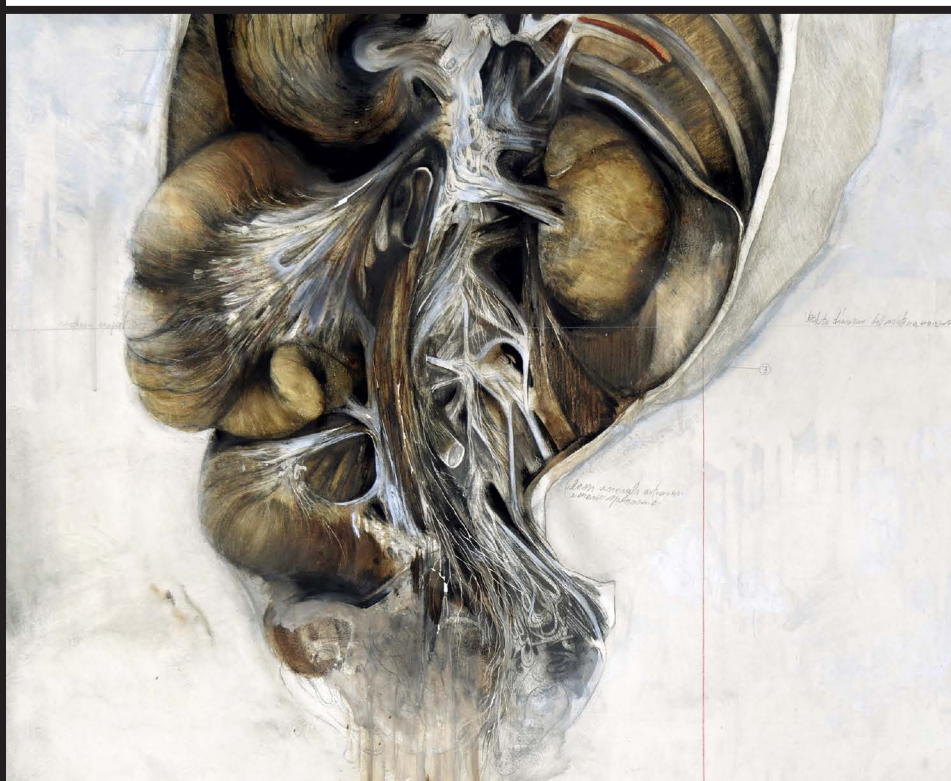
Foks AC, Bot I, Frodermann V, de Jager SCA, ter Borg MND, van Santbrink PJ, Yagita H, Kuiper J, van Puijvelde GHM. Interference of the CD30-CD30L pathway reduces atherosclerosis development. *Immunology*. 2012;137(SI1):478.

Habets KLL, Frodermann V, Foks A.C, van Puijvelde GHM, Toes REM, van Berkel TJC, Kuiper J. Foam cell formation affects MHCI processing and presentation in vitro while hypercholesteremia induces a DC-like phenotype in macrophages in vivo. *Atherosclerosis* 11/2010; 213(1):e18.

Sayedyahossein S, Chau T, Frodermann V, Toth J, Madrenas J. Plasticity of TLR2-mediated Regulation of Adaptive T Cell Responses: Learning from Staphylococcus aureus. *Clinical Immunology* 01/2010; 135.

Madrenas J, Toth JM, Hewson G, Frodermann V, Chau L. Modulation of human immune responses by ginseng extracts. *J Immunol.* 2010, 184, 89.56.

Index of Abbreviations



Abbreviations

ABCA1	ATP-Binding Cassette transporter A1
ABCG1	ATP-Binding Casette transporter G1
ACAT	Acyl-CoA cholesterol ester Transferase
AP-1	Activating Protein-1
APC	Antigen-Presenting Cell
ApoA-I	Apolipoprotein A-I
ApoB-100	Apolipoprotein B100
ApoE	Apolipoprotein E
$apop^{ctrl}$-DCs	Unpulsed control apoptotic dendritic cells
$apop^{ox}$-DCs	OxLDL-induced apoptotic dendritic cells
BM	Bone Marrow
BMT	Bone Marrow Transplantation
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcomes Study
CCL	CC chemokine Ligand
CCR	CC chemokine Receptor
CD	Cluster of Differentiation
cDC	conventional Dendritic Cell
CD11c-βcatEX3 DCs	DCs with constitutive β-catenin signaling
CFSE	CarboxyFluorescein diacetate Succinimidyl Ester
CIRT	Cardiovascular Inflammation Reduction Trial
CLR	C-type lecin receptor
CMP	common myeloid progenitor
CRP	C-Reactive Protein
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
CVD	CardioVascular Disease
CX3CL	CX3C chemokine Ligand
CX3CR	CX3C chemokine Receptor
CXCL	CXC chemokine Ligand
CXCR	CXC chemokine Receptor
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic Cell
DGAT	DiGlyceride AcylTransferase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribo Nucleic Acid
DTR	Diphtheria Toxin Receptor
EC	Endothelial Cell
ELISA	Enzyme-Linked ImmunoSorbent Assay
eNOS	Endothelial cell Nitric Oxide Synthase
ERK	Extracellular signal-Regulated Kinase
FACS	Fluorescence Activated Cell Sorting
FAS	Fatty Acid Synthase
FDA	United States Food and Drug Administration
FITC	Fluorescein IsoThioCyanate
Foxp3	Forkhead box p3
GAPDH	GlycerAldehyde-3-Phosphate DeHydrogenase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HDL	High-Density Lipoprotein
HIV	Human Immunodeficiency virus
HK-SA	Heat-Killed *Staphylococcus aureus*
HMG	3-Hydroxy-3-Methyl-Glutaryl
hLN	Draining Lymph Node of the heart
HO-1	Heme Oxygenase-1
HPRT	Hypoxanthine PhosphoRibosyl Transferase

HSP	Heat shock protein
HSPC	Hematopoietic Stem and Progenitor Cells
ICAM-1	IntraCellular Adhesion Molecule-1
IDO	Indoleamine-2,3-DiOxygenase
IFN-γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
i.p.	Intraperitoneal
iPS	induced Pluripotent Stem cells
i.v.	Intravenous
LCAT	Lecithin Cholesterol AcylTransferase
LDL	Low-Density Lipoprotein
LDLr	Low-Density Lipoprotein receptor
LN	lymph node
LPL	LipoProtein Lipase
LPS	LipoPolySaccharide
LRP1	Low density lipoprotein receptor-related protein 1
LXR	Liver X Receptor
MAL	MyD88 adaptor-like protein
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony Stimulating Factor
MDA-LDL	Malondialdehyde-modified Low-Density Lipoprotein
MDP	macrophage DC progenitor
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
miRNA	micro-RiboNucleic Acid
MMP	Matrix MetalloProteinase
moDC	monocyte-derived Dendritic Cell
Mreg	Regulatory Macrophages
mRNA	messenger RiboNucleic Acid
MS	Multiple Sclerosis
MSC	Mesenchymal Stem Cell
MTTP	Microsomal Triglyceride Transfer Protein
MyD88	Myeloid Differentiation primary-response protein 88
NADPH	Nicotinamide Adenine Dinucleotide PHosphate
NK(T)	Natural Killer (T) Cells
oxLDL	Oxidized low-density lipoprotein
OxPAPC	oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine
p53	Protein 53
PAMP	Pathogen-Associated Molecular Pattern
pDC	plasmacytoid Dendritic Cell
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll-A Protein
PD-L1	Programmed Death-Ligand 1
PI	Propidium Iodide
PI3K	Phosphoinositide 3-Kinase
PPARγ	Peroxisome Proliferator-Activated Receptor γ
PRR	Pattern Recognition Receptor
PS	PhosphatidylSerine
qPCR	quantitative Polymerase Chain Reaction
respDC	responder DC
RNA	RiboNucleic Acid
Rpl27	60S ribosomal protein L27
RXR	Retinoid X Receptor

Scd1	Stearoyl-CoA Desaturase-1
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SDF-1	Stromal cell Derived Factor-1
SEM	Standard Error of the Mean
siRNA	Small interfering RiboNucleic Acid
SMC	Smooth Muscle Cell
SNP	Single Nucleotide Polymorphisms
SOCS	Suppressor Of Cytokine Signaling
SR-A	Scavenger Receptor A
SR-BI	Scavenger Receptor BI
SREBP-1c	Sterol Regulatory Element-Binding Protein 1
STAT	Signal Transducer and Activator of Transcription
TAM	Tumor-Associated Macrophages
TCR	T Cell Receptor
TG	TriGlycerides
TGF-β	Transforming Growth Factor-β
Th	T helper
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor-α
Treg	regulatory T cell
TRAIL	Tumor necrosis factor-Related Apoptosis-Inducing Ligand
TRIF	TIR domain-containing adaptor protein inducing IFNβ
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoprotein
VSMC	Vascular Smooth Muscle Cell
WT	Wild Type
WTD	Western-type diet