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Regulation of immune responses in atherosclerosis

Foks, A.C.

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Amanda C. Foks

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Regulation of immune responses in atherosclerosis

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Chapter 1

General Introduction

Atherosclerosis

Acute cardiovascular syndromes are a major cause of death in Western society and are generally triggered by rupture of an atherosclerotic plaque.[1] In 2008, 17.3 million people died of the consequences of cardiovascular disease, accounting for 30% of all reported deaths worldwide.[2] Atherosclerosis is considered a chronic autoimmune-like disease related to high plasma cholesterol levels resulting in endothelial damage, subsequent vascular dysfunction, and cholesterol accumulation in the arterial wall.[3] Atherosclerosis mostly occurs in the medium and large sized arteries and is a slowly progressing disorder that already starts during early adolescence.[4, 5] Depending on exposure to risk factors, atherosclerotic lesions can grow event-free until the manifestation of acute thrombotic complications and subsequent clinical events, such as stroke and acute myocardial infarction (Figure 1). Risk factors include high calorie intake, hypertension, smoking, stress and physical inactivity and diseases such as obesity, diabetes and dyslipidemia correlate with the incidence of cardiovascular diseases as well.[3, 6] For many years it was believed that atherosclerosis was a lipid disorder that resulted in cholesterol accumulation within the arterial wall. However, the mechanism underlying the pathological process of atherosclerosis is much more complicated and the involvement of inflammatory cells in the development and progression of atherosclerosis was suggested after the discovery of T cells in atherosclerotic lesions in the 1980s.[7] Patients suffering from cardiovascular disease are currently treated with statins to lower low-density lipoprotein (LDL) cholesterol. However, these drugs only lead to a risk reduction of 30% in cardiovascular patients, indicating an urgent need for new therapeutic strategies to inhibit atherosclerosis and to prevent cardiovascular complications.

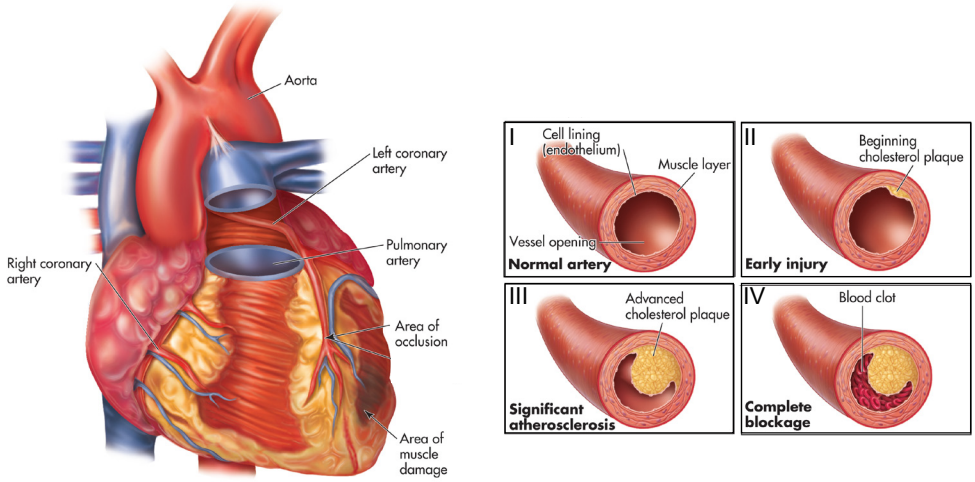


Figure 1. Atherosclerosis develops already during early adolescence, where a normal artery (I) becomes susceptible for endothelial damage, which promotes the formation of a 'fatty streak' (II). After this initial stage the lesions can develop into stable or vulnerable lesions (III). The vulnerable lesion can rupture and cause thrombus formation (IV) eventually resulting in an acute myocardial infarction and subsequent death. *Adapted and modified from The McGraw-Hill Companies, Inc.*

The development of atherosclerotic lesions

Initial lesion development

As the major regulator of vascular homeostasis, the endothelium maintains the balance between vasodilation and vasoconstriction and inhibition and stimulation of smooth muscle cell proliferation (Figure 2A).[8] When this balance is disturbed, endothelial dysfunction occurs, causing damage to the arterial wall. Endothelial damage can be induced by turbulent or oscillatory shear stress in combination with the presence of atherogenic factors such as high cholesterol levels, smoking, and hypertension. These factors enhance the permeability of the endothelial cell layer for lipoproteins and increase the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and E- and P-selectin on the surface of endothelial cells.[9, 10] Endothelial dysfunction is considered an early marker for atherosclerosis. Both the increased permeability of the endothelial cell layer and the expression of adhesion molecules correspond with the location where a lesion is formed.[11] The increased permeability causes lipoproteins, and especially LDL cholesterol particles, to migrate through the endothelial layer and to accumulate in the intima, the innermost layer of the artery.[12] LDL, also known as 'bad cholesterol', is together with 'good cholesterol', high-density lipoprotein (HDL), the most important lipoprotein in atherosclerosis.[13] Whereas HDL is anti-atherogenic because it mediates cholesterol efflux from the periphery towards the liver, modified LDL is pro-atherogenic. LDL transports the circulating cholesterol and consists of triglycerides surrounded by a shell of phospholipids and apolipoproteins. The accumulation of LDL particles within the intima is an important initiating factor in early atherosclerosis. Within the intima, these particles are prone to oxidative modifications, which lead to alterations in charge, particle size and lipid content.[14] Oxidized LDL (oxLDL), in turn, initiates innate inflammatory immune responses by activating the endothelial cells to upregulate adhesion molecules.[15]

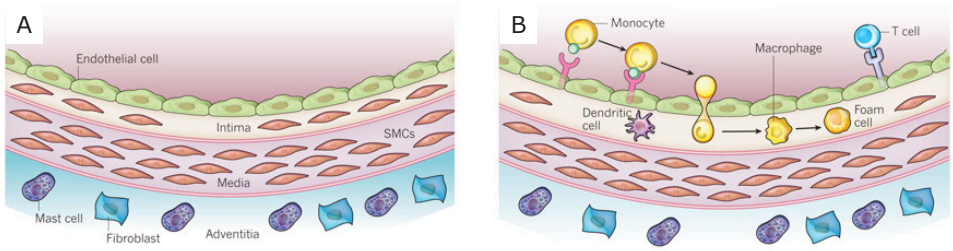


Figure 2. The normal artery consisting of the intima, media and adventitia (A) becomes damaged when the endothelial cell layer is impaired. Monocytes can enter the arterial wall and become foam cells, which accumulate and form a 'fatty streak' (B). *Adapted and modified from Libby et al. Nature 2011;473:317.*

Simultaneously with upregulation of adhesion molecules, the endothelial cells start to produce chemokines, growth factors and vasoactive molecules, which results in the recruitment of monocytes to the site of injury.[16] Monocytes attach to E- and P-selectin on the endothelium via capture and rolling.[17] Firm adhesion of the monocytes is

mediated via intracellular adhesion molecule–1 (ICAM-1) and VCAM-1.[18-20] The monocytes spread and then migrate through the endothelial layer in a process called diapedesis. The monocytes enter the subendothelial layer with help of the chemokine receptor-2 (CCR2) and the monocyte chemoattractant protein-1 (MCP-1). CCR2 is expressed on monocytes and binds to MCP-1, which is a chemoattractant released by macrophages, smooth muscle cells and other cells in response to inflammatory signals.[21] Once migrated into the subendothelial layer, the monocytes differentiate into macrophages, which subsequently engulf oxidized LDL via scavenger receptors and form lipid-rich foam cells, characteristic for the atherosclerotic lesion.[14, 22, 23] These foam cells induce inflammation via cell-cell contact and secrete inflammatory mediators, which results in the activation and recruitment of more inflammatory cells such as T cells. This process leads to the formation of a yellowish, 'fatty streak', which does not cause any clinical symptoms (Figure 2B).

Lesion progression

T cells, as well as endothelial cells, secrete cytokines and growth factors that promote the migration and proliferation of smooth muscle cells (SMCs).[3] The progression of fatty streaks into more complex lesions involves the migration of SMCs from the media into the intima. The SMCs start to cover the fatty streak and secrete extracellular matrix proteins, which results in the formation of a fibrous cap covering a necrotic core. This necrotic core contains apoptotic cells, lipid deposition, and increased proteolytic activity. When the lesion size increases by further accumulation of foam cells and the expansion of the necrotic core, the arterial wall changes in composition, eventually leading to narrowing of the artery.[24] The lesion is now called an advanced atherosclerotic lesion (Figure 3A).

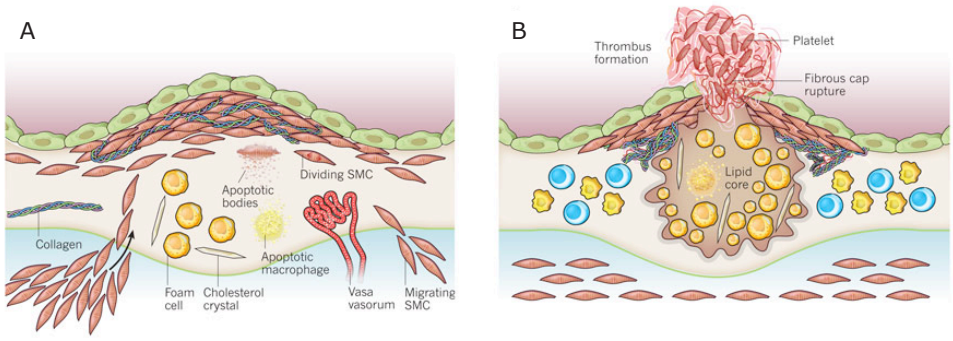


Figure 3. The progression of a fatty streak into more complex lesions involves the migration of smooth muscle cells from the media into the intima, forming a fibrous cap that covers a necrotic core (A). Lesions can develop into stable or vulnerable lesions. The vulnerable lesion (B) can rupture and cause thrombus formation eventually resulting in an acute myocardial infarction and subsequent death. *Adapted and modified from Libby et al. Nature 2011;473:317.*

Unstable lesions

Lesions that are prone to rupture, generally have a high content of lipid and necrotic

debris, a thin fibrous cap containing a low content of SMCs and collagen, and activated macrophages in the shoulder regions where rupture most often occurs.[25, 26] Thinning of the fibrous cap is caused by increased activity of matrix metalloproteinases (MMPs), which can digest collagen.[27] The production of these matrix degrading proteins can be increased by several components, such as oxidized lipids, inflammatory cytokines, heat shock proteins and hemodynamic stress. Additionally, unstable lesions contain a high content of tissue factor, which is a procoagulant and promotes thrombus formation. Upon rupture of the fibrous cap, the thrombogenic, lipid-rich core is exposed to the blood (Figure 3B), which can lead to thrombus formation and subsequent occlusion of the blood vessel, causing 75% of all myocardial infarctions.[28] Besides rupture of the fibrous cap, thrombus formation can also be initiated via erosion of the endothelial monolayer.[1] This results in exposure of collagen and von Willebrand factor to the blood, which promotes platelet adhesion and activation and eventually thrombus formation with subsequent myocardial infarction.[29] Furthermore, outward and inward arterial remodeling also play an important role in lesion stability and the occurrence of clinical symptoms.[30-32] When lesions expand outward, which means into the vessel wall, patients have a much higher risk to develop unstable angina. In contrast, inward remodeling, which means the lesion reduces the diameter of the lumen, is more common in stable angina.

Biological age of atherosclerotic lesions

Recently, using ^{14}C determination by mass spectrometry released into the atmosphere during the nuclear weapons tests in the 1950-60s, Gonçalves et al. showed that the biological age of human atherosclerotic lesion components varies between 5 and 15 years of age, with the fibrous cap being the youngest.[33] Although the lesion in it self is probably older due to the replacement of original lesion components, this study indicates that the turnover time of human atherosclerotic lesions is very slow and may explain the difficulties encountered when developing therapies to induce regression of atherosclerosis in cardiovascular intervention trials.

The immune system in atherosclerosis

Besides lipid accumulation and matrix degradation, inflammation is considered a key process in atherosclerotic plaque development and specifically in the pathogenesis of plaque rupture.[6, 34] Atherosclerosis involves both innate and adaptive immune responses. The innate immunity is the initial barrier against infections and several immune cells such as monocytes, macrophages, dendritic cells (DCs), neutrophils, natural killer (NK) cells and mast cells are programmed to detect foreign molecules of exogenous (eg lipopolysaccharide (LPS)) or endogenous (eg oxLDL and heat shock protein (HSP) 60) origin.[35] These foreign molecules are called pathogen-associated molecular patterns (PAMPs) and are recognized by the innate immune cells via pattern recognition receptors, such as Toll-Like Receptors (TLRs) and scavenger receptors (SRs).[36] The pathogens are internalized and degraded by phagocytosis.

Adaptive immune reactions are initiated when antigen-presenting cells (APCs), such as macrophages and DCs, display a surface complex consisting of an antigenic peptide bound to a major histocompatibility complex protein class I or class II (MHC I or MHC II) to a T or B cell. This leads to cytokine secretion, cytotoxicity, antibody production, memory formation and stimulation of a wide range of other components of an immune reaction.[34] A significant number of cell types is involved in the immune response during the initiation and progression of atherosclerosis (Figure 4) and studies in mice and human have revealed their roles in the pathogenesis of atherosclerosis.

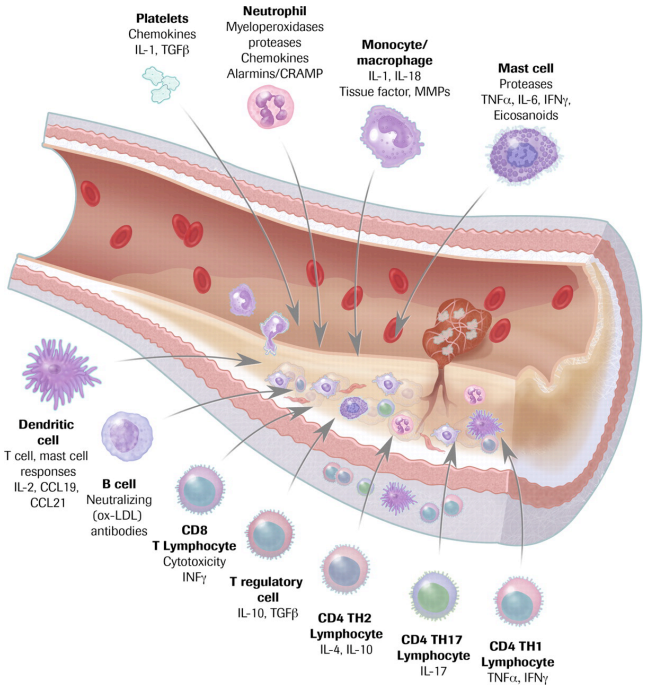


Figure 4. Atherosclerosis is considered a chronic autoimmune-like disease in which both innate and adaptive immunity play an important role. Endothelial injury results in the entry of modified LDL particles and monocytes into the arterial wall. This activates macrophages and T cells that produce cytokines such as IFN-γ that further enhance the immune response. Neutrophils and mast cells are also recruited to the site of inflammation and release their granules containing more pro-inflammatory compounds. Moreover, protective mechanisms such as B cells that secrete neutralizing antibodies and regulatory T cells that secrete IL-10 and TGF-β are active. *Adapted from Simon et al. Circ. Res. 2012;110:1036.*

To investigate the pathogenesis of atherosclerosis in mice, two mouse models, the LDL receptor deficient ($LDLr^{-/-}$) mice and apolipoprotein E ($ApoE^{-/-}$) mice, are widely used. $LDLr^{-/-}$ mice lack the LDL receptor, which impairs VLDL and LDL removal from the circulation, resulting in elevated serum cholesterol levels. Upon Western-type diet feeding $LDLr^{-/-}$ mice show strongly enhanced cholesterol levels and rapidly develop atherosclerosis. $ApoE^{-/-}$ mice do not have ApoE, the ligand for binding to the LDL receptor and lipoprotein receptor-related protein (LRP), which results in severe hypercholesterolemia even without a high-fat diet.

Monocytes

Monocytes play a crucial role in the initiation of the atherosclerotic lesion by differentiating into macrophages that engulf modified LDL and subsequently form foam cells after excessive accumulation of lipids in the arterial wall. Monocytes are recruited by inflammatory adhesion molecules and migrate into the intima as described above. Deficiencies in chemokine signaling pathways mediating this process, such as MCP-1 or its receptor CCR2, provides dramatic protection from monocyte recruitment and atherosclerotic lesion formation.[37, 38] Moreover, during atherosclerotic lesion development, monocyte accumulation correlates to the lesion size.[39] In mice two subsets of monocytes with distinct patterns of surface markers and behaviors during inflammation have been described; $CD11b^{+}Ly6C^{high}$ monocytes and $CD11b^{+}Ly6C^{low}$ monocytes. Whereas the $CD11b^{+}Ly6C^{high}$ monocyte subset infiltrates the intima via CCR2, CCR5, CXCR1 and CX3CR1 to eventually form foam cells and thus promotes inflammation[40], the $CD11b^{+}Ly6C^{low}$ monocyte subset enters the atherosclerotic lesion less frequently.[41] Depletion of monocytes in rabbits by using clodronate liposomes reduced atherosclerotic lesion formation.[42] Furthermore, $ApoE^{-/-}$ mice with individual or combined deficiencies in the chemokine receptors CCR2, CCR5 and CX3CR1 showed dramatic reductions in infiltrating monocytes and lesion development.[43-45]

Macrophages

Macrophages have an essential role in all phases of atherosclerosis, from development of the fatty streak to processes that eventually contribute to plaque rupture and myocardial infarction.[24] Macrophages express several scavenger receptors that are capable of taking up oxidized LDL, such as SR-A and CD36.[46, 47] OxLDL taken up by macrophages is delivered to lysosomes, where its cholesterol ester content is hydrolyzed to free cholesterol and fatty acids.[24] Subsequently, peptide fragments are presented on MHC class I/II molecules and lipid antigens are presented on CD1 molecules.[48] T cells that carry the appropriate T cell receptors (TCRs) are activated by binding to the MHC I/II peptide complex or CD1-lipid complex in the presence of costimulatory factors such as CD40 and CD80/CD86 molecules.

The important role of macrophages in atherosclerosis was first demonstrated by Smith et al. who showed that $M\text{-}CSF^{-/-}ApoE^{-/-}$ mice develop 86% less atherosclerosis.[49] Furthermore, Stoneman transplanted CD11b depleted bone marrow in wild type $ApoE^{-/-}$ mice and observed reduced plaque development in early atherosclerosis.[50] However, in late stages CD11b depletion did not affect atherosclerosis, which could be explained by the heterogeneity of CD11b, which is also expressed on neutrophils and dendritic cells. These cells are also implicated in atherosclerosis development.

The activation and function of macrophages residing within atherosclerotic lesions are influenced by various cytokines and microbial products in their environment. Two different macrophage subsets have been extensively characterized; classically activated (M1) and alternatively activated (M2) macrophages[51], and recently two new subsets are included; Mox macrophages and M4 macrophages.[52, 53]

M1 and M2 macrophages

M1 macrophages are activated by IL-1β, endotoxin (LPS) and IFN-γ and are considered pro-inflammatory due to their secretion of TNFα, IL-6, IL-12, MMP-1, reactive oxygen species (ROS), and nitrogen intermediates.[54-57] M2 macrophages are induced by IL-4, IL-13, adiponectin, and peroxisome proliferator-activated receptor-γ (PPAR-γ) activation and exert anti-inflammatory functions through the secretion of IL-10, transforming growth factor β (TGF-β), an IL-1 receptor antagonist and the upregulation of the mannose receptor CD206, and arginase-1.[58] Additionally, M2 macrophages can promote wound healing through matrix remodeling, efferocytosis, and the recruitment of fibroblasts.[57, 59] A number of subsets of M2 macrophages have been described: M2 macrophages activated by IL-4 or IL-13 are also called M2a macrophages, activation by immune complexes results in M2b macrophages and stimulation by glucocorticoids or IL-10 results in M2c macrophages.

Both M1 and M2 macrophages are located in human and murine atherosclerotic lesions.[52, 60] Previously, it was shown that oxLDL increases the expression of both M1 (MMP-1 and iNOS) and M2 (arginase-1) markers in macrophages.[61, 62] In addition, foam cells isolated from atherosclerotic lesions express MMP-1 and have reduced arginase-1 expression suggesting a classical M1 phenotype, but also have elevated MMP-12 expression, which is another hallmark factor for alternatively activated M2 macrophages.[63] Although these studies suggest that both M1 and M2 macrophages are involved in aggravating atherosclerosis, recent studies show that the balance between M1 and M2 macrophages may greatly affect lesion development. El Hadri et al. showed that Thioredoxin-1, an oxidative stress-limiting protein, exerts atheroprotective effects by promoting polarization of human and murine macrophages towards an anti-inflammatory M2 phenotype.[64] Furthermore, during regression of atherosclerosis, macrophages shift from an M1 phenotype towards an M2 phenotype and this shift promotes collagen synthesis, which contributes to the stabilization of the lesion after switching to a low fat diet.[65, 66] Interestingly, M2 macrophages resident in atherosclerotic lesions contained smaller lipid droplets compared with M1 macrophages and were localized far from the lipid core in comparison with M1 macrophages.[67]

Mox macrophages and M4 macrohages

In 2010, Kadl et al. showed the existence of a new macrophage subset, Mox macrophages.[52] Mox macrophages are induced by oxidized phospholipids, such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC), which generates a population of macrophages that express high levels of anti-oxidants, such as heme oxygenase-1, sufiredoxin-1 and thioredoxin reductase 1, and IL-10 and vascular endothelial growth factor (VEGF). Although Mox macrophages are found in aortas of $LDLr^{-/-}$ mice fed a Western-type diet for 30 weeks, their exact contribution to the pathogenesis of atherosclerosis remains unclear.

Another newly discovered macrophage subset, the M4 macrophage, is induced upon

CXCL4 stimulation and is identified by the expression of high levels of CD86, tumor necrosis factor superfamily (TNFSF) 10, mannose receptor C type 1 (Mrc1), CC chemokines ligand (CCL) 18, CCL22, and low levels of CD36 and IL-10.[53] It has been shown that M4 macrophages have an impaired capacity to phagocytose acLDL or oxLDL but studies showing their role in atherosclerosis are lacking.

Dendritic cells

Dendritic cells (DCs) are the most specialized antigen presenting cells that are required for the stimulation and differentiation of naive T cells and the development of antigen-specific T cell-mediated immune responses.[68] Immature dendritic cells (imDCs) patrol peripheral tissues, such as the artery wall, in search of antigens.[34, 69] Following the recognition and uptake of the antigen by phagocytosis, imDCs exit the non-lymphoid tissues and migrate via the afferent lymphatic vessels to the T cell rich area of a draining lymph node. During the migration imDCs gradually differentiate into mature DCs (maDCs). Maturation of the DCs involves the downregulation of endocytic activity and the upregulation of some surface molecules, such as chemokine receptor CCR7, costimulatory molecules CD40, CD80 and CD86 and antigen presenting molecules, including MHC I and II and CD1 molecules.[70] Once resident in the lymph nodes, the maDC presents its antigen via the MHC I/II molecules to naive and memory T cells, resulting in an adaptive immune response. The presence of both costimulatory and antigen presenting molecules on maDCs is required for T cell activation and differentiation into effector cells. MaDCs are also capable of activating natural killer T (NKT) cells by presentation of endogenous lipids via the CD1d molecule.[48] While it is generally believed that imDCs migrate towards lymphoid organs during maturation, some DCs may stay behind in the atherosclerotic lesion and form clusters with T cells or NKT cells. This cluster formation in rupture-prone regions is associated with plaque destabilization.[70, 71] The NKT cells secrete Th1 cytokines, such as IFN-γ, which may promote instability of the plaque by the inhibition of smooth muscle cells and their collagen synthesis.[72, 73]

Numerous studies have shown the importance of DCs in atherosclerosis. The number of DCs increases with the progression of atherosclerosis in $ApoE^{-/-}$ mice.[74, 75] Wu et al. showed that $CD11c^{-/-}ApoE^{-/-}$ mice fed a Western-type diet have reduced atherosclerosis with a concomitant attenuation of lesional macrophages.[76] Additionally, Paulson et al. showed that CD11c-diphtheria toxin receptor (DTR) $LDLr^{-/-}$ mice fed a cholesterol-rich diet for 5-10 days have a 55% reduced intimal lipid area in comparison with non-depleted mice.[77] In contrast, depletion of DCs in either chow fed or hypercholesterolemic CD11c-DTR $ApoE^{-/-}$ mice, does not affect atherosclerotic lesion size, while cholesterol levels are significantly enhanced.[78] However, CD11c is not a unique marker for DCs, since it can also be found on macrophages and more specifically on foam cells, which complicates the outcome of these studies.[79] Habets et al. used another approach to study DCs in atherosclerosis by vaccinating $LDLr^{-/-}$ mice with oxLDL-pulsed maDCs. These mice showed a dramatic reduction of atherosclerosis, due to increased plaque

stability, lowered plasma cholesterol levels, enhanced oxLDL-specific T cell responses and increased titers of oxLDL-specific IgG, which participates in immune-complex formation and reduces foam cell development.[80]

Several subtypes of DCs have been described. Intimal DCs can be generated either from monocytes in an Flt3/Flt3 ligand-independent manner, the so-called 'classical' DC[81], or from monocyte-independent precursors in an Flt3/Flt3 ligand-dependent manner, which represents the 'non-classical DC'.[82-84] Deficiency of Flt3 and thus a deficiency in non-classical DCs, enhances atherosclerosis in $LDLr^{-/-}$ mice through reduced Tregs and increased IFN-γ and TNFα levels.[85] This suggests that non-classical DCs are tolerogenic, which means they are capable to suppress the immune system. Because tolerogenic DCs can inhibit inflammation, they are of particular interest for the prevention of atherosclerosis. Hermansson et al. treated human ApoB-100 transgenic $LDLr^{-/-}$ mice with human ApoB-100 and IL-10 pulsed DCs.[86] Treatment with these human apoB-100 loaded tolerogenic DCs attenuated atherosclerosis with 70% through reduced splenocyte proliferation, as a consequence of induced Tregs, which dampened Th1 and Th2 responses. Another subset of DCs, the plasmacytoid DCs (pDCs), mainly responds to viruses by the production of type I interferons but can also act as tolerogenic DCs.[87] Daissormont et al. showed that depletion of pDCs by 120G8 mAb administration results in increased atherosclerosis in $LDLr^{-/-}$ mice as a consequence of increased T cell proliferation and increased IFN-γ secretion.[88]

T cells

After exposure to antigens via DCs in lymphoid organs, naive T cells undergo clonal expansion and differentiate into effector T cells. Upon re-activation of T cells by interaction with lesional macrophages or DCs, they produce large amounts of pro-atherogenic cytokines that contribute to both the growth and destabilization of lesions, which can result in rupture of the lesion. To recognize antigens presented on MHC molecules of APCs, T cells express a T cell receptor (TCR). Depending on the composition of their TCR, T cells are divided in two subsets; γδ T cells and αβ T cells. γδ T cells represent a minor subset of T cells that are mainly known for their role in innate immunity against pathogens.[89] High numbers of γδ T cells are present in gut and lung mucosa and they are triggered by antigens produced by stressed and damaged cells. Although γδ T cells also recognize lipid antigens, their role in atherosclerosis remains to be elucidated. The majority of T cells has a TCR consisting of an α- and β-chain and can in turn be divided into $CD4^+$ and $CD8^+$ T cells. Whereas $CD4^+$ T cells recognize antigens presented on MHC II molecules, $CD8^+$ T cells recognize antigens presented on MHC I. Besides the formation of the TCR-antigen complex, two other signals are required for optimal T cell activation; costimulation and cytokines. Costimulatory molecules on both the APCs and T cells must interact and can either provide costimulatory or coinhibitory signals. Signaling via costimulatory ligand/receptor pairs results in the activation of immune cells, whereas signaling through coinhibitory pairs blocks immune cell function. There are many costimulatory

and coinhibitory pathways and each pathway has its own unique effect on the fate of individual immune cells, which will be further discussed in Chapter 2. Strongly depending on the cytokine environment, naive CD4⁺ T cells differentiate into various T cell subsets, such as Th1 cells, Th2 cells, Th17 cells and Tregs, with each a distinct set of cytokines that is released upon activation (Figure 5). Nowadays, new T cell subsets are discovered, such as Th9 cells and Th22 cells and accumulating evidence suggests that the differentiation of CD4⁺ T cells displays plasticity.[90, 91]

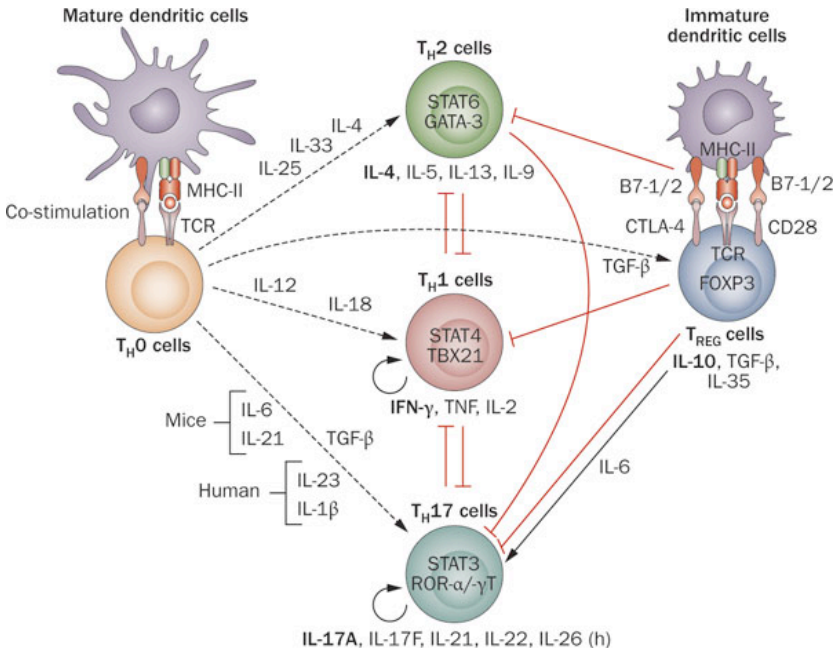


Figure 5. Naive T cells are activated by dendritic cells in lymphoid organs and differentiate into effector T cells. There are many effector T cell subsets known and a combination of costimulatory and coinhibitory molecules together with the present cytokine environment determines which T cell subset is formed. *Adapted from Lahoute et al. Nat. Rev. Cardiol. 2011;8:348.*

CD8⁺ T cells

Cytotoxic CD8⁺ T cells can induce apoptosis in target cells via the release of the cytotoxins perforin and granzymes. Once activated CD8⁺ T cells can also produce large amounts of the pro-inflammatory cytokine IFN-γ.[92] CD8⁺ T cells are present in atherosclerotic plaques of mice but their role in atherosclerosis is controversial.[93, 94] Kolbus et al. showed that CD8⁺ T cells are especially activated during initial stages of atherosclerosis in ApoE$^{-/-}$ mice[95], and in humans, CD8⁺ T cells represented up to 50% of the lymphocytes in advanced atherosclerotic lesions and upon TCR and/or pro-inflammatory cytokine activation could migrate into healthy or mildly affected sites of the vasculature.[96] Mice deficient in MHC I show enhanced atherosclerosis, which suggests a possible role for CD8⁺ T cells in the development of atherosclerosis.[97] Recently, Chyu et al. showed that CD8⁺ T cells mediate the atheroprotective effect

of ApoB-100 related peptide immunization in ApoE$^{-/-}$ mice.[98] In contrast, Elhage et al. showed that a deficiency in CD8 does not affect atherosclerosis development in ApoE$^{-/-}$ mice.[99]

CD4^{+} T cells

CD4^{+} T cells are present in different stages of human plaques[100, 101] and are also found in atherosclerotic lesions of both ApoE$^{-/-}$ and LDLr$^{-/-}$ mice.[94] A deficiency in CD4^{+} T cells[102-104] or TCRαβ cells[105] and thus a deficiency in adaptive immunity leads to reduced atherosclerosis. In addition, a transfer of CD4^{+} T cells to immune-deficient SCID$^{-/-}$ ApoE$^{-/-}$ mice accelerates atherosclerosis, indicating the importance of CD4^{+} T cells in atherosclerosis.[104] Naive CD4^{+} T cells differentiate into various T cell subsets, such as Th1 cells (producing TNFα, IFN-γ, IL-1, IL-12 and IL-18), Th2 cells (producing IL-4, IL-5 and IL-13), Th17 cells (producing IL-6, IL-17 and IL-23) and Tregs (producing TGF-β and IL-10), which will be discussed below in more detail.

Th1 cells

Th1 cells produce pro-inflammatory cytokines, such as IFN-γ and TNFα, and express the Th1 specific transcription factor T-box expressed in T cells (T-bet). The majority of the pathogenic CD4^{+} T cells in atherosclerosis are Th1 cells. High levels of IFN-γ stimulate the recruitment of macrophages, DCs and T cells to the plaque, increase lipid uptake by macrophages and activate APCs.[106, 107] In addition, IFN-γ inhibits vascular smooth muscle cell proliferation and reduces collagen production, thereby contributing to the instability of the fibrous cap. Correspondingly, LDLr$^{-/-}$ mice deficient in T-bet[108] or ApoE$^{-/-}$ mice deficient in IFN-γ[109] show attenuated atherosclerosis. In addition, deficiencies in other cytokines secreted by Th1 cells, such as TNFα[110], IL-1β[111], IL-12[112] or IL-18[113], also reduced atherosclerotic lesion development.

Th2 cells

Th2 cells are known to produce IL-4, IL-5 and IL-13, and are recognized by the expression of the transcription factor Trans-acting T-cell-specific transcription factor-3 (GATA-3). Th2 cells also provide help to B cells and promote their differentiation into plasma cells. The role of Th2 cells in atherosclerosis remains controversial and depends on the stage of the lesion, as well as on the experimental mouse model.[114, 115] Several studies show an anti-atherogenic role for Th2 cells, since IL-4 secretion inhibits pro-atherogenic Th1 responses via downregulation of IFN-γ production. Furthermore, C57Bl/6 mice injected with IL-4 and BALB/c mice prone to Th2 immune responses are protected against early fatty streak formation.[115] Other studies have shown that IL-5, another characteristic Th2 cytokine, reduces plaque formation in LDLr$^{-/-}$ mice in part by promoting oxLDL-specific IgM secretion by B1 cells.[116] Miller et al. showed that IL-33, a powerful inducer of Th2 responses, reduces the development of atherosclerosis in ApoE$^{-/-}$ mice via IL-5 and subsequent oxLDL-specific IgM antibody formation.[117] Recently, Cardilo-Reis et al. showed that IL-13 deficiency

accelerates atherosclerosis in LDLr$^{-/-}$ mice and that IL-13 administration increased collagen content and reduced VCAM-1-dependent monocyte recruitment, resulting in decreased lesional macrophages.[118] Moreover, IL-13 induced M2 macrophages, which exhibited increased clearance of oxLDL in comparison with M1 macrophages.
In contrast, Th2 cells can also fulfill a pro-atherogenic role, since LDLr$^{-/-}$ mice and ApoE$^{-/-}$ mice deficient in IL-4 have reduced atherosclerosis.[112, 119] In addition, van Wanrooij et al. showed that blockade of the costimulatory OX40-OX40L pathway reduces initial atherosclerosis in part via decreased IL-4 levels.[120] Notably, IL-5 levels and oxLDL-specific IgM responses were increased in this study, which suggests a pro-inflammatory role for IL-4 and an anti-inflammatory role for IL-5. In fact, IL-4 and IL-5 are differentially regulated at the transcriptional level. Whereas differentiation of IL-4-producing T cells is dependent on STAT6[121] and GATA-3[122], Kurowska et al. showed that IL-5-producing T cells differentiate independently of STAT6 and GATA-3.[123] This shows that Th2 cells do not exclusively secrete the complete panel of typical Th2 cytokines but may form specific subsets that secrete either IL-4 or IL-5, which might determine the pro- or anti-atherogenic fate of these cells.

Th17 cells
Another subset of CD4^{+} T cells are Th17 cells, which mainly produce the pro-inflammatory cytokine IL-17. The differentiation of Th17 cells is mainly driven by the transcription factor RORγt and the cytokines TGF-β and IL-6.[124-126] IL-17 induces tissue inflammation and is therefore commonly associated with autoimmune diseases.[127, 128] However, the role of Th17 cells in atherosclerosis has not been fully elucidated yet. In humans, IL-17 is upregulated in atherosclerotic plaques of symptomatic patients compared to plaques from asymptomatic patients.[129-131] In mice, the expression of IL-17 and RORγt also correlated to plaque size[132] and IL-17A-expressing T cells were significantly increased in the aorta and spleen of aged ApoE$^{-/-}$ mice compared with C57BL/6 mice.[133] Furthermore, a deficiency in the IL-17R[134] or blockade of IL-17 by using neutralizing antibodies[132, 135] or by use of adenovirus-produced soluble IL17-RA[133] reduces atherosclerosis, while exogenous IL-17 promotes the formation of atherosclerotic lesions. Conversely, Madhur et al. showed that an IL-17A deficiency in ApoE$^{-/-}$ mice fed a Western-type diet for 12 weeks does not affect plaque burden, whereas it does decrease aortic CD3^{+} T cells and IFN-γ production by splenocytes.[136] Taleb et al. also did not observe an effect of IL-17 neutralization on atherosclerosis, although they do describe a protective role for IL-17 in a mouse model in which suppressor of cytokine signaling (SOCS) 3 was absent in T cells.[137] These conflicting results show the need for more research to determine the exact role of Th17 cells in atherosclerosis.

Regulatory T cells
Regulatory T cells (Tregs) play an important role in the regulation of immune responses through suppression of immune cell proliferation and cytokine production. Suppression

mainly occurs through secretion of the inhibitory cytokines IL-10 and TGF-β, and cell-cell contact, mediated by membrane-bound TGF-β, CTLA-4 and/or GITR.[138, 139] 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).[140] Tregs can be divided into two subsets, natural occurring Tregs and adaptive Tregs. Natural occurring Tregs are derived from thymocytes during negative selection in the thymus as part of a tolerance mechanism.[141, 142] The tolerance mechanism deletes unmatured T- or B cells in the thymus that recognize self antigens with a high affinity. However, some self-reactive lymphocytes escape from this mechanism and have to be controlled by peripheral tolerance.[143] In the thymus, thymocytes with low affinity for self antigens are positively selected and released. Thymocytes with intermediate affinity for self antigens upregulate the transcription factor Foxp3 and become Tregs.[144] In the secondary lymphoid tissues and at sites of inflammation these Tregs function by regulating self-reactive T cell responses.[145] Naturally occurring Tregs express CD4, CD25 and Foxp3 and secrete IL-10 and TGF-β. Adaptive or induced Tregs are generated from naive T cells after stimulation by tolerogenic DCs and/or in the presence of IL-10 and TGF-β. Adaptive Tregs can be subdivided in T regulatory type 1 (Tr1) and Th3 cells. Tr1 cells suppress mainly through IL-10, whereas Th3 cells function via TGF-β production.[146-151]

Foxp3 is crucial for the development and function of Tregs. The mechanism underlying the role of Foxp3 in the suppressive function of Tregs remains to be fully understood, but recently it is shown that Foxp3 is part of a large molecular complex that represses transcription of target genes, such as nuclear factor of activated T cells (NFAT) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), that are involved in the TCR signaling pathway.[152] Additionally, Foxp3 also upregulates the expression of genes such as IL-2Rα (CD25) and glucocorticoid-induced tumor necrosis factor receptor (GITR), which are essential for the function of Tregs.[153, 154]

The delicate balance between the pro- and anti-inflammatory signals can be disturbed when Tregs are deficient or dysfunctional, ultimately leading to severe autoimmune diseases or pathogen-induced inflammation.[142, 155] In atherosclerosis an imbalance between pro-inflammatory cells, such as Th1 cells, and anti-inflammatory cells (Tregs) exists, with increased numbers of the first. In addition, oxLDL attenuates the suppressive function of Tregs.[156] Therefore, increased Treg numbers may be beneficial for patients suffering from atherosclerosis. The role of Tregs in atherosclerosis has been subject of intense investigation (Figure 6). Adoptive transfer of $CD4^+CD25^+$ T cells causes a reduction in atherosclerotic lesion development[157] while a depletion of $CD4^+CD25^+$ T cells aggravates lesion development.[157, 158] Furthermore, an increase in the number of antigen-specific Tregs via induction of oral tolerance to oxLDL and heat shock protein 60 (HSP60) was associated with reduced atherosclerosis.[159, 160] IL-10 produced by Tregs attenuates plaque formation[6, 15, 161] and similarly Tr1 induced immune responses in $ApoE^{-/-}$ mice resulted in a decreased plaque size and inflammation.[162] Deficiencies in total or T cell specific TGF-β signaling accelerates atherosclerosis and

induces an unstable plaque phenotype in hypercholesterolemic mice.[163, 164]
Whereas the atheroprotective role of Tregs in mice is well-established, the role of human Tregs in atherosclerosis remains to be clarified. Foxp3$^+$ cells are found in human atherosclerotic plaques[165, 166] and low levels of circulating human Tregs are associated with an increased risk for the development of myocardial infarction.[167] Additionally, simvastatin significantly enhanced the quantity and suppressive function of Tregs in peripheral blood mononuclear cells (PBMCs) isolated from patients with acute coronary syndrome, which might contribute to the immunomodulatory mechanism of statins.[168] It must be noted that while Foxp3 is a commonly used marker for Treg identification, Foxp3 is also expressed in CD8$^+$ T cells[169] and macrophages.[170] New markers for the characterization of Tregs are extensively investigated and the use of Tregs as a new immune therapy to modulate pro-inflammatory immune responses in atherosclerosis needs to be further explored.

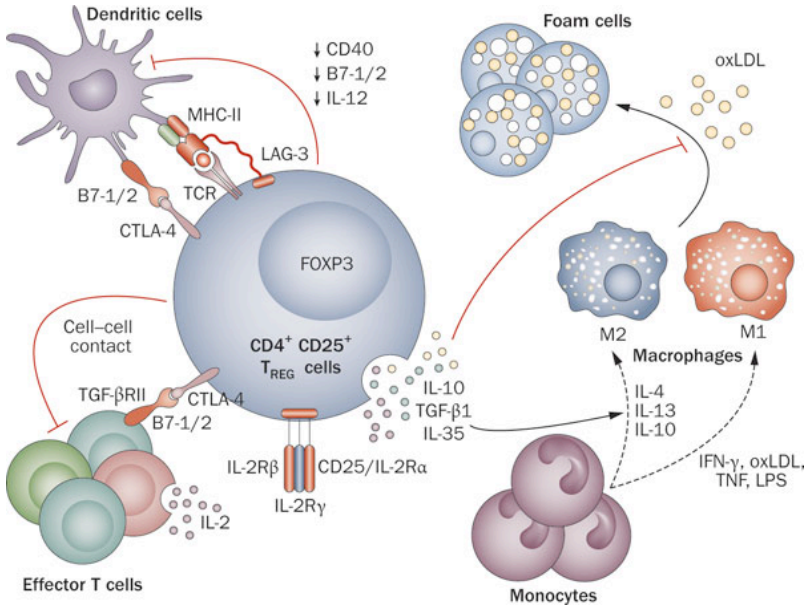


Figure 6. Tregs regulate pro-inflammatory immune responses via several mechanisms in atherosclerosis. Tregs have an elevated expression of coinhibitory molecules, such as CTLA-4, through which they can directly inhibit pro-atherogenic T cells or dendritic cells. Indirectly, Tregs inhibit immune responses via the secretion of the anti-inflammatory cytokines IL-10, TGF-β and IL-35. These cytokines can skew macrophages towards an anti-inflammatory M2 phenotype and inhibit foam cell formation. *Adapted from Lahoute et al. Nat. Rev. Cardiol. 2011;8:348.*

B cells

B cells are essential players in humoral immune responses and during atherosclerosis they are mainly present in the adventitia of lesions.[171, 172] "Atherosclerotic" B cells produce antibodies specific for self-antigens, such as oxLDL, HSP60 and β_2-glycoprotein, and for bacterial antigens, such as Streptococcus and Chlamydia. Autoantibodies against many of these antigens have been found in humans with coronary heart disease

and in animal models of atherosclerosis, and titers of oxLDL-specific antibodies are directly correlated with the severity of disease. Initial studies showed that B cells are atheroprotective, since B cell deficiency aggravates atherosclerosis in $LDLr^{-/-}$ mice[173] and adoptive transfer of splenic B cells decreases atherosclerosis in $ApoE^{-/-}$ mice.[174] In mice, several B cell subsets are identified: B1, B2 and B10 cells. B1 cells are a subset of B cells that are predominantly found in peritoneal cavities[116, 175] and secrete oxLDL-specific IgM antibodies upon IL-5 stimulation.[176] OxLDL-specific IgM plays a protective role in atherosclerosis because it prevents foam cell formation by inhibiting oxLDL uptake by macrophages, helps to clear apoptotic cells and prevents inflammatory reactions towards oxLDL and other modified lipids.[176, 177] Furthermore, IL-5 deficiency enhances atherosclerosis via decreased oxLDL-specific IgM titers and IL-33 protects against atherosclerosis through the induction of B1 cells.[117, 178] In contrast to anti-atherogenic B1 cells, B2 cells promote atherosclerotic lesion development.[179, 180] B2 cells are conventional B cells found in spleen and lymph nodes that produce low levels of IgM and high levels of IgD. Depletion of B2 cells using an anti-CD20 antibody ameliorated atherosclerosis, whereas adoptive transfer of B2 cells aggravates atherosclerosis. Therefore, future immune therapy to inhibit atherosclerosis may also focus on promoting B1 cells and inhibiting B2 cells. Recently, a third subset of B cells has been described; B10 cells or so-called IL-10 producing regulatory B cells (Bregs).[181] The exact role of Bregs in atherosclerosis has not been clarified yet, however, Bregs secrete IL-10 and thereby inhibit secretion of pro-inflammatory cytokines and support Treg differentiation, which indicates a protective role for Bregs in atherosclerosis.

Mast cells

Another important key player in atherosclerosis is the mast cell. Activated mast cells are found in the adventitia of vulnerable and ruptured lesions of patients suffering from myocardial infarction[182, 183], and mast cell numbers correlate with the incidence of plaque rupture and erosion.[182] In addition, Bot et al. showed that mast cells also play a crucial role in plaque progression and destabilization.[184] Mast cells are derived from bone marrow and circulate as precursors in the blood where they contribute to the first line of defense against pathogens, such as bacteria and parasites. Once recruited to specific tissues such as the skin, they mature into mast cells. Their most important function is the release of cytoplasmatic granules upon stimulation with various immunologic and nonimmunologic agents, such as IgE. In atherosclerotic lesions, mast cells can be activated by oxLDL-IgG immune complexes[185] and neuropeptide substance P.[186] The released granules contain inflammatory compounds, such as histamine, tryptase, chymase, TNFα, IL-6 and VEGF that promote lesion development and increase lesion instability.[187, 188] The secretion of pro-inflammatory cytokines enables mast cells to act as so-called 'non-professional' APCs and to induce T cell responses.[189] Mast cells can also secrete the cytokine IL-8 and the chemokine MCP-1, which attract immune cells to the site of inflammation and further promote the immune response.[185] In addition, mast cells are directly involved in the lipid metabolism, as

several studies show that mast cell granules and their released compounds, such as heparin, can bind to LDL particles.[190]

Neutrophils

Neutrophils are classically described as the first cells to respond to pathogens, tissue damage or inflammation. Neutrophils are characterized by their surface expression of CD11b and Gr-1, Ly6G in particular. Upon activation, neutrophils release pro-inflammatory mediators, such as myeloperoxidase (MPO), elastase, azurocidin and cathelicidin, from their granules. All these mediators are involved in atherosclerosis development. MPO for example promotes the formation of oxLDL[191] and MPO serum levels are associated with a higher risk for the development of coronary artery disease.[192, 193]

However, neutrophils are rarely detected in human and murine atherosclerotic lesions, which is possibly due to their life-span of approximately 5-8 hours and rapid apoptosis upon activation.[194] Nonetheless, neutrophils have been found in subendothelial and intimal areas of early atherosclerotic lesions in mice[195-197], as well as in plaque shoulder areas of rupture-prone lesions.[196, 197] In humans, intraplaque neutrophils are mainly located in the fibrous cap, the shoulder, the interface to media and in areas with intraplaque bleeding.[198]

Drechsler et al. showed that lesion sizes positively correlate with circulating neutrophils and depletion of neutrophils using the antibody 1A8 recognizing $CD11b^{+}Ly6G^{+}$ cells reduces early atherosclerosis in $ApoE^{-/-}$ mice.[195] Additionally, Zernecke et al. show that administration of the anti-PMN antibody also reduced initial atherosclerosis.[199] However, the development of advanced lesions remained unaffected by neutropenia as depletion of neutrophils after 3 or 11 months of high-fat diet feeding did not reduce lesion formation.[195] More specifically, neutrophils contribute in a variety of ways to the pathogenesis of atherosclerosis; neutrophils aggravate endothelial dysfunction, attract monocytes into atherosclerotic lesions, activate macrophages to produce pro-inflammatory cytokines, and can destabilize the lesion.[200] Interestingly, statins have been shown to interfere with neutrophil function via the reduction of neutrophil recruitment and activation, and lowering of neutrophil-attracting chemokines.[201, 202] More research is needed to fully describe the role of neutrophils in atherosclerosis.

MDSCs

Similar to neutrophils, myeloid-derived suppressor cells (MDSCs) express the myeloid cell markers Gr-1 and CD11b.[203] MDSCs originate from the bone marrow and consist of early myeloid progenitor cells and immature myeloid cells. In healthy individuals these immature cells mature into granulocytes, dendritic cells and macrophages. In diseased individuals this maturation is blocked and the immature cells expand to a large population of MDSCs that migrate into lymphoid organs and inflamed tissues where they strongly suppress immune responses.

Two different subtypes of MDSCs are described: granulocytic-MDSCs (gr-MSDCs),

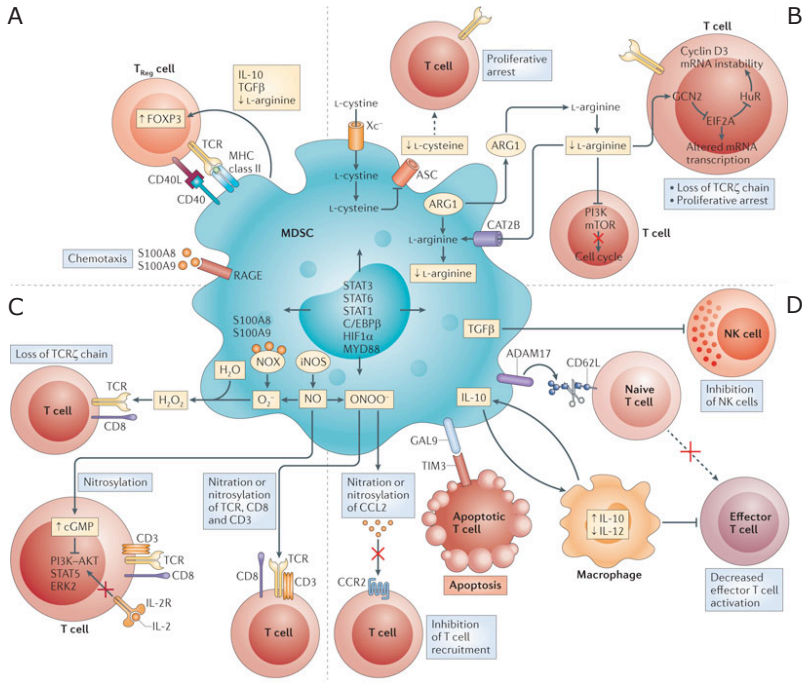


Figure 7. MDSC-mediated T cell suppression. MDSCs can suppress T cells via different mechanisms. MDSCs can induce Tregs (A), suppress T cell proliferation in an L-arginine-mediated manner (B), render T cells unresponsive for IL-2 via iNOS and NO/reactive oxygen species (C), and can induce T cell apoptosis and skews macrophages towards an M2 phenotype (D). *Adapted from Gabrilovich et al. Nat. Rev. Immunol. 2012;12:253.*

which are $CD11b^{+}Ly6G^{+}Ly6C^{low}$, and monocytic-MDSCs (mo-MSDCs), which are $CD11b^{+}Ly6G^{-}Ly6C^{hi}$.[204] Although both subtypes can suppress T cell responses, it has been well documented that mo-MDSCs exhibit more potent suppressive activity than gr-MDSCs.[204-206] Interestingly, whereas mo-MDSCs are driven by Th1 signals[207], such as IFN-γ and LPS, gr-MDSCs are driven by Th2 cytokines, such as IL-4, IL-10 and IL-13.[208]

MDSCs particularly suppress T cell function via upregulation of their expression of immune suppressive factors, such as inducible nitric oxide synthase (iNOS), arginase 1 (arg-1), nitric oxide (NO) and reactive oxygen species (ROS) (Figure 7).[209, 210] iNOS activity is mainly associated with mo-MDSCs as IFN-γ can induce iNOS expression and arg-1 is mostly linked to gr-MDSCs. Both iNOS and arg-1 compete for their common substrate L-arginine. iNOS consumes L-arginine to NO, which renders T cells non-responsive to IL-2. In contrast, arg-1 converts L-arginine to urea and polyamines, which reduces the availability of L-arginine and subsequently impairs T cell function by loss of CD3ζ expression. Both arg-1 and iNOS can generate ROS, which inhibits T cell function via nitration of the T cell receptor that impairs the T cell-antigen-MHC interaction.[209] Moreover, MDSCs can also promote *de novo* development of Foxp3, which results in elevated Tregs that also very efficiently suppress effector T cells.[211]

Besides regulating adaptive immune responses, MDSCs can also affect innate immune responses as they can stimulate macrophages to produce IL-10 and to reduce IL-12 secretion.[212]
MDSCs have been extensively investigated in the context of cancer but their contribution to other diseases is only recently appreciated. MDSCs suppressed inflammation in obese mice[213] and in a mouse model for MS[214] and prevented type 1 diabetes[215] and graft versus host disease.[216] However, no studies describe the role or existence of MDSCs in atherosclerosis.

Outline of the thesis

Patients suffering from cardiovascular disease are treated with statins to lower LDL cholesterol. However, these drugs have very little effect on established lesions, which emphasizes the requirement for novel experimental therapies to treat atherosclerosis. In this thesis, several therapeutic strategies are used to modulate the immune response in atherosclerosis. Pro-inflammatory responses responsible for atherosclerosis can be suppressed by modulation of costimulatory and coinhibitory pathways or induction of suppressor cells, such as regulatory T cells and myeloid-derived suppressor cells.

Costimulatory and coinhibitory molecules tightly control immune responses by providing positive signals that promote T cell activation or by transducing inhibitory signals that limit T cell responses. **Chapter 2** provides an overview on the current status of research on costimulatory and coinhibitory pathways in atherosclerosis.
In **Chapter 3**, a combined anti-inflammatory and lipid-lowering strategy was used to induce regression of atherosclerosis. Interruption of the costimulatory OX40-OX40L pathway reduces IgE serum levels and mast cell activation, induces atheroprotective IL-5 and oxLDL-specific IgM and in combination with dietary adjustments can induce regression of advanced lesions.
Chapter 4 describes a study in which the role of the costimulatory pathway formed by CD30 and CD30L, members of the TNF(R) superfamily involved in activation and proliferation of T and B cells, is determined in atherosclerosis. Treatment with anti-CD30L inhibits plaque development with 35% in $LDLr^{-/-}$ mice solely by modulation of T cell responses.
Chapter 5 describes a study in which the role of the coinhibitory T cell immunoglobulin and mucin domain 3 (Tim-3) in atherosclerosis was investigated. Treatment of $LDLr^{-/-}$ mice with a Tim-3 blocking antibody increased atherosclerosis development with 35% in the aortic root and with 50% in the aortic arch compared with control treatment, by increasing circulating monocytes and lesional macrophages, and by decreasing IL-10 producing regulatory T and B cells.
In **Chapter 6**, the contribution of another new-emerging negative costimulatory pathway formed by T cell immunoreceptor with Ig and ITIM domains (TIGIT) and the poliovirus receptor (PVR) was determined in the pathogenesis of atherosclerosis. Signaling through the TIGIT-PVR pathway can inhibit T cell responses in a cell-intrinsic

manner as well as via the induction of IL-10 producing tolerogenic DCs. However, although agonistic TIGIT significantly affected T cell proliferation and activation *in vitro* and *in vivo*, agonistic TIGIT treatment did not significantly affect atherosclerosis development.

Regulatory T cells are important regulators of immune responses and show great potential to be used as a therapeutic regime. In **Chapter 7**, the protective role of $Foxp3^+$ Tregs in atherosclerosis was investigated. Vaccination using dendritic cells which were electroporated with mRNA encoding for Foxp3, decreased the number of $Foxp3^+$ regulatory T cells leading to an increase in lesion formation in $LDLr^{-/-}$ mice. In addition, an increase in plaque cellularity and spleen cell proliferation was observed. In **Chapter 8**, administration of an IL-2-anti-IL-2 complex to Western-type diet fed $LDLr^{-/-}$ mice significantly expanded regulatory T cells up to 10-fold in the circulation and several (lymphoid) organs. This expansion of regulatory T cells potently suppressed effector T cells and reduced initial atherosclerotic lesion formation, whereas, in combination with a vigorous lowering of blood lipid levels, it enhanced lesion stability in $LDLr^{-/-}$ mice with pre-existing lesions.

Myeloid-derived suppressor cells (MDSCs) are potent suppressors of T cell responses in tumor immunology but their contribution to other diseases, such as EAE and type 1 diabetes, is only recently appreciated. To investigate whether and how MDSCs contribute to the development of atherosclerosis, in **Chapter 9**, we isolated bone marrow-derived $CD11b^+Gr\text{-}1^+$ cells (MDSCs) from Western-type diet fed $LDLr^{-/-}$ mice and adoptively transferred them into $LDLr^{-/-}$ mice fed a Western-type diet for 6 weeks. Mice that received MDSCs showed a 35% reduction in atherosclerotic lesion formation with a concomitant reduction in T and B cell responses.

Finally, all the results obtained in this thesis and future perspectives are summarized and discussed in **Chapter 10**.

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Chapter 2

Regulating atherosclerosis via costimulatory and coinhibitory pathways

Amanda C. Foks
Esther Lutgens[#]
Johan Kuiper

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

[#] Department of Medical Biochemistry, Amsterdam Medical Center, The Netherlands

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Abstract

Mortality from cardiovascular disease continues to increase worldwide despite the use of statins, anti-thrombotic drugs, and anti-hypertensive treatment. This indicates an urgent need for novel therapeutic strategies to inhibit atherosclerosis and to prevent cardiovascular complications and acute syndromes. T cells play a major role in the pathogenesis of atherosclerosis by promoting inflammation and destabilizing advanced lesions, and are regulated by a network of costimulatory and coinhibitory molecules. Costimulatory signals can promote T cell survival, cell cycle progression and differentiation of naive T cells to effector and memory T cells, whereas coinhibitory molecules can terminate these processes directly or indirectly via the induction of regulatory T cells. The immune system provides a large diversity of costimulatory and coinhibitory pathways and each pathway has its own unique effect on the behaviour of individual immune cells. This offers unique possibilities to regulate pro-inflammatory immune responses in atherosclerosis. In this review we provide an overview of costimulatory and coinhibitory pathways in atherosclerosis and their potential as therapeutic targets to treat or prevent cardiovascular disease.

Atherosclerosis

Acute cardiovascular syndromes, such as myocardial infarction or stroke, are a major cause of death in Western society and are generally caused by rupture of an atherosclerotic plaque.[1] Atherosclerosis is a chronic autoimmune-like disease resulting from endothelial damage and dysfunction, and cholesterol accumulation in the arterial wall. Atherosclerosis mostly occurs in medium- and large-sized arteries, already starts in early adolescence and progresses slowly.[2, 3] Depending on exposure to risk factors including high fat intake, hypertension, smoking, stress and physical inactivity, and diseases such as obesity, diabetes and dyslipidemia, atherosclerotic lesions can lead to acute thrombotic complications and subsequent clinical events.

For many years it was believed that atherosclerosis was a 'passive' lipid disorder in which cholesterol accumulated in the arterial wall. However, the mechanism underlying the pathological process of atherosclerosis is much more complicated and the involvement of inflammatory cells in the development and progression of atherosclerosis was suggested after the discovery of T cells in atherosclerotic lesions in the 1980s.[4] Patients suffering from cardiovascular disease are currently treated with statins to lower low-density lipoprotein (LDL) cholesterol. However, since these drugs lead to a 30% risk reduction in cardiovascular patients, there is an urgent need for new therapeutic strategies that target its inflammatory nature directly, to inhibit atherosclerosis and to prevent acute cardiovascular syndromes.

The immune system in atherosclerosis

Besides lipid accumulation, inflammation is considered a key process in atherosclerotic plaque development and in the pathogenesis of plaque rupture.[5, 6] Atherosclerosis involves both innate and adaptive immune responses. The innate immunity is the initial barrier against infections and several immune cells such as monocytes, macrophages, dendritic cells (DCs), neutrophils, natural killer (NK) cells and mast cells are programmed to detect foreign molecules of exogenous (eg lipopolysaccharide (LPS)) or endogenous (eg oxidized LDL (oxLDL) and heat shock protein (HSP) 60) origin.[7] These foreign molecules are called pathogen-associated molecular patterns (PAMPs) and are recognized by the innate immune cells via pattern recognition receptors such as Toll-like receptors (TLRs) and Scavenger receptors (SRs).[8] The pathogens are internalized and degraded by phagocytosis. Adaptive immune reactions are initiated when antigen-presenting cells (APCs), such as macrophages and DCs, display a surface complex consisting of an antigenic peptide bound to a major histocompatibility complex protein class I or class II (MHC I or MHC II) to a T or B cell. This leads to cytokine secretion, cytotoxicity, antibody production, memory formation and stimulation of many other components of an immune reaction.[6]

T cells in atherosclerosis

To recognize antigens presented on MHC molecules of APCs, T cells express a T cell receptor (TCR). Depending on the composition of their TCR, T cells are divided in two

subsets; γδ T cells and αβ T cells. γδ T cells represent a minor subset of T cells that are mainly known for their role in innate immunity against pathogens.[9] High numbers of γδ T cells are present in gut and lung mucosa and are triggered by antigens produced by stressed and damaged cells. Although γδ T cells also recognize lipid antigens, their role in atherosclerosis remains to be elucidated. The majority of T cells has a TCR consisting of an α- and β-chain and can be separated into $CD4^+$ and $CD8^+$ T cells. Whereas $CD4^+$ T cells recognize antigens presented on MHC II molecules, $CD8^+$ T cells recognize antigens presented on MHC I.

For sustained and optimal T cell signaling, an immunological synapse is assembled at the interface of the T cell and an APC after the interaction of MHC-antigen complexes with the TCR. Initially, these MHC-antigen-TCR complexes are accumulated in a ring surrounding a central cluster of intercellular cell adhesion molecule-1 (ICAM-1) interactions with the integrin leukocyte function-associated molecule-1 (LFA-1).[10, 11] This immature immunological synapse transforms in a mature immunological synapse when the MHC-antigen-TCR complexes accumulate in the center via actin-mediated transport and are surrounded by a ring of ICAM-1/LFA-1 interactions.

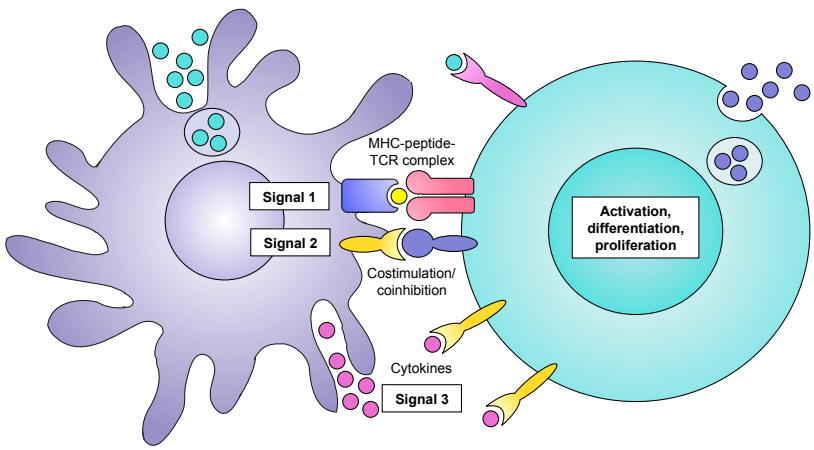


Figure 1. Schematic overview of optimal T cell activation. Three signals are required: (1) antigen presentation via MHC molecules on APCs to the TCR on T cells, (2) costimulatory or coinhibitory signaling, (3) environmental cytokines that can influence the T helper cell differentiation.

Besides the formation of the MHC-antigen-TCR complex (signal 1), two other signals are required for optimal T cell activation; costimulation (signal 2) and cytokines (signal 3, Figure 1). Costimulatory molecules on both the APCs and T cells must interact and can either provide costimulatory or coinhibitory signals. Signaling via costimulatory ligand/receptor pairs results in the activation and proliferation of immune cells, whereas signaling through coinhibitory pairs blocks immune cell function. Naive T cells that only receive signal 1 without signal 2 become anergic or die through apoptosis. Moreover, it has been shown that engagement of costimulatory ligand/

receptor pairs triggers an accumulation of receptors and protein complexes at the center of the immunological synapse, which then amplifies and enhances the duration of TCR signaling.[12] Strongly depending on signal 3, the cytokine environment, naive $CD4^+$ T cells differentiate into various T cell subsets, such as Th1 cells, Th2 cells, Th17 cells and Tregs, with each a distinct set of cytokines that is released upon activation. Thus, after exposure of antigens via APCs within the lesions or in lymphoid organs, this 'three signal model' of T cell activation ensures the clonal expansion and differentiation of effector T cells. Upon activation T cells produce large amounts of pro-atherogenic cytokines that contribute to both the growth and destabilization of lesions, which can result in rupture of the lesion.

Costimulation and coinhibition of T cells

The immune system provides a large diversity of costimulatory and coinhibitory pathways (signal 2) and each pathway has its own unique effect on the fate of individual immune cells. Depending on the activation status of the cell, each costimulatory and coinhibitory molecule has its own expression pattern and thereby tightly regulates T cell function. Costimulatory signals can promote T cell survival, cell cycle progression and differentiation to effector and memory T cells, whereas coinhibitory molecules can terminate these processes directly or indirectly via the induction of regulatory T cells (Tregs). Tregs play an important role in the regulation of immune responses through suppression of immune cell proliferation and cytokine production.[13, 14]

Costimulation can be provided in *cis*, meaning that both signal 1 and 2 are provided by the same APC, or in *trans*, in which signal 2 is provided by a different or so-called 'bystander' APC than signal 1. During *cis*-costimulation both signal 1 and 2 are in close proximity, which results in a rapid and more efficient activation of the T cell compared with *trans*-costimulation.[15] However, some studies suggest that *trans*-costimulation is as effective as *cis*-costimulation in the activation of T cells.[16, 17]

Furthermore, several studies suggest that costimulatory molecules can elicit bidirectional signals that benefit both the interacting T cell and APC.[18, 19] This 'reverse signaling' can provide costimulatory signals or cause anergy. Bidirectional signal transduction has been described for CD40L, CD137, OX40L, CD30L and LIGHT but the exact mechanism remains to be elucidated.[19]

Most of the costimulatory and coinhibitory molecules belong to either the B7[20] or tumor necrosis factor (TNF)[21] superfamilies. The B7 superfamily plays an important role in regulating T cell function and its members such as CD28, CTLA-4 and PD-1 are promising therapeutic targets. Members of the TNF superfamily, such as CD40L and OX40, also provide costimulation but additionally comprise molecules with cytoplasmic death domains which are involved in apoptosis. Not surprisingly, many of the known costimulatory and coinhibitory receptor-ligand pairs are expressed in atherosclerotic lesions[22, 23] and all affect atherogenesis in various ways. In the next section, we will provide an overview of the different costimulatory and coinhibitory pathways and their role in the pathogenesis of atherosclerosis.

Costimulatory pathways in atherosclerosis

Figure 2 provides an overview of costimulatory molecules of both the B7 and TNF(R) family that upon interaction contribute to the development of atherosclerosis. Each costimulatory molecule pair results in the proliferation and differentiation of T cells in a unique manner, which will be described in more detail.

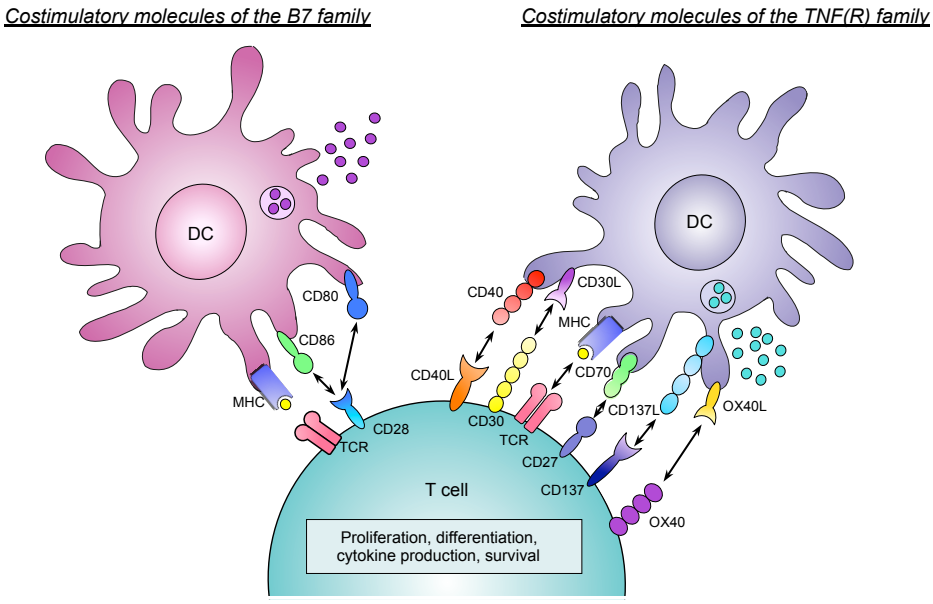


Figure 2. Signaling via costimulatory pathways in atherosclerosis promotes T cell proliferation, differentiation and survival.

The CD28-CD80/86 pathway

The first identified and best characterized costimulatory molecules belonging to the B7 superfamily are CD80 (B7.1) and CD86 (B7.2) expressed on APCs, which bind to their receptor CD28 expressed on T cells, B cells, thymocytes and macrophages.[24] CD28 colocalizes with the TCR to form a TCR-CD28 microcluster which amplifies the phosphorylation of several molecules that play a role in T cell activation.[25] CD28-CD80/CD86 signaling is essential for the activation, proliferation, cytokine secretion and survival of T cells and in absence of CD28 costimulation, T cells become anergic and may eventually die.[26] Several costimulatory molecules, such as OX40, ICOS and CD27, only exhibit low expression on naive T cells but are upregulated following TCR and CD28 signaling. To maintain homeostasis, CD28 can also bind CTLA-4, which in contrast to CD80/CD86 ligation can inhibit T cell responses. This pathway will be further discussed in another section.

CD28, CD80 and CD86 are expressed on macrophages in human atherosclerotic lesions.[22] Additionally, 5-15% of $CD3^+$ T cells present in atherosclerotic lesions expressed CD80 and CD28. Furthermore, Dopheide et al. showed that monocyte-derived DCs from patients with coronary artery disease have elevated expression of

CD80 and CD86 compared with DCs from healthy controls.[27]
In experimental atherosclerosis it was shown that LDLr$^{-/-}$ mice deficient in CD80/CD86 develop smaller atherosclerotic lesions in both initial and progressed atherosclerosis models compared with wild-type LDLr$^{-/-}$ mice.[28] In initial lesions of LDLr$^{-/-}$CD80$^{-/-}$ CD86$^{-/-}$ mice reduced MHC II expression was found, whereas advanced lesions of these mice had reduced smooth muscle cell and collagen content compared with lesions from wild-type LDLr$^{-/-}$ mice. Additionally, Buono et al. showed that CD4^{+} T cells from LDLr$^{-/-}$CD80$^{-/-}$CD86$^{-/-}$ mice produced lower amounts of IFN-γ compared with LDLr$^{-/-}$ mice upon restimulation with HSP60. Interestingly, a two-fold increase in lesion size was observed in chimeric LDLr$^{-/-}$CD80$^{-/-}$CD86$^{-/-}$ and LDLr$^{-/-}$CD28$^{-/-}$ mice due to decreased Tregs.[29] This remarkable difference between a CD80/CD86 deficiency from birth and bone marrow chimeras of CD80 and CD86 was explained by the fact that in the bone marrow transplantation model CD80 and CD86 could still be expressed on nonhematopoietic cells after irradiation. Moreover, a deficiency in CD80 and CD86 from birth could affect proliferation and activation of naive T cells, while T cells in chimeric LDLr$^{-/-}$CD80$^{-/-}$CD86$^{-/-}$ are less dependent on costimulation.

The CD40-CD40L pathway

CD40 (TNFRSF5) and CD40L (TNFSF5, CD154) form a special costimulatory dyad that not strictly mediate T cell costimulation, but mainly functions to activate APCs which subsequently upregulate the expression of other costimulatory molecules.[30, 31] Moreover, the expression of CD40L is not restricted to T cells and the expression of CD40 is not confined to the APC, suggesting functions of the CD40L-CD40 dyad other than mere costimulation.[32, 33]
The CD40L-CD40 pathway is one of the best-described costimulatory molecule pairs in the pathogenesis of atherosclerosis. In the mid '90s, it was found that both CD40L and CD40 are expressed on the vast majority of immune cells (T and B cells, monocytes, macrophages, DCs, neutrophils and mast cells) and non-immune cells (eg endothelial cells and vascular smooth muscle cells) present in the plaque, as well as on platelets in the circulation.[33-35] Both CD40L and CD40 are already expressed in early stages of atherosclerosis. Their expression increases with plaque progression and is the highest in lesions with a thin fibrous cap and in ruptured plaques.[35]
In 1998, Mach et al. showed in hyperlipidemic LDLr$^{-/-}$ mice that treatment with an anti-CD40L antibody significantly reduced the size and lipid content of aortic atherosclerotic lesions.[36] In 1999, it was shown that CD40L$^{-/-}$ApoE$^{-/-}$ mice exhibited a 5.5 fold decrease in plaque area[37], and these mice displayed a remarkable plaque phenotype. Advanced atherosclerotic plaques of CD40L$^{-/-}$ApoE$^{-/-}$ mice contained increased amounts of collagen and smooth muscle cells, while plaque lipid levels and the number of inflammatory cells were strongly reduced.[37] These plaques resemble clinically favorable, stable plaques in humans. In a follow-up study an antagonizing anti-CD40L antibody was administered to ApoE$^{-/-}$ mice on chow diet, either at the onset of atherosclerosis or when established atherosclerotic lesions were present,

as the equivalent for the situation in patients. In both treatment groups, anti-CD40L antibody treatment did not result in a decrease in plaque area but resulted in the development of lipid-poor, collagen-rich, stable plaques.[38] Schonbeck et al. showed similar results with a different anti-CD40L antibody (M158, Immunex) in high-fat diet fed $LDLr^{-/-}$ mice.[39] Transplantation of $CD40L^{-/-}$ bone marrow into $LDLr^{-/-}$ mice did not significantly alter atherosclerosis.[40,41] Interestingly, repeated administration of $CD40L^{-/-}$ platelets in $ApoE^{-/-}$ mice did prevent accelerated atherosclerosis that is induced by administration of wild type platelets.[42]

Besides the membrane-associated form, CD40L also exists in a truncated soluble form, sCD40L. sCD40L is cleaved from the CD40L protein upon activation, especially in platelets.[43] Numerous studies have proven sCD40L to be a useful biomarker for cardiovascular disease severity. Elevated levels of sCD40L have been associated with hypercholesterolemia, diabetes, ischemic stroke and acute coronary syndromes and predict increased restenosis after percutaneous coronary and carotid interventions.[44-49]

For CD40, the receptor for CD40L, the results are contradictory. In $CD40^{-/-}ApoE^{-/-}$ mice, atherosclerosis was decreased, and plaques contained only few inflammatory cells and showed increased levels of fibrosis. This phenotype was due to CD40 expressing hematopoietic cells, since bone marrow transplantation of $CD40^{-/-}$ bone marrow into irradiated $LDLr^{-/-}$ recipients resulted in similar results.[50] Zirlik et al. reported that atherosclerosis development is not affected in $CD40^{-/-}LDLr^{-/-}$ mice and claims that CD40 is not the only receptor for CD40L, but that CD40L can interact with the integrin Mac-1.[51]

The mechanisms and pathways, as well as the interacting cell-types that modulate CD40L-CD40 signaling in vascular biology, are still under investigation, but many *in vitro* studies have provided insights how CD40L-CD40 interactions affect atherosclerosis.[52] CD40L-CD40 endothelial-leukocyte interactions mediate expression of chemokines and adhesion molecules, thereby affecting leukocyte adhesion and diapedesis.[53, 54] Platelet-monocyte CD40L-CD40 interactions facilitates leukocyte recruitment and induces the expression of cytokines, whereas platelet CD40L affects platelet aggregation.[42] Activation of CD40-CD40L interactions seems to polarize T cells towards the pro-atherogenic Th1 phenotype, and CD40 is an important player in the activation of classically activated macrophages and DCs, which upon activation release a plethora of pro-atherogenic chemokines and cytokines.[50]

CD40-CD40L interactions thus play a crucial role in the development and progression of atherosclerosis, are involved in the many different aspects of the disease, and constitute promising biomarkers for cardiovascular disease.

The OX40-OX40L pathway

OX40 (TNFRSF4, CD134) and OX40L (TNFSF4, CD252) are members of the TNF/TNF receptor family, respectively.[55, 56] In contrast to other costimulatory molecules, OX40 and OX40L are expressed 2-3 days after activation. OX40 is present on activated T cells, Tregs and NKT cells, whereas OX40L is mainly expressed on APCs but is also

found on vascular endothelial cells. The OX40-OX40L interaction is important for T cell proliferation, in particular Th2 cells[57, 58], and mediates crosstalk between APCs, mast cells, smooth muscle cells and endothelial cells.[59] The OX40-OX40L axis also plays a key role in the survival of effector and memory T cells. OX40 deficient mice have reduced $CD44^{hi}CD62L^{lo}$ memory $CD4^+$ T cells, whereas OX40L transgenic mice have increased levels of memory $CD4^+$ T cells.[60, 61] Furthermore, activated $OX40^+$ T cells drive isotype switching of B cells via interaction with OX40L and interruption of the OX40-OX40L pathway.[62]

Modulation of the OX40-OX40L pathway potently ameliorates autoimmune-like diseases, such as EAE[63], GVHD[64], asthma[65] and arthritis.[66] The role of the OX40-OX40L pathway in cardiovascular disease was first discovered in the 1980s, when Wang and Paigen et al. showed that OX40L is located in Ath-1 on chromosome 1, a quantitative trait locus (QTL) that affects the differences in atherosclerosis susceptibility in Bl6 mice versus C3H/He and BALB/C mice.[67, 68] In humans, single nucleotide polymorphisms (SNPs) in both the OX40 and OX40L genes have shown to affect the incidence of cardiovascular disease.[67, 69, 70] Furthermore, patients suffering from acute coronary syndrome have increased expression of OX40 and OX40L on $CD4^+$ T cells.[71] Interestingly, elevated soluble OX40L levels in serum were associated with higher risk for cardiovascular events such as acute coronary syndrome, sudden death and recurrent angina. In addition, serum soluble OX40L correlated with carotid intima-media thickness[72], indicating sOX40L is a promising biomarker to predict occurrence of cardiovascular disease.

The role of the OX40-OX40L pathway has also been established in several mouse models of atherosclerosis. OX40L deficiency renders mice less susceptible to atherosclerosis, whereas OX40L overexpression enhances the development of atherosclerosis.[67] In addition, cholesterol levels are correlated to upregulation of OX40 expression on T cells and interruption of the OX40-OX40L interaction using an OX40L-blocking antibody leads to a 53% reduction in initial atherosclerotic lesion development.[73] Another study showed that $ApoE^{-/-}OX40L^{-/-}$ mice have, in addition to a reduction in atherosclerotic lesion formation, a reduced vascular endothelial growth factor-induced angiogenesis compared with $ApoE^{-/-}$ mice.[74] Recently, we showed that interference in the OX40-OX40L signaling pathway combined with decreases in dietary cholesterol induces regression of atherosclerosis through induction of IL-5-producing T cells and oxLDL-specific IgM and reduction in Th2 responses and mast cell numbers (*unpublished data*). These data demonstrate that OX40 and OX40L are not only promising biomarkers to predict cardiovascular events, they are also potential therapeutic targets to reduce well-established atherosclerotic lesions.

The CD27-CD70 pathway

Another costimulatory pair of the TNF(R) family involved in T cell activation is formed by CD27 and CD70 (TNFSF7). CD27 is a transmembrane homodimer constitutively expressed on naive $CD4^+$ and $CD8^+$ T cells, early thymocytes, B cells and NK cells.[75,

[76] CD27 is upregulated on T cells following TCR stimulation and is downregulated when T cells differentiate towards effector T cells.[77] Additionally, binding of CD70 to CD27 can result in proteolytic cleavage of a truncated form of CD27.[78] CD70 is a type II transmembrane glycoprotein expressed on activated DCs, macrophages, B cells and activated T cells.[22, 79, 80] Interaction of CD27 with CD70 activates the NF-κB and JNK pathways[81] and enhances the proliferation and survival of effector and memory T cells.[82] Signaling through CD27-CD70 especially induces IFN-γ and IL-2 producing $CD4^+$ T cells which in turn may promote the accumulation of $CD8^+$ memory T cells.[83] Furthermore, CD27-CD70 signaling is also involved in humoral responses as CD27 signaling enhances plasma cell formation and promotes IgG production[75], whereas CD70 engagement inhibits IgG secretion.[84]

In humans, $CD4^+CD25^+$ T cells expressing CD27 represent a highly suppressive Treg subset.[85, 86] Sardella et al. reported that patients with myocardial infarction have decreased $CD27^+$ Tregs compared with healthy individuals.[87] Both CD27 and CD70 are expressed on T cells and macrophages in human atherosclerotic lesions.[22] The role of the CD27-CD70 pathway in atherosclerosis has been investigated by Olffen et al. who constitutively triggered CD27 signaling on T cells through transgenic overexpression of CD70 on B cells in ApoE*3-Leiden mice.[88] Previously, they showed that B cell-specific CD70 transgenic mice have increased numbers of IFN-γ producing T cells which subsequently deplete B cells.[89] Surprisingly, B cell-specific CD70 transgenic ApoE*3-Leiden mice are protected against atherosclerosis despite enhanced numbers of IFN-γ producing effector T cells and inflammatory $Ly6C^{hi}$ monocytes. This anti-atherogenic effect of excessive CD27-CD70 signaling was ascribed to the increased susceptibility of monocytes to undergo apoptosis. In addition, B cell-specific CD70 transgenic ApoE*3-Leiden mice show extremely reduced oxLDL-specific antibodies as a consequence of B cell loss, which might also contribute to reduced atherosclerosis. No atherosclerosis studies have been performed using blocking or agonistic CD27 or CD70 antibodies.

The CD137–CD137L pathway

CD137 (4-1BB, TNFRSF9) and CD137L (4-1BBL, TNFSF9) are also members of the TNF(R) superfamily. CD137 is a glycoprotein and exists as a monomer or dimer on activated $CD4^+$ and $CD8^+$ T cells, NK cells, NKT cells and mast cells, and is constitutively expressed on resting monocytes, DCs, neutrophils and Tregs.[90-94] Upon activation, CD137 expression is slowly increased within hours, peaks round 60 hours, and remains stably expressed.[91] CD137 binds to its ligand CD137, which is a type II membrane glycoprotein and expressed on monocytes, macrophages, dendritic cells, activated B cells and T cells.[95, 96] Similar to other costimulatory molecules, CD137 can also be secreted in a soluble form.[97] Interaction of CD137 with CD137L initiates costimulatory responses via downstream signaling through TNF receptor-associated factor-2 and activation of NF-κB, which results in enhanced T cell proliferation and IL-2 production.[98] Interestingly, CD137-CD137L signaling affects $CD8^+$ T cell

proliferation more vigorously than $CD4^+$ T cell proliferation.[99] Moreover, CD137-CD137 signaling also affects humoral responses since engagement of CD137L induces B cell proliferation and immunoglobulin synthesis.[100]

Agonistic CD137 antibodies have been extensively used in several autoimmune diseases. Surprisingly, agonistic CD137 greatly reduced EAE[101], systemic lupus erythematosus[102] and collagen-induced arthritis[103] in mice. This rather unexpected protective effect was attributed to elevated activation-induced cell death of $CD4^+$ T cells with a subsequent suppression of autoantibody production.

A possible role for the CD137-CD137L pathway in the pathogenesis of cardiovascular disease was first shown by Drenkard et al. who showed that CD137 is expressed on endothelial cells at sites of inflammation in the arterial wall and enhances the migration of monocytes into the intima.[104] Both CD137 and CD137L are also expressed on smooth muscle cells and cardiac myocytes, which are also involved in cardiovascular disease.[105, 106] Olofsson et al. showed that human atherosclerotic arteries contain 17 times higher levels of CD137 mRNA in comparison with healthy arteries and that CD137 is mainly colocalized with endothelial cells and $CD8^+$ T cells.[107] Additionally, atherosclerotic lesions of $ApoE^{-/-}$ mice showed a 10-fold increase in CD137 mRNA expression compared with C57BL/6 mice. *In vitro* experiments demonstrated that pro-atherogenic cytokines induce CD137 expression on endothelial and smooth muscle cells and that activation of the CD137-CD137L pathway upregulates adhesion molecules on endothelial cells and reduces proliferation of smooth muscle cells. Despite the protective effect of an agonist for CD137 described in numerous autoimmune diseases, treatment with agonistic CD137 enhanced atherosclerosis development in $ApoE^{-/-}$ mice via increased inflammation, $CD8^+$ T cell infiltration and MHC II expression in lesions. Aortic expression of pro-inflammatory molecules, such as ICAM-1, IL-1β and TNFα was also increased. In another study, CD137 deficiency in hyperlipidemic $LDLr^{-/-}$ and $ApoE^{-/-}$ mice attenuated atherosclerosis, which was attributed to reduced pro-inflammatory cytokines, such as IFN-γ, MCP-1 and TNFα, released by endothelial cells and monocytes/macrophages.[108]

Furthermore, CD137 expression, either soluble or membrane-bound, is elevated in patients with acute coronary syndromes (ACS) compared with healthy controls.[109] Recently, Dumitriu et al. showed that patients with ACS have elevated levels of CD137 and OX40 on $CD4^+CD28^{null}$ T cells, a distinct subset of T cells known to expand in the circulation and atherosclerotic plaques of these patients.[110] Blockade of CD137 reduced the IFN-γ, TNFα and perforin secretion of $CD4^+CD28^{null}$ T cells isolated from PBMCs from patients with ACS. Additionally, CD137L is highly present in human atherosclerotic lesions.

These studies show an important role for CD137-CD137L signaling in atherosclerosis and future studies should identify approaches to interfere in the CD137-CD137L pathway to prevent atherosclerosis.

The CD30-CD30L pathway

CD30 (TNFRSF8) and CD30L (TNFSF8, CD153) also belong to the TNF(R) superfamily and similar to OX40 and OX40L, are mainly expressed on activated immune cells. Whereas CD30 and CD30L are both present on activated B and T cells, and especially $CD4^+$ T cells of both Th1 and Th2 phenotypes, CD30L is also expressed on other cell types, such as mature DCs, macrophages and mast cells. Signal transduction via CD30-CD30L induces activation and proliferation of T cells via TRAF1- and TRAF2-mediated NF-κB activation.[111, 112] However, under certain circumstances CD30-CD30L signaling can also promote cell cycle growth arrest and apoptosis.[113, 114] Furthermore, the CD30-CD30L pathway plays a major role in secondary humoral immune responses. Mice deficient in CD30 have impaired follicular germinal center responses and reduced secondary antibody responses.[115, 116] In addition, CD30L transgenic mice show increased numbers and activity of splenic germinal centers and have elevated serum antibody levels, such as IgG2b and IgE.[117]

The *in vivo* role of the interaction between CD30 and CD30L can be investigated using anti-CD30L antibodies, which interrupt the CD30-CD30L pathway. CD30 deficiency or treatment with a CD30L blocking antibody (RM153) significantly reduced airway inflammation in a murine asthma model by inhibition of splenocyte proliferation, reducing serum IgE levels and diminishing the Th2 cytokines IL-5 and IL-13.[118] Blazar et al. showed that anti-CD30L prolongs survival of mice in graft versus host disease.[119] Furthermore, administration of anti-CD30L completely suppressed the development of spontaneous/type I diabetes in NOD mice.[120] Interestingly, CD30 and CD30L can also be cleaved by metalloproteinases and circulate as a soluble form.[121, 122] Elevated concentrations of serum CD30 correlate with disease activity in cancer[123], systemic lupus erythematosus[124], rheumatoid arthritis[125], and HIV.[126]

Only limited research is performed to establish the CD30-CD30L pathway in cardiovascular diseases. Macrophages bearing CD30 have been identified in ruptured plaques of patients with coronary artery disease[127] and recently, we showed that treatment with a blocking anti-CD30L antibody reduced atherosclerosis with 35% in $LDLr^{-/-}$ mice fed a Western-type diet for 8 weeks, independent of plasma cholesterol levels and plaque macrophage and collagen content.[128] This reduction in atherosclerosis coincided with reduced adventitial T cell numbers, reduced percentages of $CD4^+$ T cells in the spleen and lymph nodes and with strongly reduced splenocyte proliferation. In particular, $CD4^+$ T cells isolated from anti-CD30L-treated mice proliferated less vigorously after αCD3/CD28 stimulation than $CD4^+$ T cells from control mice, whereas their adhesion and migration capacity remained unaffected. This identifies anti-CD30L treatment as a novel therapeutic modality in the inhibition of atherosclerotic lesion development and the prevention of acute cardiovascular syndromes.

The TL1A-DR3 pathway

Another costimulatory pair of the TNF-TNFR family is formed by TNF-like protein A (TL1A, VEGI, TNFSF15) and Death receptor 3 (DR3, TNFRSF12).[196] TL1A is mainly

expressed on endothelial cells and is induced on APCs[196-198] after stimulation with for example FcyR or microbial antigens.[199, 200] DR3 is expressed on a variety of cells involved in atherosclerosis such as macrophages, T cells, NK cells and endothelial cells, and regulates cell apoptosis and activation by NF-κB.[198, 201-203] Signaling via TL1A and DR3 can enhance Th1, Th2 and Th17 effector functions.[203-205]

Both TL1A and DR3 are present in macrophage/foam cell rich regions of human atherosclerotic lesions and exposure of IFN-y-stimulated human monocytes to anti-DR3 or recombinant TL1A induces the release of pro-atherogenic mediators, such as MMP-9 and IL-8.[198, 206] Furthermore, activation of human PBMCs with TNFα and LPS induced DR3 expression. More recently, McLaren et al. showed that TL1A and DR3 regulate foam cell formation by increasing cholesterol uptake via upregulation of SR-A, SR-B1, CD36 and LPL, and by reducing cholesterol efflux via ApoE, ABCA1 and ABCG1.[207] These data suggest that the TL1A-DR3 pathway might be involved in atherosclerosis via the induction of pro-inflammatory mediators, by promoting foam cell formation and by decreasing plaque stability through the induction of extracellular matrix degrading enzymes but the exact role of TL1A and DR3 in atherosclerosis remains to be elucidated.

Coinhibitory pathways in atherosclerosis

Figure 3 provides an overview of coinhibitory molecules of both the B7 and TNF(R) family that upon interaction suppress the development of atherosclerosis. Their specific contribution to the suppression of pro-atherogenic immune responses will be discussed in more detail.

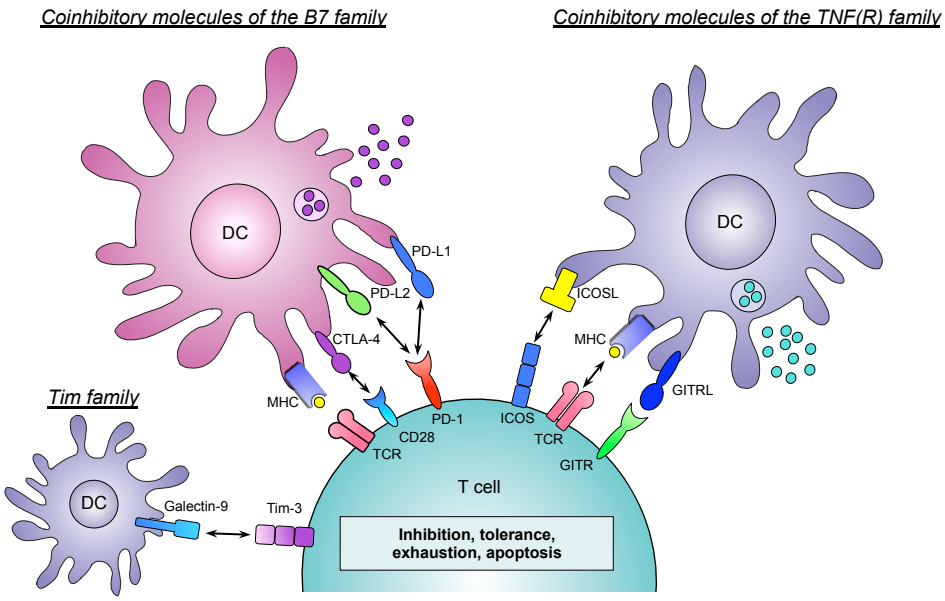


Figure 3. Signaling via coinhibitory molecules in atherosclerosis inhibits T cell function, induces tolerance and can induce apoptosis.

The CTLA-4-CD80/CD86 pathway

Similar to CD28, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152), a member of the B7-family, binds to the costimulatory molecules CD80 and CD86. Whereas the interaction between CD80/CD86 and CD28 promotes T cell responses, CTLA-4 binding to CD80/CD86 inhibits T cell activation and proliferation.[129, 130] CTLA-4 is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily[131] and is mainly expressed on Tregs and on activated $CD4^+$ and $CD8^+$ T cells[130, 132, 133] but can also be found on a variety of other cells such as monocytes[134], activated B cells[135] and embryonic stem cells.[136] CTLA-4 is predominantly an intracellular antigen whose surface expression is not detected until 24-48 hours after activation.[132] The importance of CTLA-4 in the regulation of lymphocyte homeostasis became evident when Waterhouse et al. showed that CTLA-4 deficient mice die already at 3-4 weeks of age due to severe lymphoproliferative disease.[137] Peripheral lymphoid organs of CTLA-4 deficient mice contain 5-10 times the normal amount of lymphocytes, and immunoglobulin levels, such as IgE and IgG1, are highly increased as a consequence of enhanced B cell activation. Two mechanisms are described for CTLA-4-mediated T cell inhibition. A number of studies showed that CTLA-4 competes with CD28 for the ligands CD80 and CD86 and since CTLA-4 has a 10 times greater affinity for CD80/CD86 than CD28, this limits CD28-mediated signaling and induces T cell anergy.[138, 139] CTLA-4 can also directly inhibit T cell proliferation through negative signaling via for example phosphorylation of tyrosine motifs on the cytoplasmic tail.[138, 140] However, the exact mechanism of this direct T cell inhibition remains to be elucidated.

Since CTLA-4 plays a dominant role in T cell homeostasis, CTLA-4 provides a promising therapeutic target to inhibit autoimmune diseases. Two recombinant CTLA-4-Ig soluble fusion proteins, abatacept and belatacept, are approved by the FDA for the treatment of rheumatoid arthritis and renal allograft rejection.[141] These fusion proteins consist of the extracellular CD80/CD86 binding portion of CTLA-4 coupled to IgG1 to elongate the circulating half-life, and disrupt the CD28-CD80/CD86 signaling pathway. However, limited information is available on the role of CTLA-4 in atherosclerosis. CTLA-4-Ig treatment has been shown to prevent experimental hypertension by reducing the activation and cytokine production of T cells and by abrogation of vascular T cell accumulation.[142] Increased mRNA levels of CTLA-4 have been associated with reduced atherosclerotic lesions and increased Tregs in several studies.[143-145] Ma et al. showed that CTLA-4 IgG (abatacept) ameliorates homocysteine-accelerated atherosclerosis by inhibiting T cell overactivation in $ApoE^{-/-}$ mice.[146] Abatacept treatment alone, for 2 or 4 weeks, did not reduce atherosclerosis. However, it must be noted that homocysteine induces very small atherosclerotic lesions whereas for example Western-type diet induced atherosclerosis results in much greater lesion sizes. Recently, Ewing et al. showed that abatacept treatment of hypercholesterolemic ApoE3*Leiden mice prevents intimal thickening by 59% in a femoral artery cuff mouse model for post-interventional remodeling.[147] Furthermore, treatment with abatacept resulted in a 78% reduction in accelerated atherosclerosis development, whereas treatment with a

CTLA-4 blocking antibody strongly increased lesion size by 67%. The atheroprotective effect of abatacept was attributed to a reduction in the activation of Th1 and elevated Tregs, with concomitant reduced IFN-γ and elevated IL-10 serum levels. In contrast, our group treated 30 week old or Western-type diet fed ApoE$^{-/-}$ mice with abatacept and we did not observe any effect on atherosclerotic lesion size in comparison with control IgG treatment (*unpublished data*). In conclusion, promoting CTLA-4 activity has the potential to be a useful strategy for prevention of atherosclerosis, but the mechanism-of-action in different stages of atherosclerosis is still unclear and needs to be further investigated.

The PD-1-PD-L1/2 pathway

Another coinhibitory pair in the B7-CD28 family is formed by programmed death 1 (PD-1, CD279)[148], which binds to programmed death-ligand 1 (PD-L1, B7-H1, CD274)[149, 150] and 2 (PD-L2, B7-DC, CD273).[151, 152] PD-1 is mainly expressed on activated T cells, including CD4$^+$ T cells, CD8$^+$ T cells, NKT cells and NK cells, but is also expressed on B cells and activated monocytes.[153, 154] PD-L1 is constitutively expressed on T cells, B cells, DCs, macrophages and bone-marrow derived mast cells and can also be found on non-hematopoietic cell types, such as vascular endothelial cells, epithelial cells and muscle cells.[154, 155] In contrast, expression of PD-L2 is highly restricted to DCs and activated macrophages.[151, 156] While PD-L1 and PD-L2 bind with a similar affinity to PD-1, they do cross-compete for binding to PD-1.[157] Interestingly, PD-L1 has also been shown to bind to CD80 in both mice and humans.[158, 159]

The exact mechanism by which the interaction between PD-1 and PD-L1/2 inhibits costimulation-mediated T cell proliferation and cytokine secretion remains to be fully understood. PD-1 engagement has shown to inhibit ZAP70/CD3ζ signaling and decreases the phosphorylation of ERK and PKCΘ, eventually resulting in downmodulation of TCR signaling and IL-2 secretion.[160] In addition, association of protein tyrosine phosphatases, SHP-1 and 2, to immunoreceptor tyrosine-based motifs (ITSM and ITIM) present on the cytoplasmic tail of PD-1, may inhibit T cell responses. Furthermore, PD-1 has shown to upregulate a basic leucine transcription factor, ATF-like (BATF), which impairs T cell responses.[161] In contrast, some studies report a pro-inflammatory role of PD-L1/2; PD-L2 induces IL-12 producing DCs and subsequent T cell activation.[162]

Modulation of the PD-1/PD-L1/2 pathway may be a potent approach to regulate T cell responses in autoimmune diseases such as atherosclerosis. Previously, it has been shown that PD-1 is upregulated on aortic T cells in hypercholesterolemic LDLr$^{-/-}$ mice[163], whereas PD-L1 and PD-L2 are present on macrophages and dendritic cells in aortic lesions.[164] Combined deficiency of PD-L1 and PD-L2 in LDLr$^{-/-}$ mice aggravated atherosclerosis and increased numbers of lesional CD4$^+$ and CD8$^+$ T cells.[164] In addition, LDLr$^{-/-}$PD-L1/2$^{-/-}$ mice showed increased numbers of activated CD4$^+$ T cells in lymphoid organs and serum TNFα was elevated. Both *in vitro* and *in vivo*, PD-L1/2 deficient APCs showed increased capability to activate CD4$^+$ T cells under hypercholesterolemic

conditions. Furthermore, LDLr$^{-/-}$ mice deficient in PD-1 or LDLr$^{-/-}$ mice treated with a blocking anti-PD-1 antibody also showed increased atherosclerosis development.[163] Similar to PD-L1 and PD-L2 deficiency, lesions of LDLr$^{-/-}$PD1$^{-/-}$ mice contained high numbers of CD4^{+} T cells, CD8^{+} T cells and macrophages, and TNFα levels in serum were increased. Interestingly, both PD-L1/2 and PD-1 deficiencies induced CD8^{+} T cells, which are rarely present in lesions of LDLr$^{-/-}$ mice. Bu et al. showed that CD8^{+} T cells of LDLr$^{-/-}$PD1$^{-/-}$ mice have increased cytotoxic capacity. Moreover, PD1$^{-/-}$CD8^{+} T cells are potent killers of SMC *in vitro* and could thereby play an important role in lesion stability.

There are also indications that this coinhibitory pathway regulates T cell responses in humans with cardiovascular disease. Patients suffering from coronary artery disease showed significantly decreased expression of PD-1 and PD-L1 on T cells and myeloid DCs. As a result, CD4^{+} and CD8^{+} T cell proliferation and production of pro-inflammatory cytokines was enhanced.[165]

Stimulation of the PD-1/PD-L1/2 pathway by using agonistic antibodies could be a successful approach to inhibit atherosclerosis and to further unravel the role of PD-L1 and PD-L2 in atherosclerosis, since the differential roles of PD-L1 and PD-L2 in atherosclerosis remain unclear.

The ICOS-ICOSL pathway

CD28/B7 family member inducible costimulatory molecule (ICOS) is rapidly induced on activated T cells and resting memory T cells, and its ligand ICOSL (B7h) is expressed on B cells, a small subset of T cells, monocytes, macrophages, dendritic cells and in non-lymphoid tissues, such as the lung.[166-168] ICOS$^{-/-}$ mice show impaired humoral immune responses and germinal center reactions[169, 170] and both ICOS- and ICOSL-deficient T cells show impaired proliferative capacity and IL-2 production.[170, 171] Furthermore, IL-4 production by these T cells was greatly reduced upon restimulation, which is in line with several studies that report a role for ICOS-ICOSL in Th2 differentiation.[171-173] Stimulation of ICOS-ICOSL signaling in CD4^{+} T cells cultured with αCD3 increased not only the production of IL-4 but also of IFN-γ, the hallmark cytokine of Th1 responses.[174] In line with this finding, ICOS-ICOSL interruption enhances Th1 differentiation. Moreover, CD4^{+} T cells expressing ICOS are shown to produce a great amount of anti-inflammatory IL-10.[174, 175]

The role of the ICOS-ICOSL pathway in autoimmune diseases remains controversial. ICOS$^{-/-}$ mice do not develop collagen induced arthritis, with great reductions in anti-collagen IgM and IgG2A antibodies and reduced T cell proliferation.[171] In an EAE model, ICOS deficiency or ICOS blockade during disease onset greatly exacerbated EAE, which was associated with enhanced IFN-γ production.[176] In contrast, ICOS blockade after the manifestation of EAE is protective.[177]

Afek et al. showed that ICOS and ICOSL are present in murine atherosclerotic plaques.[178] Spleens from ApoE$^{-/-}$ mice contained reduced percentages of CD3^{+}ICOS^{+} cells but surprisingly when the splenocytes were cultured in the presence of 0-50 μg/

mL oxLDL, $CD3^+ICOS^+$ cells increase dose-dependently. Subsequently, blockade of the ICOS-ICOSL interaction by immunization with a human ICOS-Ig fusion protein increased early/spontaneous atherosclerosis with 77% in $ApoE^{-/-}$ mice and increased atherosclerosis with 36% in $ApoE^{-/-}$ mice fed a high-fat diet for 8 weeks. They observed increased splenocyte proliferation after stimulation with oxLDL with concomitant increased levels of IFN-γ and reduced IL-10. However, a mechanism for this ICOS-mediated atherosclerosis induction was provided in another study by Gotsman et al. who showed that ICOS deficiency on bone marrow-derived cells significantly increased atherosclerotic lesion development with 53% in the aortic root.[179] A 3-fold increase in $CD4^+$ T cells was observed in the intima and in line with this finding, $CD4^+$ T cells isolated from $ICOS^{-/-}$ mice showed enhanced proliferation, which was ascribed to reduced Treg numbers with reduced suppressive capacity. Furthermore, $ICOS^{-/-}CD4^+$ T cells produced more Th1 and Th2 cytokines, whereas the anti-inflammatory TGF-β was decreased. In addition, Tregs and $ICOS^+$-Treg subsets are decreased in patients with myocardial infarction and stable angina.[180]

All together, these data suggest a coinhibitory role for the ICOS-ICOSL pathway in atherosclerosis through its effect on Tregs. Modulation of the ICOS-ICOSL pathway with agonistic antibodies might have a therapeutic potential to prevent cardiovascular diseases.

Tims

T cell immunoglobulin and mucin domain (Tim) proteins are type 1 transmembrane proteins expressed on various immune cells and are similar to PD-1/PD-L1/2 negative regulators of immune responses. Four functional TIM genes have been identified in the murine genome (TIM-1-4), whereas the human genome only contains three TIM genes (TIM-1,3 and 4).[181] The genes encoding Tim proteins are located on chromosome 11 (mouse) and chromosome 5 (human), which are associated with enhanced susceptibility to allergy and several autoimmune diseases, such as EAE and diabetes.[181]

Tim-1 ligation can costimulate T cell proliferation and cytokine production, and is present at very low levels on naive T cells.[182] Tim-1 is upregulated upon activation and interacts either with Tim-4 or can homodimerize with itself. Besides the direct effect on T cell proliferation, Tim-1 is also involved in Treg function. Tim-4 is preferentially expressed by APCs and upregulated upon activation.[183] Tim-4 specifically phosphorylates Tim-1 and induces T cell expansion by enhancing cell division and reducing apoptosis.[183] Although Tim-1 and Tim-4 are interesting candidates to modulate pro-atherogenic immune responses in atherosclerosis, their contribution to atherosclerosis remains to be elucidated.

Tim-3 was first discovered as a specific marker for Th1 cells[184] but is also expressed on a variety of immune cells such as NK cells, monocytes, macrophages and mast cells.[185] During innate immune responses, Tim-3 promotes inflammation via TNFα secretion by monocytes and APCs[186] and enhances macrophage clearance of intracellular

pathogens.[187] However, in adaptive immune responses, Tim-3 terminates IFN-γ driven inflammation by inducing cell death of T cells after binding to its ligand galectin-9, a soluble molecule that is upregulated by IFN-γ.[188] In addition, Tim-3 can induce Treg activity[189] and induce expansion of myeloid-derived suppressor cells, which play an important role in tumor immunology.[190] Recently, Zhang et al. showed that Tim-3 can also negatively regulate innate immune responses, since reduced Tim-3 signaling by antibody blockade or knock-down with siRNA increases the activation of monocytes.[191] The *in vivo* role of Tim-3 can be investigated using anti-Tim-3 antibodies, which interrupt the Tim-3-galectin-9 interactions. Previously, it was shown that blocking Tim-3 with either a Tim-3 blocking antibody or a Tim-3-Ig fusion protein, enhances type 1 diabetes in NOD-mice and prevents the generation of immunological tolerance in a transplantation model, by dampening the function of Tregs.[192] Furthermore, *in vivo* administration of a Tim-3 blocking antibody enhances inflammation and demyelination in a mouse model of EAE by increasing the number and activation of macrophages.[184] Blockade of the Tim-3-galectin-9 interaction also accelerates graft versus host disease via enhanced activation of Th1 cells and cytotoxic T cells.[193] In addition, blocking Tim-3 signaling aggravates inflammatory heart disease in BALB/c mice by decreasing CD80 expression on macrophages and mast cells and by reducing Tregs.[194]
Hou et al. showed that patients with atherosclerosis have augmented Tim-3 expression on NK cells, which might affect NK cell function during atherosclerosis.[195] Recently, we showed that Tim-3 acts as a negative regulator of atherosclerosis, since blockade of Tim-3 augmented atherosclerotic lesion development and enhances immune responses by increasing circulating monocytes and plaque macrophages. Additionally, Tim-3 blockade decreased IL-10 producing Tregs and Bregs (*unpublished data*). In the future, approaches to promote the Tim-3 pathway, such as treatment with agonistic Tim-3 antibodies or galectin-9, may represent novel therapeutic strategies to inhibit atherosclerotic lesion development and prevent cardiovascular diseases.

The GITR-GITRL pathway

Glucocorticoid-induced TNF receptor family related protein (GITR, TNFRSF18) and its ligand GITRL (TNFSF18) are members of the TNFR superfamily. GITR is expressed at low levels on a range of cell types such as $CD4^+$ and $CD8^+$ T cells, NK cells, B cells, macrophages, DCs, endothelial cells and smooth muscle cells, whereas Tregs constitutively express high levels of GITR.[208, 209] GITR is upregulated after T cell activation, with a peak expression after 24 hours, and induces T cell proliferation and cytokine production.[210, 211] GITR-GITRL signaling also inhibits TCR-induced apoptosis and promotes T cell survival in a MAPK and NF-κB-dependent manner.[211, 212] Additionally, GITR signaling in Tregs abrogates the suppressive capacity of Tregs.[208, 213] GITRL is mainly expressed on APCs and endothelial cells[210, 214, 215] and after activation with pro-inflammatory stimuli induces the production of IL-1β, IL-8, TNFα, MCP-1, iNOS and MMP-9.[216, 217]
Similar to the TL1A-DR3 pathway, the GITR-GITRL pathway has not been extensively

investigated in atherosclerosis. GITR and GITRL are mainly expressed in macrophage rich areas of human atherosclerotic lesions and human and mouse monocytes/macrophages produce MMP-9 and TNFα upon GITR/GITRL signaling in a NF-κB and MAPK-dependent manner.[217, 218] Furthermore, GITR positive cells are found in human atherosclerotic lesions and overlap with Foxp3$^+$ T cells.[219] Notably, the frequency of GITR and Foxp3$^+$ T cells was significantly higher in healthy arterial walls compared with atherosclerotic lesions, which is in line with the general consensus that atherosclerosis is a chronic autoimmune disease resulting from an imbalance between pro-inflammatory cell types and anti-inflammatory Tregs.

Clinical implications

In cardiovascular disease, modulation of costimulatory and coinhibitory molecules can be a powerful tool to target specific stages of atherosclerosis or specific cell types involved in the pathogenesis of atherosclerosis. A highly relevant feature of costimulatory and coinhibitory pathways is that they individually have their unique effect on the behaviour of specific immune cells and thus on the outcome of disease. For example, interference in OX40-OX40L and CD30-CD30L signaling both reduce atherosclerosis via different pathways; anti-OX40L specifically targets Th2 responses and mast cell activity, whereas anti-CD30L limits all CD4$^+$ T cell responses without affecting a specific T cell subset or other immune cells. In addition, several costimulatory and coinhibitory molecules are involved in the induction and function of Tregs. This enables the development of a treatment that particularly targets different subsets of T cells. Ultimately, it would be ideal to modulate antigen-specific pro-atherogenic T cells using blocking costimulatory antibodies and agonistic coinhibitory antibodies without affecting the general T cell population and other immune cells to limit any adverse effects on the immune system. Although several candidates of atherosclerosis specific antigens have been investigated, such as oxLDL, HSP60 and ApoB100, to date the exclusively atherosclerosis-associated antigen is not identified yet, which complicates the approach to specifically target the pro-atherogenic T cells. However, some costimulatory molecules, such as OX40, are virtually absent on naive T cells but are upregulated on activated T cells. Targeting these costimulatory molecules with blocking antibodies could specifically eliminate the pathogenic T cells without causing any side effects. Furthermore, the ligand of OX40, OX40L, is expressed on endothelial cells which upon blockade can also reduce the attraction of OX40$^+$ T cells to the site of inflammation.

Blocking and agonistic antibodies for costimulatory and coinhibitory molecules have already been extensively explored in cancer and allograft rejections. Blocking antibodies for CTLA-4 and PD-1 to boost T cell responses are approved for treatment of patients with several types of cancer.[220] In contrast to cancer where T cell activity is highly appreciated, the unwanted activation of the immune system needs to be suppressed in atherosclerosis. Therefore, whereas in cancer for example a blocking

PD-1 antibody to promote T cell activity is beneficial, in atherosclerosis an agonistic PD-1 antibody is needed to suppress T cells. CTLA-4-Ig has already been established as an effective treatment for human autoimmune diseases including rheumatoid arthritis[221] and psoriasis.[222] At present, one clinical trial has been completed using anti-OX40L in the prevention of allergen-induced airway obstruction in adults with mild asthma.[223] However, no study results are reported yet.

Although many antibodies against costimulatory and coihibitory molecules have been approved and are used in clinical settings, caution is needed when translating animal experiments to the clinic, as a Phase I clinical trial with an agonistic monoclonal anti-CD28 antibody induced a strong cytokine storm (IFN-γ, TNFα, IL-2) several hours after drug infusion, which caused multiorgan failure in six human volunteers who ended up on the intensive care unit.[224] Moreover, blocking costimulatory pathways and stimulating coinhibitory pathways may enable opportunistic infections to emerge. However, treatment can be adjusted in a way that patients will only receive blocking antibodies for costimulatory molecules or agonistic antibodies for coinhibitory molecules temporarily until the lesion is stabilized.

No clinical trials investigating blocking antibodies for costimulatory molecules and agonistic antibodies for coinhibitory molecules in cardiovascular disease have been started yet. In fact, only recently the first clinical trial involving interference of inflammatory pathways to reduce major cardiovascular events in persons with preexisting coronary artery disease was launched.[225, 226] This CANTOS trial is a large-scaled study in which over 17.000 subjects will be included to test three different doses of Canakinumab, a humanized monoclonal antibody specific for IL-1β, compared with placebo. Canakinumab is already approved in other autoimmune diseases where IL-1β plays a major role, such as Muckle-Wells syndrome and familial cold autoinflammatory syndrome.[227] This study will provide the first evidence whether interference in inflammatory pathways can reduce clinical events in cardiovascular patients and will possibly initiate numerous clinical trials focused on modulating immune responses in atherosclerosis.

Interestingly, some anti-tumor therapy trials have indicated that treatment with only a single costimulatory agonist, in addition to existing cytostatic therapy or cancer-antigen vaccination, is not effective or induces adverse immunological events. The co-administration of a second agonist or another factor that stimulates T cell function is necessary to achieve a greater anti-tumor reactivity. For example, a clinical trial with an anti-CTLA-4 antibody (MDX-010) in conjunction with anti-cancer antigen vaccination resulted in regression of cancer but unfortunately also induced severe autoimmune diseases in melanoma patients.[228] Kocak et al. showed that a combination therapy in mice with pre-existing tumors with anti-CTLA-4 and anti-4-1BB enhances anti-tumor immunity without any adverse effects on the immune system.[229] The mechanism through which the combination of anti-CTLA-4 and anti-41BB reduce each other's side effects is not fully explained but it is shown that they synergistically enhance the

suppressive capacity of regulatory T cells. Currently, a phase I clinical trial is carried out in which anti-CTLA-4 (Ipilimumab) is combined with anti-PD-1 (BMS-936558) to treat melanoma patients.[230]

It may be very likely that a combinatorial therapy may also be very effective in atherosclerosis. For example, previous studies reported a synergistic effect of OX40L and CD30L on T cell responses. Blocking CD30 together with OX40 signaling prevented lethal X-linked CD4 T cell-dependent Th1- and Th2-driven autoimmune disease in mice lacking regulatory T cells[231] and affected effector and memory T cell formation and function.[115] Moreover, a combined blockade of costimulatory signals, e.g. anti-OX40L or anti-CD30L, with activation of coinhibitory signals, e.g. PD-1 or Tim-3 agonists, could be explored to suppress for example pro-atherogenic T cells while stimulating athero-protective Tregs. More research should be performed to identify the most relevant combinations of blocking and agonistic antibodies for costimulatory and coinhibitory molecules respectively, which could be used as an immunotherapy to inhibit atherosclerosis.

Costimulatory and coinhibitory molecules are also essential for the establishment and maintenance of immunological tolerance via the induction of tolerogenic DCs and Tregs. A frequently used method to induce tolerance is by oral immunization. In atherosclerosis, oral tolerance induction to oxLDL[144], HSP60[143], β2-glycoprotein I[232] and ApoB100 peptide[233] has been shown to suppress atherosclerosis. Van Puijvelde et al. showed that oral tolerance induction against oxLDL and HSP60 increased Tregs and their CTLA-4 expression.[143, 144] Possibly, oral tolerance induction against auto-antigens such as oxLDL, can be combined with blocking antibodies against costimulatory molecules or agonists for coinhibitory molecules to achieve T cell non-responsiveness against these auto-antigens and to promote the induction of antigen-specific Tregs.

In conclusion, modulation of costimulatory and coinhibitory molecules provides novel approaches to dampen the immune response in atherosclerosis. However, further characterization of these potential new drug targets are necessary before they can be applied in clinical research.

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Chapter 3

Interruption of the OX40-OX40L pathway in LDL receptor-deficient mice causes regression of atherosclerosis

Amanda C. Foks
Gijs H.M. van Puijvelde
Ilze Bot
Mariëtte N.D. ter Borg
Kim L.L. Habets
Hideo Yagita[#]
Theo J.C. van Berkel
Johan Kuiper

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

[#] Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

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Abstract

Objective: Patients suffering from cardiovascular disease have well-established atherosclerotic lesions, rendering lesion regression of therapeutic interest. The OX40 (TNFRSF4)-OX40L (TNFSF4) pathway is important for the proliferation and survival of T cells, stimulates B cells, and is associated with cardiovascular disease. We hypothesized that interference with the OX40-OX40L pathway, in combination with decreases in cholesterol, may induce regression of atherosclerosis.

Methods and Results: LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks, after which they received chow diet and were treated with anti-OX40L or PBS for 10 weeks. A significant regression of lesions was observed in the aorta and aortic arch of anti-OX40L-treated mice compared with control mice. Interference of the OX40-OX40L pathway reduced Th2 responses, as shown by decreases in GATA-3 and IL-4 levels. Also, IgE levels were decreased, as demonstrated by reduced mast cell presence and activation. Notably, IL-5 production by T cells and B1 cells was increased, thus enhancing atheroprotective oxLDL-specific IgM production. The increase in IL-5 production and IgM was mediated by IL-33 production by APCs upon OX40L blockade.

Conclusions: We conclude that interruption of the OX40-OX40L signaling pathway, combined with decreases in dietary cholesterol, induces the regression of atherosclerosis through induction of IL-5-producing T cells and oxLDL-specific IgM and reductions in Th2 and mast cell numbers.

Introduction

Atherosclerosis is a chronic autoimmune-like disease resulting from endothelial damage, subsequent vascular dysfunction, and cholesterol accumulation in the arterial wall. The development of experimental therapies for the treatment of atherosclerosis has focused mainly on preventing the initiation and, to a lesser degree, the progression of atherosclerosis. Patients suffering from cardiovascular disease, however, have well-established lesions and could benefit from a therapy that decreases the extent of disease. Currently, patients are usually treated with statins to lower LDL cholesterol, but these drugs have very little effect on established lesions.[1, 2] Several strategies to induce regression via modulation of lipid homeostasis have been tested in mouse models of atherosclerosis. Reintroducing apolipoprotein E (ApoE) into ApoE$^{-/-}$ mice via ApoE gene transfer induces regression.[3, 4] Furthermore, LXR compounds induce atherosclerotic regression by promoting reverse cholesterol transport in macrophages.[5] Another approach to study regression was introduced by Feig et al., who used a model in which atherosclerosis-containing aortic segments from ApoE$^{-/-}$ mice were transplanted into wild-type recipient mice.[6] However, the mechanisms underlying regression of atherosclerosis are not completely understood. While Feig et al. argued that promoting the emigration of CCR7-dependent monocytes/macrophages (CD68^{+} cells) is required for maximal regression, Potteaux et al. hypothesized that enhanced suppression of monocyte recruitment and increased macrophage apoptosis is essential for regression of atherosclerosis.[7]

In addition to lipids, immune responses play a pivotal role in the pathogenesis of atherosclerosis. Oxidized LDL-cholesterol (oxLDL) is generally accepted as an antigen that activates the adaptive immune system after recognition by APCs such as macrophages and dendritic cells (DCs).[8] Although antibody treatment against a specific epitope of oxLDL, ApoB100, has been shown to induce regression of atherosclerosis, the effect of modulating immune responses on regression has not been explored.[9] T cells are important in the process of atherosclerosis and affect all stages of the disease process. For maximal activation, T cells require T cell receptor-mediated antigen stimulation and costimulatory signals provided by costimulatory molecules on APCs. OX40 and OX40L are costimulatory molecules and belong to the TNF/TNF receptor family, respectively. OX40 is mainly present on activated T cells, whereas OX40L is mostly expressed on APCs but is also found on vascular endothelial cells. The OX40-OX40L interaction is important for T cell proliferation and survival; in particular, it promotes Th2 responses.[10] Furthermore, activated OX40^{+} T cells drive isotype switching of B cells via interaction with OX40L.[11]

Modulation of the OX40-OX40L pathway potently ameliorates autoimmune-like diseases, such as EAE[12], GVHD[13], asthma[10], and arthritis.[14] We have previously linked increased levels of cholesterol to upregulation of OX40 expression on T cells and showed that interruption of the OX40-OX40L interaction using an OX40L-blocking antibody leads to a reduction in the initiation of atherosclerosis.[15] In addition, Nakano et al. showed that the OX40-OX40L pathway plays an important role in *vasa vasorum*

formation.[16] OX40L is located in Ath-1 on chromosome 1, a QTL that affects the differences in atherosclerosis susceptibility in Bl6 mice versus C3H/He and BALB/C mice.[17, 18] OX40L deficiency renders mice less susceptible to atherosclerosis, whereas OX40L overexpression enhances the development of atherosclerosis.[17] In humans, SNPs in both the OX40 and OX40L genes affect the incidence of cardiovascular disease.[17, 19, 20]

In the present study, we aimed to induce the regression of atherosclerotic lesions by a combined anti-inflammatory and lipid-lowering strategy. Atherosclerosis-prone LDL receptor-deficient ($LDLr^{-/-}$) mice that had previously been fed a Western-type diet for 10 weeks were placed back on a chow diet to lower plasma cholesterol levels and given anti-OX40L treatment, after which the degree of atherosclerosis was quantified.

Materials and Methods

Animals

Male $LDLr^{-/-}$ mice, 10-12 weeks old, were obtained from Jackson Laboratories and male C57BL/6J mice were obtained from Charles River 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, Witham, Essex, UK). Diet and water were provided *ad libitum*. All animal work was approved by the regulatory authority of Leiden University and performed in compliance with the Dutch government guidelines.

Regression of atherosclerosis

To study regression of atherosclerosis, mice were initially fed a Western-type diet for 10 weeks. At week 10, a baseline group (n=13) 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 300 µg of anti-OX40L antibody (RM134L) in 150 µl of sterile PBS twice a week during 10 weeks (n=14). 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. Pittsburg, USA). Tissues were snap frozen in liquid nitrogen and stored at -80 °C until further use.

Serum cholesterol levels

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

Histological analysis and morphometry

Cryosections of the aortic root (10 µm) were collected and stained with Oil-Red-O.

Lesion size was determined in 5 subsequent sections of the heart within the three aortic valves. Lesion collagen content was determined with a Masson trichrome staining (Sigma-Aldrich, Zwijndrecht, The Netherlands). 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, Serotec). 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 adventitial T cells, a CD3 staining was performed using anti-mouse CD3 (1:50, BD Biosciences Pharmingen, San Diego. CA). Mast cells were visualized by staining with Chloro-Acetate Esterase (CAE, Sigma-Aldrich) according to manufacturer's protocol. Mast cell numbers and the extent of mast cell degranulation were assessed manually. 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. Collagen content was determined with a Masson trichrome staining. Morphology was studied using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK).

Flow cytometry

After sacrificing the mice, blood, spleen and peritoneal cells were isolated (n=5 per group). Single cell suspensions were obtained by squeezing the cells through a 70 µm cell strainer. 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). Subsequently, cells were stained for the following surface markers: CD4, OX40, CD5, IgM, CD3 and NK1.1 (0.20 µg Ab/200.000 cells). For intracellular staining, cells were fixed and permeabilized according to manufacturer's protocol (eBioscience, Belgium). Subsequently, the cells were stained for IL-5, GATA-3, Foxp3, T-bet or RORγt. All antibodies were purchased from eBioscience (Belgium) or Beckton Dickinson (Mountain View, CA). FACS analysis was performed on a FACSCantoII (Beckton Dickinson). Data were analyzed using FACSDiva software.

Serum antibody detection

IgM levels against oxLDL were detected in serum using Abs recognizing mouse IgM and HRP-labeled goat anti-rat Ig (BD Pharmingen). OxLDL (5 µg/mL) was dissolved in $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and was coated o/n onto a flat-bottom 96-well high binding plate (Corning, NY). Serum samples were 1:1 diluted in PBS and absorbance was detected at 450 nm. Total IgE in serum was determined by a mouse IgE quantitative ELISA according to manufacturer's protocol (Bethyl Laboratories, Montgomery TX, USA).

Cytokine determination in serum and supernatant of splenocytes

To detect IL-5 in serum an ELISA was performed according to manufacturer's protocol

(eBioscience, Belgium). Serum samples were 1:1 diluted in assay diluent and absorbance was detected at 450 nm. Eotaxin levels were measured with an eotaxin ELISA kit (R&D Systems) and used according to manufacturer's protocol. Absorbance was measured at 450 nm. For the detection of IL-4, IL-5 and IL-10, splenocytes isolated from mice at sacrifice (n=5 per group) were cultured for 48 hours in triplicate at $2x10^5$ cells/well in the presence of 2 μg/mL αCD3 and αCD28. Supernatant was collected and cytokine concentrations were determined by ELISA (eBioscience, Belgium).

Real-time PCR assays
Spleens from baseline mice (n=13), control mice (n=14) and anti-OX40L mice (n=14) were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (RevertAid M-MuLV reverese transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, CA) using SYBR green technology. The following primer pair was used: 5'-GATGGGAAGAAGGTGATGGGTG-3' and 5'-TTGTGAAGGACGAAGAAGGC-3' for IL-33. Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyltransferase (HPRT) were used as the endogenous references.

Cell culture
Bone marrow cells were harvested from the femora and tibia of C57BL/6J mice and were cultured for 10 days in complete IMDM supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) to obtain immature DCs. To obtain immature macrophages, bone marrow cells were cultured for 7 days in complete RPMI supplemented with M-CSF (L929 supernatant). Immature DCs and macrophages were stimulated with copper-oxidized LDL in the absence or presence of 1, 5 or 10 μg/mL anti-OX40L (RM134L). IL-33 production was analyzed with flow cytometry by using IL-33-PE (R&D Systems).

Statistical analysis
All data are expressed as mean±SEM. An unpaired two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Probability values of $P<0.05$ were considered significant.

Results

Interruption of the OX40-OX40L pathway induces regression of atherosclerosis
To study the regression of atherosclerotic lesions, we investigated the effect of lowering cholesterol combined with an immunomodulatory treatment on pre-formed atherosclerotic lesions. To this end, we put $LDLr^{-/-}$ mice that had been fed a Western-type-type diet for 10 weeks on a chow diet for another 10 weeks, combined with simultaneous administration of anti-OX40L or PBS. A baseline group was sacrificed after 10 weeks on the Western-type diet to determine the effect of treatment on atherosclerotic lesion size. No significant differences in weight and cholesterol levels

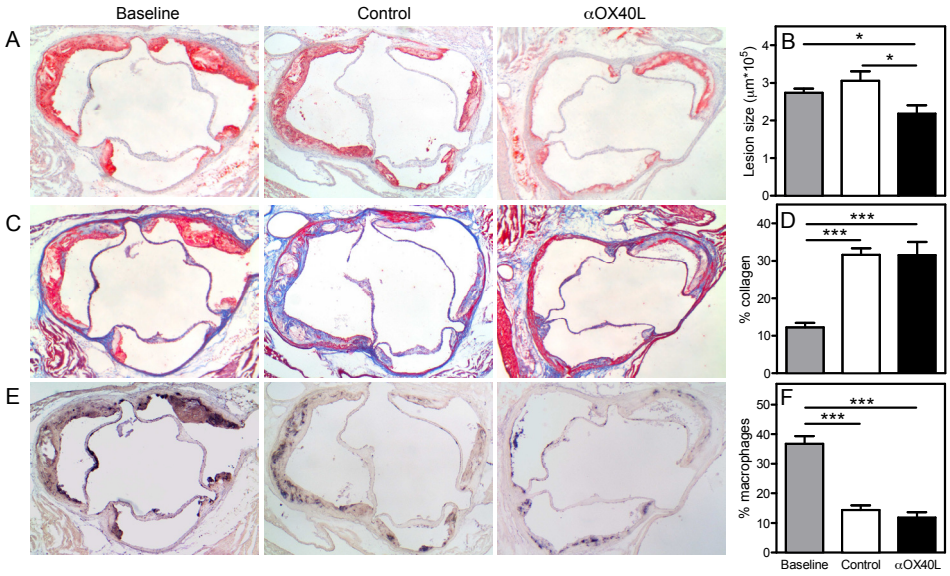


Figure 1. Anti-OX40L treatment induces regression of atherosclerosis. LDLr$^{-/-}$ mice received Western-type diet for 10 weeks and were subsequently put on chow diet for 10 weeks and simultaneously treated with anti-OX40L (n=14) or PBS (n=14). A baseline group was sacrificed after 10 weeks of Western-type diet (n=13). Sections of the aortic root were stained with Oil-Red-O and hematoxylin (A) and subsequently lesion size was determined (B). Corresponding sections on separate slides were also stained for collagen using Masson's trichrome staining (C). The percentage of collagen relative to the lesion size was determined (D). Furthermore, relative macrophage content was determined with a MOMA-2 staining (E) and quantified (F). *P<0.05, ***P<0.001

were found between baseline, control and anti-OX40L-treated mice after 10 weeks of Western-type diet (data not shown). In addition, no differences in weight and cholesterol levels were observed between the control and anti-OX40L-treated mice 10 weeks after switching to chow diet. After 10 weeks on the chow diet, the mice were sacrificed, and the degree of atherosclerosis was analyzed at a number of sites in the vascular bed. Figure 1A shows representative cross-sections of lesions in the three-valve area of the aortic root. We observed a significant 29% reduction in the aortic root lesion size in anti-OX40L-treated mice ($2.18\times10^5\pm0.22\times10^5$ μm^2) compared with control mice ($3.06\times10^5\pm0.25\times10^5$ μm^2, P<0.05) and a 20% reduction compared to baseline mice ($2.74\times10^5\pm0.10\times10^5$ μm^2, P<0.05, Figure 1B). With respect to the composition of the lesion, no differences were found in lesion collagen content between anti-OX40L-treated mice (31.5±3.5%) and control mice (31.6±1.8%). We did observe a significant increase in the relative collagen content (P<0.001) in both groups of mice that were put on a low-fat diet compared to baseline-sacrificed mice (collagen content 12.2±1.2%, Figure 1C-D). Furthermore, the relative macrophage content did not significantly differ in the anti-OX40L-treated group (13.4±2.3%) versus the control group (14.3±1.6%, Figure 1E-F). 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).

Analysis of lesion formation in the aortic arch showed an even greater capacity of anti-OX40L to induce regression when administered in combination with cholesterol lowering (Figure 2A-B). The lesion size in anti-OX40L-treated mice ($1.73\times10^5\pm0.22\times10^5$ μm^2) was reduced by 38% in comparison with control mice ($2.78\times10^5\pm0.39\times10^5$ μm^2, $P<0.05$) and by 27% compared with baseline mice ($2.38\times10^5\pm0.22\times10^5$ μm^2, $P<0.05$).

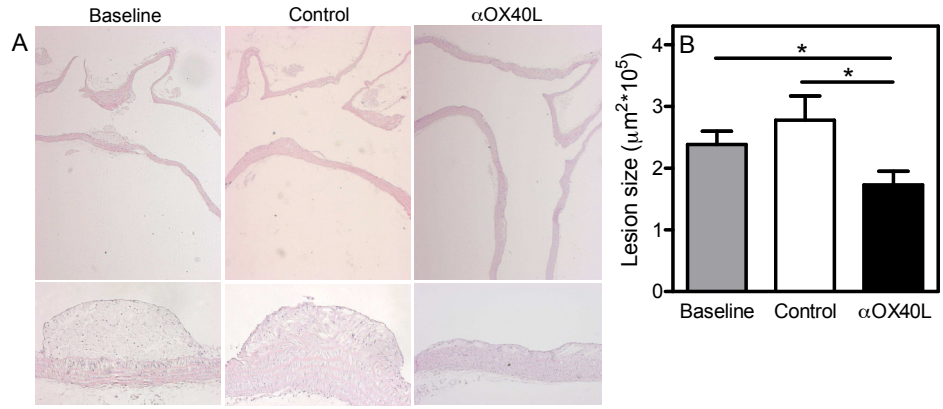


Figure 2. Anti-OX40L treatment induces regression of atherosclerosis in the aortic arch. $LDLr^{-/-}$ mice received Western-type diet for 10 weeks and were subsequently put on chow diet for 10 weeks and simultaneously treated with anti-OX40L (n=14) or PBS (n=14). A baseline group was sacrificed after 10 weeks of Western-type diet (n=13). The aortic arch and its main branch points were excised, fixed, and embedded in paraffin. Longitudinal sections of the aortic arch were stained with hematoxylin and eosin (A) to analyze lesion extent (B). *$P<0.05$

Anti-OX40L treatment reduces OX40 expression and adventitial T cell numbers

To determine whether anti-OX40L treatment effectively interrupted the OX40-OX40L pathway, OX40 expression on T cells in the blood was determined using flow cytometry. As shown in Figure 3A, a 47% decrease in $CD4^+OX40^+$ T cells within the $CD4^+$ T cell population was observed in the blood of mice treated with anti-OX40L (9.9±0.8%) compared with control mice (18.5±1.3%, $P<0.001$). Furthermore, we analyzed the aortic root to identify $CD3^+$ T cells within lesions and found almost no T cells in the lesions of the baseline, control and anti-OX40L-treated mice. However, we observed a significant 56% reduction in the number of $CD3^+$ T cells within the adventitia of anti-OX40L-treated mice (17.6±1.4 T cells/section) compared with control mice (40.3±6.3 T cells/section, $P<0.05$, Figure 3B-C). Notably, both groups showed an accumulation of adventitial $CD3^+$ T cells compared with baseline mice (7.7±1.8 T cells, $P<0.01$) after the switch to the chow diet.

Interruption of the OX40-OX40L pathway reduces Th2 responses

OX40/OX40L costimulatory molecules modify T cell polarization; in particular, they promote Th2 responses.[10] In addition, several studies indicate that OX40 expression

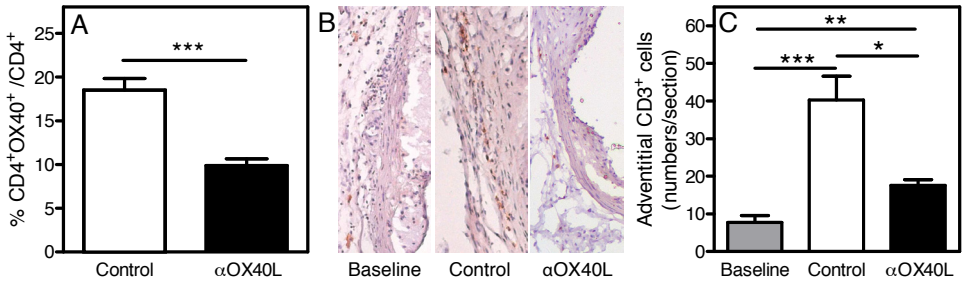


Figure 3. Decreased OX40 expression and adventitial $CD3^+$ T cell infiltration in anti-OX40L-treated mice. $LDLr^{-/-}$ mice received 10 weeks Western-type diet and were subsequently put on chow diet for 10 weeks and treated with anti-OX40L (n=14) or PBS (n=14). A baseline group was sacrificed after 10 weeks of Western-type diet (n=13). At sacrifice, OX40 expression on T cells in the blood was determined by flow cytometry (A). Sections of the aortic root were stained for CD3 (red) to determine the number of infiltrating T cells (B-C). **P*<0.05, ***P*<0.01, ****P*<0.001

on T cells inhibits Treg differentiation and may thus enhance inflammation.[21] The differentiation of naive T cells into Th1, Th2, Th17 or Treg cells following anti-OX40L treatment was analyzed using flow cytometry. Anti-OX40L-treated mice had a significantly reduced number of GATA-3^+ cells within the $CD4^+$ T cell population of the blood (5.0±0.6% versus 7.0±0.4% (control), *P*<0.05, Figure 4A) and spleen (3.6±0.4% versus 5.3±0.4% (control), *P*<0.05). The other T cell subsets, Th1, Th17 and Tregs, remained unaffected under hypercholesterolemic conditions following anti-OX40L treatment (data not shown). To further define the reduced Th2 response, splenocytes isolated from anti-OX40L-treated mice and control mice were cultured for 48 hours in the presence of αCD3/CD28 stimulation. Secretion of the Th2 cytokines IL-4 and IL-10 was significantly (*P*<0.05) decreased by splenocytes from anti-OX40L-treated mice, whereas IL-5 secretion was unexpectedly increased in these mice, compared with IL-5 production by splenocytes from control mice (Figure 4B).

Anti-OX40L treatment increases the production of atheroprotective oxLDL-specific IgM via IL-5

Previous studies have shown that IL-5 exerts its atheroprotective functions in part by promoting oxLDL-specific IgM secretion by B1 cells, a subset of B cells that is predominantly found in peritoneal cavities.[22, 23] To determine whether the increased IL-5 levels observed in anti-OX40L-treated mice induced oxLDL-specific IgM production in B1 cells, we quantified the serum IL-5 levels. As shown in Figure 4C, IL-5 was elevated in the serum of anti-OX40L-treated mice (229.5±52.6 pg/mL) compared with control mice (123.7±4.1 pg/mL, *P*<0.05), which is in agreement with our previous findings on OX40L inhibition.[15] In addition, increased percentages of IL-5-producing cells were found in the blood of anti-OX40L-treated mice (3.0±0.7% versus 0.8±0.1% (control), *P*<0.05, Figure 4D). Most importantly, IL-5 production was increased in the peritoneum of anti-OX40L-treated mice (17.4±3.2%) compared with controls (7.1±1.1%, *P*<0.05, Figure 4D), and increased numbers of B1 cells ($CD4^-$

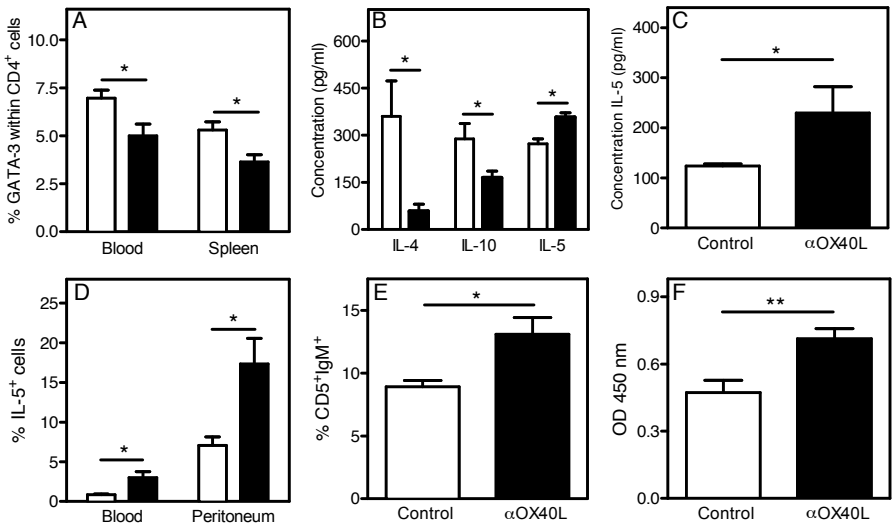


Figure 4. Reduced Th2 response and increased oxLDL-specific IgM production by B1 cells in anti-OX40L treated mice. LDLr$^{-/-}$ mice received 10 weeks Western-type diet and were subsequently put on chow diet for 10 weeks and treated with anti-OX40L (n=14) or PBS (n=14). The treated mice were sacrificed 20 weeks after initiation of the experiment. At sacrifice, GATA-3 expression in CD4$^+$ T cells from the blood and spleen was determined by flow cytometry (A). Detection of IL-4, IL-5 and IL-10 was determined by ELISA in the supernatant of splenocytes stimulated with αCD3 and αCD28 for 48 hours (B). IL-5 present in serum of treated mice was determined by ELISA (C). At sacrifice, IL-5 production by cells in blood and the peritoneum was determined by flow cytometry (n=5 per group) (D). In the peritoneum B1 cells (CD4$^-$ CD5$^+$IgM$^+$) were stained (E). oxLDL-specific IgM production was detected in serum of control (n=9) and anti-OX40L treated mice (n=8) (F). *P<0.05, **P<0.01

CD5$^+$IgM$^+$) were found in the peritoneum of anti-OX40L-treated mice (13.1±1.3% versus 8.9±0.5% in control mice, P<0.05, Figure 4E). Furthermore, a significant 51% increase in oxLDL-specific IgM was found in the serum of anti-OX40L-treated mice, as shown in Figure 4F (P<0.01). We previously showed that the OX40-OX40L interaction also affects T cell-dependent humoral responses.[15]

Anti-OX40L treatment induces IL-5-producing T helper cells

Most researchers agree that a single T cell population secretes a complete panel of typical Th2 cytokines. However, splenocytes from anti-OX40L-treated mice produced increased levels of IL-5 but had a vast decrease in IL-4 secretion (Figure 4B), which may indicate that a specific subset of T cells is responsible for the production of IL-5. Therefore, we investigated which cells produced IL-5, independent of IL-4, in anti-OX40L-treated mice. Notably, we observed increased levels of IL-5-producing CD4$^+$ T cells in the peritoneum of anti-OX40L-treated mice compared with control mice (1.1±0.2% versus 2.9±0.4%, respectively, P<0.01, Figure 5A). A newly described IL-1 family member, IL-33, induces antigen-specific IL-5$^+$ T cells and oxLDL antibody production.[24] We therefore analyzed IL-33 production in anti-OX40L-treated mice; quantitative PCR demonstrated a 4-fold higher relative expression of IL-33 in the spleen of anti-OX40L-treated mice compared with control mice (P<0.05, Figure 5B).

IL-33 is produced by a variety of cells, including macrophages and dendritic cells.[25] We determined the effect of anti-OX40L on cultured, oxLDL-stimulated macrophages and dendritic cells; anti-OX40L dose-dependently increased IL-33 secretion by both macrophages (Figure 5C) and DCs (Figure 5D), as measured using flow cytometry.
Finally, high levels of IL-5 are associated with increased amounts of eosinophils.[26] Eotaxin is the most potent chemoattractant for eosinophils, and using an ELISA, we found that eotaxin levels did not differ between control and anti-OX40L-treated mice, whereas the reduction in cholesterol upon the switch to chow diet did reduce eotaxin levels (data not shown).

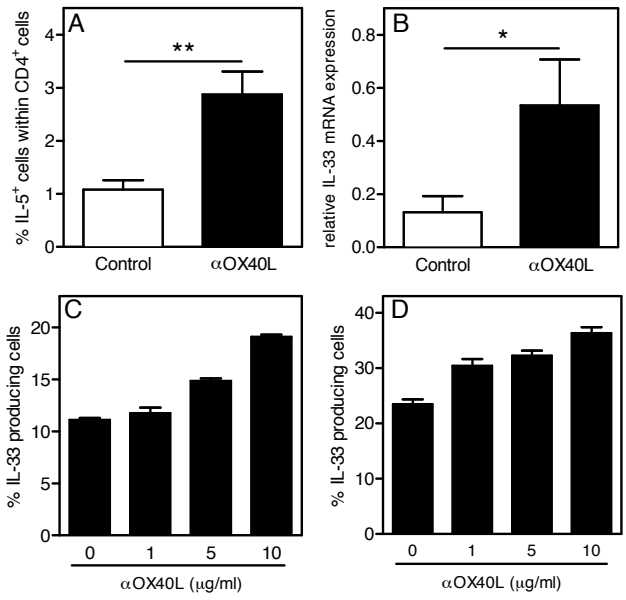


Figure 5. Anti-OX40L treatment induces Th5 cells via IL-33. LDLr$^{-/-}$ mice received 10 weeks Western-type diet and were subsequently put on chow diet for 10 weeks and treated with anti-OX40L (n=14) or PBS (n=14). At sacrifice, CD4$^+$ T cells secreting IL-5 in the peritoneum were determined with flow cytometry (A). mRNA expression of IL-33 in the spleen was determined in response to anti-OX40L treatment (B). Expression of IL-33 is expressed relative to 36B4 and HPRT, and subsequently compared with the expression in control mice. Bone-marrow derived macrophages (C) and DCs (D) were cultured in the presence of 2.5 μg/mL oxLDL with increasing concentrations of anti-OX40L. IL-33 secretion was measured with flow cytometry. *P<0.05, **P<0.01

Decreased mast cell activity in anti-OX40L-treated mice

Besides promoting the development of naive T cells into Th2 cells, IL-4 also affects IgG and IgE isotype switching in B cells. Because interruption of the OX40-OX40L pathway reduces IL-4 levels and Th2 development (as indicated by the number of GATA3$^+$ T cells), we determined whether IgE secretion was affected by anti-OX40L treatment. We found a 68% decrease in IgE levels in anti-OX40L-treated mice (36±7 ng/mL) compared with control (113±25 ng/mL, P<0.01) and baseline (110±23 ng/mL, P<0.01, Figure 6A) mice. Paralleling the reduction in IgE serum levels, a 20% reduction in mast cells (MC) was found in the atherosclerotic lesions of the aortic root in anti-OX40L-treated mice (18.5±1.6 MC/mm^2) compared with control (23.2±1.1 MC/mm^2, P<0.05) and baseline (24.4±1.6 MC/mm^2, P<0.05, Figure 6B-C) mice. Moreover, 40% and 63% decreases in activated mast cells were found in anti-OX40L-treated mice (2.1±0.32 MC/mm^2) compared with control (3.5±0.35 MC/mm^2, P<0.01) and baseline (5.6±0.66 MC/mm^2, P<0.001, Figure 6D) mice, respectively.

Notably, cholesterol reduction alone reduces mast cell degranulation but does not change the total number of mast cells.

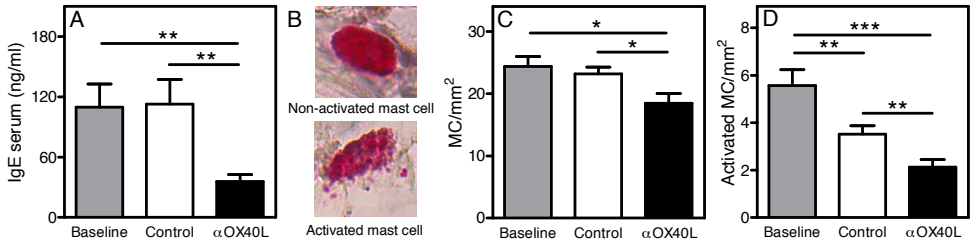


Figure 6. Reduction of IgE and mast cell activation in anti-OX40L treated mice. LDLr$^{-/-}$ mice received Western-type diet for 10 weeks and were subsequently put on chow diet for 10 weeks and simultaneously treated with anti-OX40L (n=14) or PBS (n=14). A baseline group was sacrificed after 10 weeks of Western-type diet (n=13). IgE was measured in the serum of baseline, control and anti-OX40L treated mice (A). Sections of the aortic root were stained with CAE to detect mast cells (B). Mast cell numbers (C) and the extent of mast cell degranulation (D) were assessed manually. *P<0.05, **P<0.01, ***P<0.001

Discussion

Experimental therapies aimed at achieving regression of atherosclerotic lesions in both human and animal models currently focus on lowering plasma LDL levels using statins[2] and improving reverse lipid transport and plasma HDL levels using agents such as phosphatidylcholine (PC) liposomes[27], CETP inhibitors[28], LXR agonists[6], and more recently, modulators of miR-33 expression.[29] Atherosclerosis, however, results not only from lipid accumulation but is also a chronic autoimmune-like disease and immune responses promote disease in every stage of atherosclerosis. Immune cells such as APCs, T cells, B cells and mast cells contribute to lesion initiation and progression, and modulation of these cells may yield an unexplored therapeutic strategy to induce regression of well-established atherosclerotic lesions. Therefore, in the present study, we aimed to simulate the effect of plasma lipid lowering (mimicking statin treatment) by switching mice from a Western-type diet to a chow diet in combination with reducing the immune response by interfering with the OX40-OX40L pathway.

Here, we show that lipid lowering alone increased lesion stability, as demonstrated by increases in collagen content, but only additional anti-inflammatory treatment with an anti-OX40L antibody induced lesion stabilization and regression. Furthermore, a robust loss in macrophage content was observed in both control and anti-OX40L-treated mice compared with baseline mice. The biological processes underlying the morphological changes of a regressing lesion are subjects of discussion. Whether a decrease in lesion macrophage content during regression results from enhanced macrophage efflux[6], reduced monocyte influx[7], increased macrophage apoptosis[7], or enhanced influx of functional phagocytes that clear debris from the lesions is currently being debated.[30] Notably, we show herein that after the switch to chow diet, control mice show a 5-fold increase in adventitial CD3$^+$ T cells compared with the number of adventitial T cells before the diet switch. This large increase suggests that T cells are

attracted to the lesion and parallels the decrease in the number of macrophages, which may indicate that macrophage death within the lesion results in the attraction of these adventitial T cells. These newly infiltrating T cells may support a new, fibrosis-like inflammatory process and thereby prevent lesion regression in control mice, whereas anti-OX40L-treated mice, which have a significantly lower influx of adventitial $CD3^+$ T cells, show significant lesion regression in the aortic root and arch. The reduction in T cell numbers in the regressed lesions correlates with the significant 47% reduction in circulating OX40-expressing $CD4^+$ T cells and the reduced mast cell activation, although the number of adventitial $CD3^+$ T cells in these mice was still higher than in baseline mice.

Previously, we demonstrated that anti-OX40L treatment reduces initial atherosclerosis in part via a reduced Th2 response.[15] We now confirm that interruption of the OX40-OX40L pathway also induces elevated levels of IL-5, a characteristic Th2 cytokine, and enhances oxLDL-specific IgM levels in serum when plasma cholesterol levels are normal. We also show that the source of the enhanced IL-5 levels is a specific subset of T helper cells. Consistent with previous findings, interruption of the OX40-OX40L pathway reduced the Th2 response, as shown by decreases in GATA-3, IL-4 and IL-10 expression in anti-OX40L-treated mice. Notably, we demonstrate that the two typical Th2 cytokines, IL-5 and IL-4, are differentially expressed after anti-OX40L treatment, which may result from the fact that IL-4 and IL-5 are differentially regulated at the transcriptional level. Whereas differentiation of IL-4-producing T cells is dependent on STAT6[31] and GATA-3[32], Kurowska et al. showed that IL-5-producing T cells differentiate independently of STAT6 and GATA-3.[24] These $CD4^+$-IL-5 producing T cells can be induced by IL-33, a novel cytokine and a member of the IL-1 family. IL-33 binds to a receptor complex composed of ST2L and IL-1RAcP, which is expressed on Th2 cells and mast cells.[25] Recently, Miller et al. showed that IL-33 reduces the development of atherosclerosis in $ApoE^{-/-}$ mice via induction of IL-5 and anti-oxLDL-specific IgM antibody formation.[33] Furthermore, IL-33 inhibits foam cell formation via a decrease in oxLDL uptake and an increase in cholesterol efflux[34] and can protect against the development of adipose tissue inflammation during obesity.[35] Indeed, a 4-fold increase in IL-33 expression in the spleen was observed in anti-OX40L-treated mice compared with control mice. In addition, *in vitro* experiments showed that IL-33 production is dose-dependently increased by anti-OX40L treatment of DCs and macrophages exposed to oxLDL. We also found an increase in the numbers of B1 cells, which subsequently produce oxLDL-specific IgM. These findings are consistent with a study by Binder et al., who showed that IL-5-mediated stimulation of B1 cells is responsible for the increased secretion of natural IgM that is specific to oxLDL.[22] OxLDL-specific IgM plays a protective role in atherosclerosis because it prevents foam cell formation by inhibiting oxLDL uptake by macrophages, helps clear apoptotic cells and prevents inflammatory reactions towards oxLDL and other modified lipids.[36, 37] Furthermore, IL-5 deficiency leads to decreased oxLDL-specific IgM titers and enhanced atherosclerosis.[22]

The increases in the atheroprotective factors IL-33, IL-5 and oxLDL-specific IgM in anti-OX40L-treated mice likely contribute to the observed regression of atherosclerosis. The exact mechanism of IL-33 secretion is unknown; however, IL-33 acts as an alarmin and is secreted by necrotic cells.[38] Blockade of the OX40-OX40L pathway induces anergy in cells[39], which may induce secretion of the alarmin IL-33, and IL-33 may directly contribute to lesion regression by enhancing cholesterol efflux from macrophages within the lesion.

An additional pathway via which OX40-OX40L blockade may facilitate lesion regression is a reduction in IgE levels and subsequent mast cell activation. IL-4 induces isotype switching of B cells from IgM- to IgE- and IgG-producing cells. Previously, we showed that anti-OX40L treatment reduces IgG1 levels[15]; we now show that interruption of OX40-OX40L treatment also induces a strong reduction in serum IgE, which is consistent with several studies that show reduced IL-4 production and IgE serum levels upon anti-OX40L treatment[40, 41] or in $OX40L^{-/-}$ mice following H. polygyrus exposure.[42] IgE is a common mast cell activator that binds to the high-affinity Fc epsilon receptor present on mast cells and subsequently induces the release of inflammatory compounds, such as histamine, TNFα and IL-6. This IgE-induced mast cell activation is of particular interest with respect to atherosclerosis and lesion regression because mast cells are implicated in cardiovascular disease. Activated mast cells are found in the adventitia of vulnerable and ruptured lesions of patients suffering from myocardial infarction[43, 44], and mast cell numbers correlate with the incidence of plaque rupture and erosion.[43] Our lab has previously shown that mast cells also play a crucial role in plaque progression and destabilization *in vivo*.[45] We now demonstrate that a reduction in plasma IgE levels reduces mast cell numbers and activation in anti-OX40L-treated mice. Mast cells not only contribute to atherosclerosis by releasing proteases and histamine but also secrete pro-inflammatory molecules that directly interact with T cells and can function as so-called "non-professional" APCs. Nakae et al. showed that anti-OX40L treatment reduces IgE/Ag-dependent mast cell-mediated T cell proliferation and cytokine production.[46] Thus, reductions in mast cell numbers and activation may contribute to the reduction in adventitial $CD3^+$ T cells observed in anti-OX40L-treated mice compared with control mice. Furthermore, switching mice to a low-fat diet does not affect mast cell numbers but reduces mast cell activation, thereby possibly reducing the recruitment of new immune cells to the site of inflammation. In addition, enhanced IgE levels were observed in patients with unstable angina pectoris and in dyslipidemia[47, 48] and recently, Wang et al. showed that IgE promotes atherosclerosis in $ApoE^{-/-}$ mice.[49] In line with these findings, we suggest that IgE may play an important role in atherosclerosis and show that modulation of the OX40-OX40L pathway reduces IgE levels and thereby contributes to lesion regression.

Conversely, IL-33 enhances inflammatory responses in other autoimmune diseases, such as rheumatoid arthritis. IL-33 is abundantly expressed in synovial fluid[50] and induces the expression of pro-inflammatory cytokines such as TNFα, IL-1β and IFN-γ.[51] Furthermore, IL-33 exacerbates collagen-induced arthritis via the activation of mast

cells.[52] Notably, in this study, plasma IgE levels were strongly reduced, and vascular mast cell activation was inhibited by anti-OX40L treatment, despite the upregulation in IL-33. These results may indicate that the anti-OX40L-dependent reductions in IL-4 and IgE contribute more strongly to mast cell activation than does IL-33. Notably, although Miller et al. observed a reduction in atherosclerosis upon IL-33 treatment, they did not determine the numbers of mast cells present in the atherosclerotic lesions. In conclusion, modulation of the OX40-OX40L pathway, combined with cholesterol reduction, induces regression of atherosclerosis via (1) the induction of IL-5-producing T cells and oxLDL-specific IgM and (2) Th2 reduction and subsequent mast cell inhibition. It must be noted that interruption of the OX40-OX40L pathway does not induce maximal regression of lesions. Further research into modulating immune responses to induce regression must be explored and, in combination with lipid lowering, may hold the key to therapies for cardiovascular patients with well-established lesions.

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Chapter 4

Interference of the CD30-CD30L pathway reduces atherosclerosis development

Amanda C. Foks
Ilze Bot
Vanessa Frodermann
Saskia C.A. de Jager
Mariëtte N.D. ter Borg
Peter J. van Santbrink
Hideo Yagita[#]
Johan Kuiper
Gijs H.M. van Puijvelde

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

[#] Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

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Abstract

Objective: Costimulatory molecules tightly control immune responses by providing positive signals that promote T cell activation or by transducing inhibitory signals that limit T cell responses. CD30 and CD30L are members of the TNF(R) superfamily and are involved in activation and proliferation of T and B cells, which have been implicated in the initiation and progression of atherosclerosis. In the present study, we thus aimed to determine the role of the CD30-CD30L pathway in the development of atherosclerosis.

Methods and Results: Western-type diet fed LDL receptor deficient ($LDLr^{-/-}$) mice were treated with an anti-CD30L antibody for 8 weeks, which resulted in a reduction of atherosclerotic lesion formation in the aortic root by 35%. Reduced numbers of adventitial $CD3^{+}$ T cells were found in anti-CD30L-treated mice, whereas no differences were observed in collagen and macrophage content of the atherosclerotic lesions. B cell and mast cell responses were also not affected upon anti-CD30L treatment. Interestingly, splenocyte proliferation was reduced with 53%, while T cell numbers were concomitantly reduced in anti-CD30L-treated mice compared with control mice. These data thus indicate that the CD30-CD30L pathway solely exerts its function via inhibition of T cell responses.

Conclusions: In the present study, we are the first to show that interruption of the CD30-CD30L pathway reduced initial atherosclerosis development by modulating T cell function.

Introduction

Atherosclerosis is considered a chronic autoimmune-like disease resulting from endothelial damage and subsequent cholesterol accumulation in the arterial wall.[1, 2] Within the atherosclerotic lesion a chronic inflammation manifests by a continuous infiltration of immune cells. Antigen presenting cells, such as dendritic cells (DCs) and macrophages, present antigens such as oxidized LDL-cholesterol to T cells, resulting in their activation. As a result both T cells and macrophages secrete cytokines, and more immune cells are attracted to the site of inflammation, which aggravates atherosclerotic lesion development.

T cell activation is tightly controlled by a complex network of costimulatory molecules, which can either provide positive or negative signals. Two large families of costimulatory molecules are known; the B7-CD28 superfamily, that includes CD28/CD80/CD86 and PD-1/PD-L1/2, and the TNF-TNFR superfamily, including OX40/OX40L and CD40/CD40L. Numerous studies have shown the crucial role of costimulatory molecules in the pathogenesis of atherosclerosis.[3-5] Previously, our lab showed that interruption of the OX40-OX40L interaction using an OX40L-blocking antibody leads to a reduction in the initiation of atherosclerosis.[4] Signaling of CD40-CD40L has been shown to affect advanced atherosclerosis, as lesions of $CD154^{-/-}ApoE^{-/-}$ mice contained fewer lipids, showed increased collagen levels and reduced numbers of immune cells such as T cells and macrophages, compared to $ApoE^{-/-}$ mice.[3] In addition, Gotsman et al. showed that the negative costimulatory pathway PD-1/PD-L1/2 downregulates pro-atherogenic T cell responses and atherosclerosis, since PD-L1/2 LDLr double knockout mice developed significantly larger atherosclerotic lesions compared with $LDLr^{-/-}$ mice.[5] CD30 (TNFRSF8) and CD30L (TNFSF8, CD153) also belong to the TNF-TNFR superfamily. Whereas CD30 and CD30L are both present on activated B and T cells, CD30L is also expressed on other cell types, such as mature DCs, macrophages and mast cells. Triggering via CD30-CD30L has been shown to induce activation and proliferation of T cells.[6, 7] Furthermore, the CD30-CD30L pathway has been implicated as a major player in secondary humoral immune responses. $CD30^{-/-}$ mice have impaired follicular germinal center responses and reduced secondary antibody responses.[8, 9] In addition, CD30L transgenic mice show increased numbers and activity of splenic germinal centers and have elevated serum antibody levels, such as IgG2b and IgE.[10]

The *in vivo* role of the interaction between CD30 and CD30L can be investigated using anti-CD30L antibodies, which interrupt the CD30-CD30L pathway. CD30 deficiency or treatment with a CD30L blocking antibody (RM153) significantly reduced airway inflammation in a murine asthma model[11], while Blazar et al. showed that anti-CD30L prolongs survival of mice in graft versus host disease.[12] Furthermore, administration of anti-CD30L completely suppressed the development of spontaneous/type I diabetes in NOD mice.[13]

Although macrophages bearing CD30 have been identified in ruptured plaques of patients with coronary artery disease[14], the involvement of the CD30-CD30L pathway in the development of atherosclerosis has not been investigated. In the present study,

we therefore investigated the role of the CD30-CD30L pathway in the initiation of atherosclerosis by treatment of LDLr$^{-/-}$ mice with a CD30L blocking antibody.

Methods

Animals

Female 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, Witham, Essex, UK). 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.

CD30L expression during atherosclerosis

After 2 weeks of Western-type, atherosclerosis was induced in LDLr$^{-/-}$ mice by collar placement (2 mm long, inner diameter 0.3 mm) around both carotid arteries and continuous Western-type diet feeding.[15] Mice were sacrificed at 0, 2, 4, 6, 8 and 10 weeks after collar placement and tissues were harvested after *in situ* perfusion using PBS. Carotid arteries and spleens (n=4-6 per timepoint) 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, CA) using SYBR green technology. The following primer pairs were used: 5′-CCAAGAAGTCATGGGCCTACCTCCAA-3′ and 5′-GCAAACGATGAAGTACAAGCCAGGGAA-3′ for CD30L, 5′-GAGCTCTTGTTGGTTG-GGAA-3′ and 5′-CGAACATCTGTGAAGGCAAA-3′ for CD4 and 5′-GTTGGGGCAGT-TGTAGGAAG-3′ and 5′-TGTGAAGCCAGAGGACAGTG-3′ for CD8. The following primers were used as endogenous references: 5′-GGACCCGAGAAGACCTCCTT-3′ and 5′ GCACATCACTCAGAATTTCAATGG-3′ for acidic ribosomal phosphoprotein PO (36B4) and 5′-TTGCTCGAGATGTCATGAAGGA-3′ and 5′-AGCAGGTCAGCAAAGAACTTATAG-3′ for hypoxanthine phosphoribosyltransferase (HPRT). Protein levels of CD30L were determined in blood of LDLr$^{-/-}$ mice fed a Western-type diet (n=5) or a chow diet (n=5) for 0, 4 and 8 weeks. Red blood cells were removed from blood using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Cells were stained with CD4 and CD30L and positive cells were determined with flow cytometry. All antibodies were purchased from eBioscience (Vienna). FACS analysis was performed on a FACSCantoII (Beckton Dickinson, Mountain View, CA). Data were analyzed using FACSDiva software (Beckton Dickinson).

Functionality of the anti-CD30L antibody under hypercholesterolemic conditions

To determine the effect of anti-CD30L on splenocyte proliferation, splenocytes from Western-type diet fed mice (n=3) were cultured for 24 hours in triplicate in a 96-wells round-bottom plate (2×10^5 cells/well, Greiner Bio-One) in RPMI 1640 supplemented

with L-Glutamine, 100 U/mL streptomycin/penicillin and 10% FCS. Splenocytes were cultured in the absence or presence of αCD3 and αCD28 (2 μg/mL) with anti-CD30L (0.1–10 μg/mL). Proliferation was measured by addition of ^{3}H-thymidine (0.5 μCi/well, Amersham Biosciences, The Netherlands) 16 hours prior to cell lysis. 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 αCD3/CD28 stimulation to triplicate cultures without stimulation.

Atherosclerosis

Atherosclerosis was induced in LDLr$^{-/-}$ mice by feeding a Western-type diet for 8 weeks. Mice were treated i.p. with 250 μg anti-mouse CD30L (RM153) (n=12) or sterile PBS (n=12) twice a week. Anti-mouse CD30L was kindly provided by Hideo Yagita and prepared as previously described.[16] At week 8 mice were sacrificed and tissues were harvested after *in situ* perfusion using PBS. Tissues for histology were fixed in Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). 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, 4, 6 and 8 after start of the Western-type diet feeding. The concentrations of serum cholesterol were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). 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. To determine the number of adventitial T cells, a CD3 staining was performed using anti-mouse CD3 (1:100, SP7, Immunologic, The Netherlands). Lesion 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. Mast cells were visualized by staining with Chloro-Acetate Esterase (CAE, Sigma-Aldrich) according to manufacturer's protocol. Mast cell numbers and the extent of mast cell degranulation were assessed manually. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. 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. Spleen sections were stained with hematoxylin and eosin. Morphology was studied using a

Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK).

Flow cytometry

At sacrifice, blood, spleen and mediastinal lymph nodes (LN) were isolated (n=5 per group). Single cell suspensions were obtained by squeezing the organs through a 70 µm cell strainer. Red blood cells were removed from blood and splenocytes using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Cells were stained with CD4, CD8 and CD19 to detect T cells and B cells. For intracellular staining, cells were fixed and permeabilized according to manufacturer's protocol (eBioscience, Vienna). Subsequently, the cells were stained for the transcription factors T-bet, GATA-3, RORγt or Foxp3 and the cytokines IFN-γ, IL-4, IL-5, IL-10 and IL-17. FACS analysis was performed as described above.

Spleen cell proliferation

At sacrifice, splenocytes (n=5 per group) were cultured for 72 hours in quintuplicate in a 96-wells round-bottom plate (2×10^5 cells/well, Greiner Bio-One) in RPMI 1640 supplemented with L-Glutamine, 100 U/mL streptomycin/penicillin and 10% FCS. As a positive control cells were stimulated with αCD3 and αCD28 (2 µg/mL). Proliferation was measured as described above.

Cytokine determination in serum and supernatant of splenocytes

To detect IL-8 in serum an ELISA was performed according to manufacturer's protocol (Biosource). Serum samples were 1:1 diluted in assay diluent and absorbance was detected at 450 nm. To detect IL-4 and IL-5 in the serum an ELISA was performed according to manufacturer's protocol (eBioscience, Vienna). Serum samples were 1:1 diluted in assay diluent and absorbance was detected at 450 nm.

Serum antibody detection

IgM, IgG1, IgG2a and IgG2b levels against oxLDL were detected in serum using Abs recognizing mouse IgM, IgG1, IgG2a and IgG2b and HRP-labeled goat anti-rat Ig (BD Pharmingen). OxLDL (5 µg/mL) was dissolved in $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and was coated o/n onto a flat-bottom 96-well high binding plate (Corning, NY). Serum samples were 1:1 diluted in PBS and absorbance was detected at 450 nm. Total IgE in serum was determined by a mouse IgE quantitative ELISA according to manufacturer's protocol (Bethyl Laboratories, Montgomery TX, USA).

Statistical analysis

All data are expressed as mean±SEM. An unpaired two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Probability values of $P<0.05$ were considered significant.

Results

CD30L is upregulated in the initial stages of atherosclerosis

Since we aimed to interrupt the CD30-CD30L pathway via blockade of CD30L, we first monitored the expression of CD30L in atherosclerotic lesions and the lymphoid tissue. While it is known that costimulatory molecules such as OX40[4] and PD-1[17] are upregulated in atherosclerotic lesions, it is unclear whether CD30L is regulated as well. LDLr$^{-/-}$ mice were fed a Western-type diet and slightly constrictive perivascular collars were placed around the carotid arteries, which leads to the development of shear stress induced atherosclerotic lesions at the proximal site of the collars.[15] At 0-10 weeks after collar placement, RNA was isolated from the atherosclerotic lesions. As shown in Figure 1A, induction of atherosclerotic lesion development is reflected by an increase in CD68 expression over time (P<0.01). CD30L was also induced during lesion development and showed the highest expression after 2 weeks of Western-type diet feeding (P<0.05), coinciding with the influx of CD68 positive macrophages, but also with the influx of various immune cells, such as CD4 and CD8 positive T cells (Figure 1B). Parallel to the data on the lesion, CD30L mRNA levels in the spleen of LDLr$^{-/-}$ mice significantly increased during Western-type diet feeding (P<0.05, Figure 1C). In addition, we determined protein levels of CD30L and as shown in Figure 1D and E, CD4$^+$ T cells expressing CD30L increase in blood of Western-type diet fed mice (P<0.05).

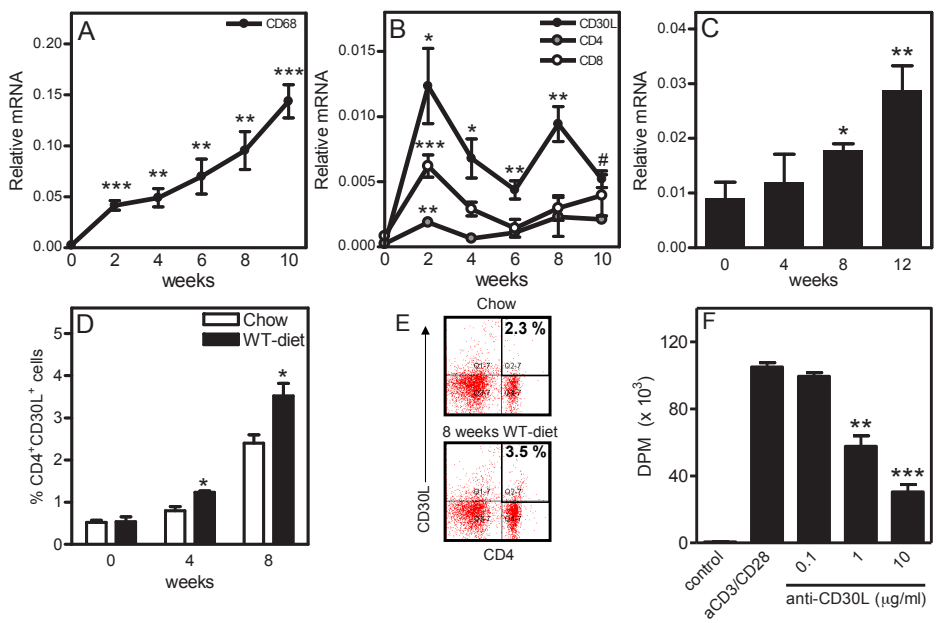


Figure 1. Relative mRNA levels of CD68 (A), CD30L, CD4 and CD8 in lesions of the carotid arteries were determined with RT-PCR (B). Relative mRNA levels of CD30L in spleens were determined with RT-PCR (C). At 0, 4 and 8 weeks CD30L expression was determined on CD4$^+$ T cells in blood from LDLr$^{-/-}$ mice fed a Western-type diet (n=5) and a chow diet (n=5) by flow cytometry (D-E). Splenocytes were cultured with αCD3/CD28 in the presence of anti-CD30L (0.1-10 μg/mL) and proliferation was assessed by the amount of ^{3}H-thymidine incorporation (F).* P<0.05, ** P<0.01, *** P<0.001, $^{\#}$ P<0.01 for all celltypes

Blockage of CD30L reduces proliferation of splenocytes from Western-type diet fed mice

To determine whether interruption of the CD30-CD30L pathway impairs T cell function of LDLr$^{-/-}$ mice fed a Western-type diet for 8 weeks *ex vivo*, we stimulated splenocytes with αCD3/CD28 in the presence or absence of RM153, a blocking CD30L antibody. This antibody was generated by Shimozato et al.[16] and potently inhibits the binding of CD30 to CD30L and the proliferation of αCD3/CD28 activated T cells. As shown in Figure 1F, blockage of the CD30-CD30L pathway by using RM153 dose-dependently reduced splenocyte proliferation of Western-type diet fed LDLr$^{-/-}$ mice (P<0.01).

Impaired T cell numbers and function in anti-CD30L treated mice

RM153 has been shown to reduce murine autoimmune diabetes[13], prolong survival of mice in a graft versus host disease model[12], and together with OX40L blockade reduced autoimmune disease in Foxp3 deficient mice[18] when administered i.p. twice a week during the experiments (200-500 µg/dose). To study the effect of CD30-CD30L interruption on T cells *in vivo*, we therefore treated LDLr$^{-/-}$ mice with 250 µg of RM153 twice a week, while the mice were fed a Western-type diet for 8 weeks. The relative number of CD4$^+$ T cells (Figure 2A) in spleen and mediastinal heart lymph nodes of

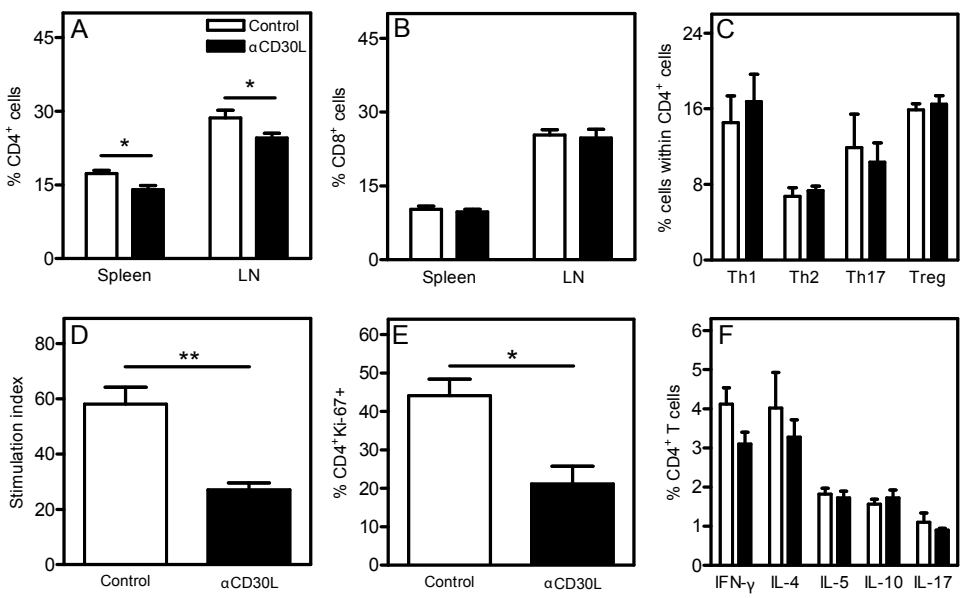


Figure 2. Atherosclerosis was induced in LDLr$^{-/-}$ mice by feeding a Western-type diet for 8 weeks. Mice were treated with anti-mouse CD30L (RM153) (n=12) or sterile PBS (n=12) twice a week. At sacrifice, spleen and LN cells were isolated and stained for CD4 (A) and CD8 (B) and analyzed by flow cytometry (n=5 per group). T-bet, GATA-3, RORγt and Foxp3 expression in T cells from the spleen were also determined by flow cytometry (C). Splenocytes of control and anti-CD30L-treated mice (n=5 per group) were cultured in the presence or absence of CD3/CD28 stimulation. Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells and is expressed as stimulation index (D). CD4$^+$ T cells isolated from splenocytes were cultured for 72 hours in the presence of CD3/CD28 stimulation and stained for CD4 and Ki-67, a proliferation marker. Positive cells were assessed with flow cytometry (E). IFN-γ, IL-4, IL-5, IL-10 and IL-17 secretion by these CD3/CD28-stimulated CD4$^+$ T cells were determined by flow cytometry (F). * P<0.05, ** P<0.01

anti-CD30L-treated mice was reduced compared with control mice ($P<0.05$), whereas $CD8^+$ T cell numbers were not affected by the anti-CD30L treatment (Figure 2B). Furthermore, the differentiation of naive $CD4^+$ T cells into Th1, Th2, Th17 or Treg cells was unaffected by anti-CD30L treatment (Figure 2C). To determine the proliferative capacity of T cells from anti-CD30L-treated mice in comparison with control mice, splenocytes from both groups were cultured for 72 hours in the presence of αCD3/αCD28 stimulation. A significant 53% decrease in splenocyte proliferation was observed in mice treated with anti-CD30L (stimulation index of 27.0±2.5) compared to control mice (stimulation index of 58.1±6.1, $P<0.01$, Figure 2D). As shown in Figure 2E, we demonstrate that $CD4^+$ T cells are the main effector cells, as $CD4^+$ T cells isolated from anti-CD30L-treated mice (n=5) showed an 52% reduction in αCD3/αCD28-mediated proliferation in comparison with $CD4^+$ T cells isolated from control mice (n=5, $P<0.05$), whereas $CD8^+$ T cell proliferation was unaffected (data not shown). In addition, we determined cytokine secretion by these $CD4^+$ T cells with flow cytometry; no significant differences in cytokine profiles between control and anti-CD30L-treated mice were observed (Figure 2F).

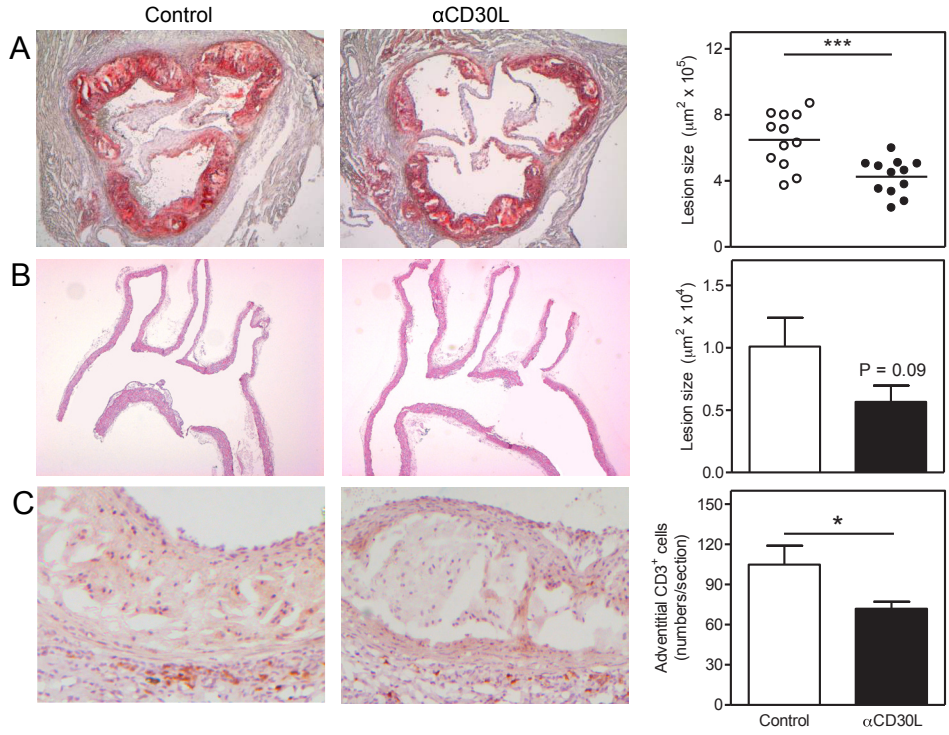


Figure 3. Anti-CD30L treatment reduces atherosclerosis development in $LDLr^{-/-}$ mice (n=12) fed a Western-type diet for 8 weeks in comparison with control treatment (n=12). Representative cross-sections of lesion formation in the three valves area of the aortic root stained with Oil-Red-O and hematoxylin are shown and lesion size was determined (A). The aortic arch and its main branch points were excised, fixed, and embedded in paraffin. Longitudinal sections of the aortic arch were stained with hematoxylin and eosin to analyze lesion extent (B). Corresponding sections of the aortic root on separate slides were stained for CD3 (red) to determine the number of infiltrating T cells (B). *$P<0.05$, *** $P<0.001$

Interference in the CD30-CD30L pathway reduces the development of atherosclerosis

To determine whether the anti-CD30L-mediated reduction in T cell responses affects atherosclerosis development, we determined atherosclerotic lesion sizes upon treatment with anti-CD30L. Figure 3A shows representative cross-sections of lesions in the three-valve area of the aortic root. We observed a significant 35% reduction in the aortic root lesion size in anti-CD30L-treated mice ($4.3\times10^5\pm0.3\times10^5$ μm^2) compared with control mice ($6.5\times10^5\pm0.5\times10^5$ μm^2, $P<0.001$). Treatment with rat IgG (isotype control for RM153) did not alter atherosclerotic lesion size in comparison with PBS treatment (data not shown). In addition, lesion formation in the aortic arch was reduced in anti-CD30L-treated mice compared with control mice (P=0.09, Figure 3B). During the experiment, anti-CD30L treatment did not affect body weight and total plasma cholesterol levels (data not shown). In line with reduced T cell percentages and splenocyte proliferation following anti-CD30L treatment, we observed a significant 31% reduction in the number of $CD3^+$ T cells within the adventitia of anti-CD30L-treated mice (71.9±5.3 T cells/section) compared with control mice (104.9±14.1 T cells/section, $P<0.05$, Figure 3C). This reduction in adventitial T cells in anti-CD30L-

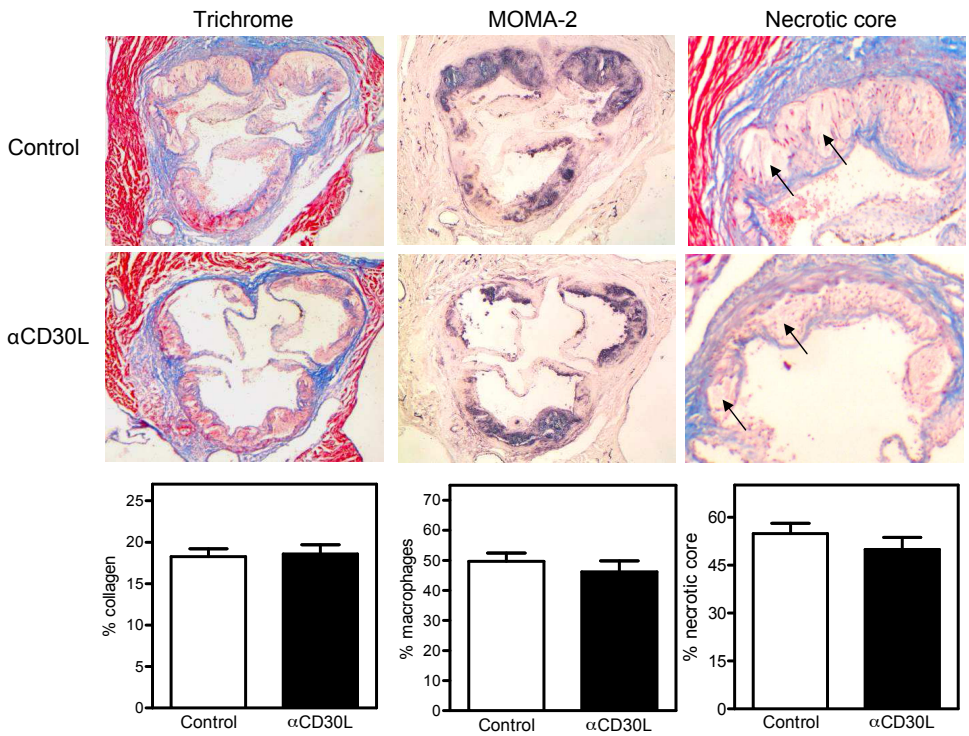


Figure 4. No differences in lesion composition after anti-CD30L treatment. Sections of the aortic root were stained for collagen using Masson's trichrome staining. The percentage of collagen relative to the lesion size was determined. Furthermore, relative macrophage content was determined with a MOMA-2 staining and quantified. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area.

treated mice was not a consequence of impaired migration and adhesion of $CD4^+$ and $CD8^+$ T cells (data not shown). In addition, we determined T cell subsets and cytokine expression locally in the plaque with qPCR. In line with our previous findings, we did not find any differences in T cell subsets and their cytokines (data not shown).

Anti-CD30L treatment does not affect lesion composition

With respect to the composition of the lesion (Figure 4), no differences were found in lesion collagen content (18.6±1.3% vs. 19.3±1.3%) and macrophage content (46.2±3.6% vs. 49.7±2.7%) between anti-CD30L-treated mice and control mice, respectively. In addition, no differences in necrotic cores were observed between anti-CD30L-treated mice (49.9±3.7%) and control mice (54.8±3.2%).

Humoral responses in anti-CD30L treated mice are not affected

The CD30-CD30L pathway is described to be involved in germinal center responses and secondary antibody responses.[8, 9] However, in our study both the percentage of B cells in blood and spleen (Figure 5A) and the levels of oxLDL-specific IgM, IgG1, IgG2a and IgG2b in serum did not differ between control mice and anti-CD30L-treated mice (Figure 5B). Furthermore, we did not observe any differences in spleen morphology in anti-CD30L-treated mice compared with control mice (Figure 5C).

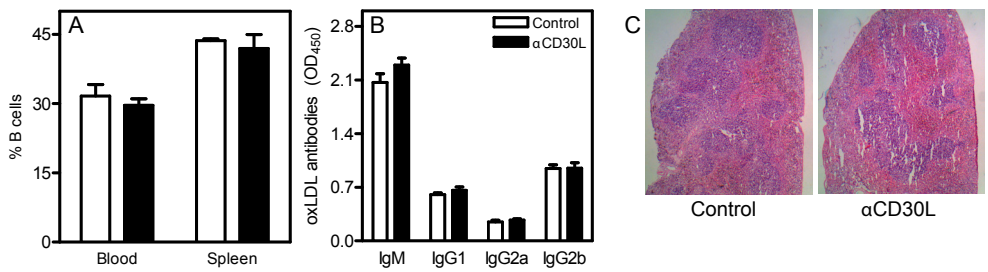


Figure 5. CD30-CD30L pathway interruption does not affect humoral responses under hypercholesterolemic conditions. At sacrifice, blood and spleen cells were isolated and stained for CD19 and analyzed by flow cytometry (n=5 per group, A). oxLDL-specific IgM, IgG1, IgG2a and IgG2b production was detected in serum of control (n=12) and anti-CD30L treated mice (n=12) (B). Spleen sections were stained with hematoxylin and eosin. Representative sections are shown (C).

Anti-CD30L treatment does not inhibit mast cells

Treatment with anti-CD30L significantly reduced serum IgE levels in a murine asthma model[11] and several other studies reported decreased IgE levels following CD30-CD30L pathway interruption.[8, 9] In our study, a trend towards lowered serum IgE was observed in anti-CD30L-treated mice (363±90 ng/mL) compared with control mice (609±132 ng/mL, Figure 6A). Since IgE is a common mast cell activator and mast cells can aggravate atherosclerosis[19], we analyzed the number of adventitial mast cells. However, the numbers of activated mast cells and total number of mast cells (MC, Figure 6B) in the aortic root remained unaffected by anti-CD30L treatment (activated: 13.8±1.9 MC/mm^2 and total: 29.4±2.4 MC/mm^2 versus control

treatment; activated: 12.5±1.6 MC/mm^2 and total: 28.1±3.0 MC/mm^2). In addition, the percentage of activated mast cells did not differ (control: 44.2±2.8% versus anti-CD30L: 45.1±4.5%, Figure 6C). Interestingly, CD30-CD30L signaling can induce degranulation-independent mast cell activation via the secretion of IL-8.[20] However, anti-CD30L treatment also did not influence serum levels of KC, the mouse analogue of IL-8 (Figure 6D).

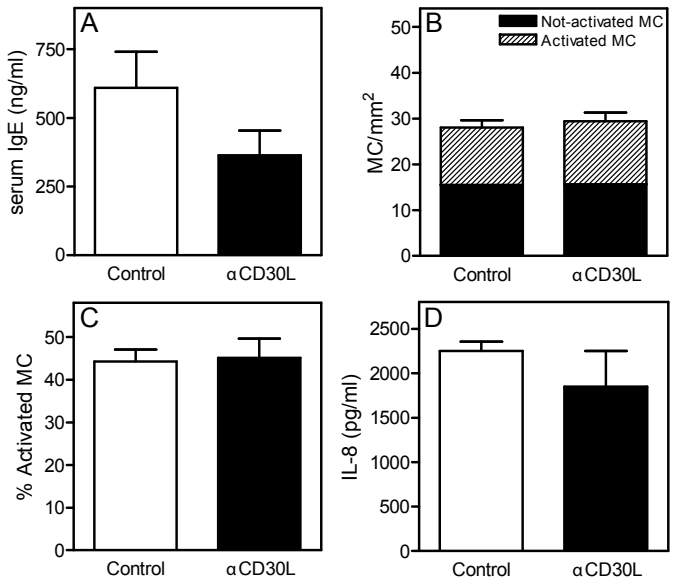


Figure 6. Mast cells are unaffected in anti-CD30L treated mice. IgE was measured in the serum of control and anti-CD30L treated mice (A). Sections of the aortic root were stained with CAE to detect mast cells. Mast cell numbers (activated and not-activated, B) and the percentage of mast cell degranulation (C) were assessed manually. Serum IL-8 was measured by ELISA (D).

Discussion

Optimal T cell activation is regulated by costimulatory signals and modulation of these signals provides a very promising therapeutic strategy to improve the outcome of autoimmune diseases. T cells play an important role in atherosclerosis and whereas the CD30-CD30L pathway has been implicated in various autoimmune diseases, such as asthma[11], GVHD[12] and type I diabetes[13], no studies describe a role for the CD30-CD30L axis in atherosclerosis.

In the present study, we found that CD30L expression within the atherosclerotic lesion highly correlated with CD4$^+$ and CD8$^+$ T cell infiltration and that CD30L expression in the spleen was upregulated after 8 and 12 weeks of Western-type diet feeding. In addition, CD4$^+$ T cells expressing CD30L are increased in Western-type diet-fed mice, suggesting a pro-atherogenic role of the CD30-CD30L pathway. We therefore chose to intervene with the CD30-CD30L pathway during the development of atherosclerosis as a therapeutic approach. Interruption of CD30-CD30L by using the CD30L blocking antibody RM153 reduced atherosclerosis development in LDLr$^{-/-}$ mice with 35% and coincided with a 31% reduction in adventitial T cell numbers. CD30L signaling is reported to enhance proliferation of T cells[16] and blocking CD30L therefore

diminishes proliferation of T cells as shown by several studies.[11, 13, 21] We showed that anti-CD30L inhibited the proliferation of splenocytes from Western-type diet fed mice *ex vivo*. Interruption of the CD30-CD30L interaction also potently reduced T cell numbers *in vivo*, as we found reduced percentages of $CD4^+$ T cells in the spleen and LN of anti-CD30L-treated mice compared with control mice. Furthermore, a 56% reduction in splenocyte proliferation was observed following anti-CD30L treatment, which was particularly due to reduced $CD4^+$ T cell proliferation. Additionally, we show that anti-CD30L does not interfere with the migration and adhesion capacity of T cells. The role of T cells in atherosclerosis has been established already in several studies.[22-24] $CD4^+$ T cells can be subdivided in several subclasses; Th1, Th2, Th17 and Treg cells. However, no differences were found in T cell subsets following anti-CD30L treatment, which indicates that interruption of the CD30-CD30L pathway under hypercholesterolemic conditions impairs T cell numbers and function but does not influence their differentiation. In line with our findings, Chakrabarty et al. showed that anti-CD30L (RM153) treatment reduced T cell proliferation in response to islet antigens and markedly inhibited the development of spontaneous diabetes in NOD mice. Furthermore, they showed that anti-CD30L inhibited the incidence of diabetes in NOD-SCID mice after diabetogenic T cell transfer.[13]

Signaling via CD30-CD30L may also affect humoral responses. Mice deficient in CD30 mice show reduced levels of several immunoglobulins, such as IgG1, IgG2c and IgE.[8] CD30L Tg mice show increased numbers and activity of splenic germinal centers and elevated basal serum concentrations of IgG2a, IgG2b and IgE.[10] In addition, Shanebeck et al. showed that mouse splenic B cells stimulated via CD30L induced increased amounts of a number of immunoglobulins, such as IgG1 and IgE.[9] However, under hypercholesterolemic conditions, we did not find any significant difference in immunoglobulin production or spleen morphology in anti-CD30L-treated mice compared with control mice.

In a murine asthma model, CD30 deficiency or treatment with anti-CD30L significantly reduced airway inflammation, splenocyte proliferation, Th2 responses and serum IgE levels.[11] Whereas in the present study we also observed a reduction in splenocyte proliferation and a trend towards reduced serum IgE, we did not observe reduced Th2 responses as shown by GATA-3 expressing $CD4^+$ cells and serum IL-4 and IL-5 levels (data not shown). IgE may induce activation of mast cells, which are correlated with the incidence of plaque rupture and erosion[25] and also play a crucial role in plaque progression and destabilization *in vivo*.[19] Furthermore, mast cells are the predominant CD30L-expressing cells in Hodgkin's disease, which are involved in tumorigenesis and tumor progression.[26] However, despite a reduction in serum IgE, anti-CD30L-treated mice did not have reduced numbers of mast cells or activated mast cells as shown by adventitial mast cells and IL-8 release.

In conclusion, we are the first to demonstrate that anti-CD30L treatment inhibits plaque development in LDL receptor deficient mice independent of plasma cholesterol levels and lesional macrophage and collagen content. Given the profound inhibition

of anti-CD30L treatment on T cell proliferation and activation, we propose that anti-CD30L treatment, at least partly, exerts its protective effects by modulating this process. These data thus identify anti-CD30L treatment as a novel therapeutic modality in the inhibition of atherosclerotic lesion development and the prevention of acute cardiovascular syndromes.

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Chapter 5

T cell immunoglobulin and mucin domain 3 acts as a negative regulator of atherosclerosis

Amanda C. Foks
Ingrid A. Ran
Loes Wasserman
Vanessa Frodermann
Mariëtte N.D. ter Borg
Hideo Yagita[#]
Hisaya Akiba[#]
Ilze Bot
Johan Kuiper
Gijs H.M. van Puijvelde

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

[#] Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

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Abstract

Objective: Atherosclerosis is a chronic autoimmune-like disease in which lipids and fibrous elements accumulate in the arterial blood vessels. T cells are present within atherosclerotic plaques, and their activation is partially dependent on costimulatory signals, which can either provide positive or negative signals that promote T cell activation or limit T cell responses, respectively. T cell immunoglobulin and mucin domain 3 (Tim-3) is a coinhibitory type I transmembrane protein, which affects the function of several immune cells involved in atherosclerosis, such as monocytes, macrophages, effector T cells and regulatory T cells (Tregs). In the present study, we determined the role of Tim-3 in the development of atherosclerosis.

Methods and Results: Western-type diet fed $LDLr^{-/-}$ mice were treated with an anti-Tim-3 antibody for 8 weeks. Anti-Tim-3 administration increased atherosclerotic plaque formation with 35% in the aortic root and with 50% in the aortic arch. Furthermore, blockade of Tim-3 signaling increased percentages of circulating monocytes with 33% and lesional macrophages with 20%. Additionally, anti-Tim-3 administration increased $CD4^+$ T cells with 17% and reduced percentages of Tregs with 18% and regulatory B cells (Bregs) with 37%.

Conclusions: In the present study, we show that Tim-3 acts as a negative regulator of atherosclerosis, since anti-Tim-3 treatment augments lesion development by enhancing monocyte and macrophage expansion and by decreasing IL-10 producing Tregs and Bregs.

Introduction

Costimulatory and coinhibitory molecules are important regulators of the immune system by fine-tuning innate and adaptive immune responses. Studies on atherosclerosis, a chronic inflammatory disease[1], show that modulation of costimulatory and coinhibitory pathways affects its development by regulating T cell responses.[2] In secondary lymphoid tissues but also in the arterial wall, antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, present antigens, such as oxidized LDL-cholesterol, to T cells. The T cells can become either activated in the presence of costimulatory molecules, or inhibited when T cells and APCs interact via coinhibitory molecules. Therefore, stimulating coinhibitory molecules may provide a novel therapeutic approach to prevent the activation or function of autoreactive immune cells in atherosclerosis. Previously, Gotsman et al. showed that the coinhibitory pathway PD-1/PD-L1/2 inhibits pro-atherogenic T cell responses and atherosclerosis, since PD-L1/2$^{-/-}$LDLr$^{-/-}$ mice develop significantly larger atherosclerotic lesions compared with LDLr$^{-/-}$ mice.[3]

T cell immunoglobulin and mucin domain (Tim) proteins, are type 1 transmembrane proteins expressed on various immune cells and are similar to PD-1/PD-L1/2 negative regulators of immune responses. Four functional TIM genes have been identified in the murine genome (TIM-1-4), whereas the human genome only contains three TIM genes (TIM-1,3 and 4).[4] The genes encoding Tim proteins are located on chromosome 11 (mouse) and chromosome 5 (human), which are associated with enhanced susceptibility to allergy and several autoimmune diseases, such as EAE and diabetes.[4] Tim-3 was first discovered as a specific marker for Th1 cells[5] but is also expressed on a variety of immune cells such as NK cells, monocytes, macrophages and mast cells.[6] During innate immune responses, Tim-3 promotes inflammation via TNFα secretion by monocytes and APCs[7] and enhances macrophage clearance of intracellular pathogens.[8] However, in adaptive immune responses, Tim-3 terminates IFN-γ driven inflammation by inducing cell death of T cells after binding to its ligand galectin-9, a soluble molecule which is upregulated by IFN-γ.[9] In addition, Tim-3 can induce Treg activity[10] and induce expansion of myeloid-derived suppressor cells, which play an important role in tumor immunology.[11] Recently, Zhang et al. showed that Tim-3 can also negatively regulate innate immune responses, since reduced Tim-3 signaling by antibody blockade or knock-down with siRNA increases the activation of monocytes.[12] The *in vivo* role of Tim-3 can be investigated using anti-Tim-3 antibodies, which interrupt the Tim-3-galectin-9 interactions. Previously, it was shown that blocking Tim-3 with either a Tim-3 blocking antibody or a Tim-3-Ig fusion protein, enhances type 1 diabetes in NOD-mice and prevents the generation of immunological tolerance in a transplantation model, by dampening the function of Tregs.[13] Furthermore, *in vivo* administration of a Tim-3 blocking antibody enhances inflammation and demyelination in a mouse model of EAE by increasing the number and activation of macrophages.[5] Blockade of the Tim-3-galectin-9 interaction also accelerates graft versus host disease via enhanced activation of Th1 cells and cytotoxic T cells.[14] In addition, blocking Tim-3

signaling aggravates inflammatory heart disease in BALB/c mice by decreasing CD80 expression on macrophages and mast cells and by reducing Tregs.[15]
Recently, Hou et al. showed that patients with atherosclerosis have augmented Tim-3 expression on NK cells, which might affect NK cell function during atherosclerosis.[16] However, to date, the exact role of Tim-3 in atherosclerosis has not been investigated. In the present study, we therefore examined the role of Tim-3 during atherosclerosis development by treatment of LDLr$^{-/-}$ mice with a Tim-3 blocking antibody. This research provides novel information on the importance of the Tim-3 pathway in regulating immune responses and provides possibly new therapeutic targets to prevent atherosclerosis.

Materials and Methods

Animals
Female 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 Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). 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.

TIM-3 expression during atherosclerosis
After 2 weeks of Western-type diet, atherosclerosis was induced as previously described by collar placement (2 mm long, inner diameter 0.3 mm) around both carotid arteries and continuous Western-type diet feeding.[17] Mice were sacrificed at 0, 2, 4, 6, 8 and 10 weeks after collar placement and tissues were harvested after *in situ* perfusion using PBS and subsequent perfusion using Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). Carotid arteries (n=4-6 per timepoint) 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, CA) using SYBR green technology. The following primer pair was used for Tim-3: 5′-TGGAGTGGGAGTCTCTGCTGGGT-3′ and 5′-GCTCCTGCATTTGCCAACCCTCC-3′. The following primers were used as endogenous references: 5′-GGACCCGAGAAGACCTCCTT-3′ and 5′-GCACATCACTCAGAATTTCAATGG-3′ for acidic ribosomal phosphoprotein PO (36B4), 5′-TTGCTCGAGATGTCATGAAGGA-3′ and 5′-AGCAGGTCAGCAAAGAACTTATAG-3′ for hypoxanthine phosphoribosyltransferase (HPRT), 5′-AGCAGGTCAGCAAAGAACTTATAG-3′ and 5′-AGCAGGTCAGCAAAGAACTTATAG-3′ for Rps13 and 5′-AACCGTGAAAAGATGACCCAGAT-3′ and 5′-CACAGCCTGGATGGCTACGTA-3′ for α-actin.

Atherosclerosis
Atherosclerosis was induced in LDLr$^{-/-}$ mice by feeding a Western-type diet for 8 weeks. Mice were treated twice a week i.p. with 250 μg anti-Tim-3 antibody (RMT3-

23, n=12) or sterile PBS (n=11). At week 8, mice were sacrificed and tissues were harvested after *in situ* perfusion using PBS. Tissues for histology were fixated in Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). Tissues for RNA analysis were frozen in nitrogen and stored at -20 °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, 4, 6 and 8 after start of the Western-type diet feeding. The concentrations of serum cholesterol were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). 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 to determine lesion size. Lesion 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. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. To determine apoptosis, TUNEL positive nuclei were counted manually and the percentage of apoptotic cells was determined as percentage of total cells. In addition, the aortic arch and its main branch points were excised, fixed, and embedded in paraffin. Longitudinal sections of the aortic arch (4 µm) 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, Cambridge, UK).

Flow cytometry

At sacrifice, blood, spleen and peritoneal cells were isolated (n=5 per group). Single cell suspensions were obtained by squeezing the organs through a 70 µm cell strainer. Red blood cells were removed from blood and splenocytes using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Different immune cells were analyzed with flow cytometry: T cells (CD4, CD8), B cells (CD19), DCs (CD11c), neutrophils ($CD11b^+Ly6G^+$), monocytes ($CD11b^+Ly6G^-$), NK cells ($CD3^-NK1.1^+$), Bregs (splenic subset: $CD5^+CD1d^{hi}CD21^+$, peritoneal subset: $CD5^+CD1d^{hi}$), Tregs ($CD4^+CD25^+Foxp3^+$) and IL-10^+ cells. To detect Tregs and IL-10 producing cells, cells were fixed and permeabilized according to manufacturer's protocol (eBioscience, Vienna). Subsequently, the cells were stained for Foxp3 or IL-10. All antibodies were purchased from eBioscience (Vienna) and Beckton Dickinson (Mountain View, CA). To detect apoptotic cells, an Annexin-V/PI staining was performed on splenocytes

according to manufacturer's protocol (eBioscience, Vienna). FACS analysis was performed on a FACSCantoII (Beckton Dickinson). Data were analyzed using FACSDiva software (Beckton Dickinson).

MCP-1 determination in serum and supernatant of oxLDL-loaded macrophages

To detect MCP-1 in serum an ELISA was performed according to manufacturer's protocol (eBioscience, Vienna) and absorbance was detected at 450 nm. To determine MCP-1 secretion by oxLDL-loaded macrophages, bone marrow cells were harvested from the femora and tibia of C57BL/6J mice and were cultured for 7 days in complete RPMI supplemented with M-CSF (L929 supernatant) to obtain macrophages. Immature macrophages were stimulated with copper-oxidized LDL (2.5 µg/mL) in the absence or presence of 0, 2, 10, 25 or 50 µg/mL anti-Tim-3 (RMT3-23) for 24 hours. MCP-1 production was measured in the supernatant with ELISA as described above.

Statistical analysis

All data are expressed as mean±SEM. An unpaired two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Probability values of $P<0.05$ were considered significant.

Results

TIM-3 is upregulated during atherosclerosis development

To determine TIM-3 expression in atherosclerotic lesions, LDLr$^{-/-}$ mice were fed a Western-type diet and slightly constrictive perivascular collars were placed around the carotid arteries, which leads to the development of shear stress induced atherosclerotic lesions at the proximal site of the collars. As shown in Figure 1A, TIM-3 expression was elevated following placement of collars around carotid arteries of LDLr$^{-/-}$ mice fed a Western-type diet ($P<0.05$), with the highest induction 10 weeks after collar

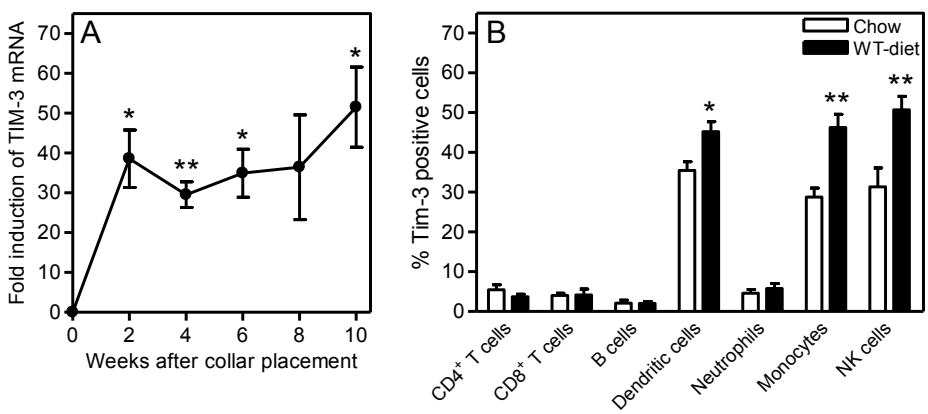


Figure 1. Tim-3 expression in lesions of the carotid arteries was determined with RT-PCR. Fold induction of Tim-3 mRNA relative to Tim-3 mRNA expression before collar placement is shown (A). Protein levels of Tim-3 were determined on immune cells in the circulation of mice fed a chow diet (n=5) or a Western-type diet (n=5) for 8 weeks (B). * $P<0.05$, ** $P<0.01$

placement (52-fold increase, $P<0.05$). To investigate which circulating cell types express Tim-3 and upregulate Tim-3 during Western-type diet feeding, we determined Tim-3 expression on the surface of several immune cells of mice fed a chow or Western-type diet for 8 weeks with flow cytometry (n=5 per group, Figure 1B). Only minor populations of T cells, B cells and neutrophils express Tim-3 on their surface, while larger populations of DCs, monocytes and NK cells express Tim-3. Western-type diet feeding significantly enhanced the percentage of Tim-3$^+$ cells from 35.5±2.2% (chow) to 45.2±2.5% in DCs ($P<0.05$), from 28.8±2.3% to 46.3±3.3% in monocytes ($P<0.01$) and from 31.4±4.7% to 50.7±3.4% in NK cells ($P<0.01$).

Blocking Tim-3 signaling aggravates atherosclerosis

Because interference of the Tim-3 signaling pathway aggravates autoimmune diseases such as EAE[5], type 1 diabetes[13] and GVHD[14] by dampening regulatory cells and enhancing monocyte and macrophage activation, we determined the role of Tim-3 in the initiation of atherosclerosis. We treated LDLr$^{-/-}$ mice with a Tim-3 blocking antibody (RMT3-23) and determined atherosclerotic lesion development. Figure 2A shows representative cross-sections of lesions in the aortic valve area. We observed a significant 35% increase in the aortic root lesion size in anti-Tim-3-treated mice (5.2±0.5×10^5 μm^2) compared with control mice (3.8±0.2×10^5 μm^2, $P<0.05$). Previously, we showed that treatment with rat IgG, the isotype control for RMT3-23, did not alter atherosclerotic lesion size in comparison with PBS treatment.[18] During the experiment, anti-Tim-3 treatment did not affect body weight and total plasma cholesterol levels (data not shown).

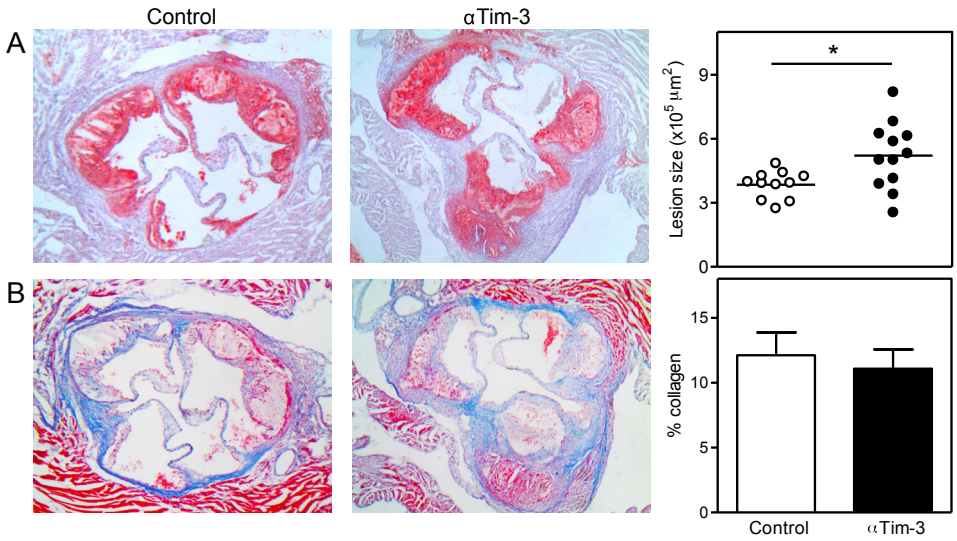


Figure 2. Anti-Tim-3 treatment exacerbates atherosclerosis development compared with PBS (n=11) treatment in LDLr$^{-/-}$ mice (n=12) fed a Western-type diet for 8 weeks. Representative cross-sections of lesion formation in the aortic valve area stained with Oil-Red-O and hematoxylin are shown and lesion size was determined (A). Sections of the aortic root were stained for collagen using Masson's Trichrome staining. The percentage of collagen relative to the lesion size was determined (B). *$P<0.05$

Furthermore, no differences were found in lesion collagen content (anti-Tim-3 mice: 11.1±1.5% and control mice: 12.1±1.8%, Figure 2B). To check whether anti-Tim-3 treatment also affects atherosclerosis development at another site of the vasculature, we determined lesion size in the aortic arch. As shown in Figure 3, anti-Tim-3 treatment ($1.1\pm0.2\times10^4$ μm^2) aggravated atherosclerosis with 50% compared with control treatment ($0.5\pm0.1\times10^4$ μm^2).

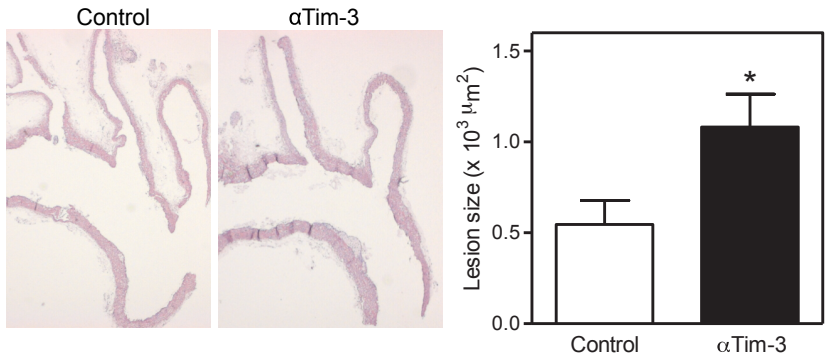


Figure 3. Anti-Tim-3 treatment aggravates atherosclerosis development in the aortic arch compared with PBS treatment in LDLr$^{-/-}$ mice (n=11/12 per group) fed a Western-type diet for 8 weeks. Representative cross-sections of lesion formation in the aortic arch stained with hematoxylin are shown and lesion size was determined. *P<0.05

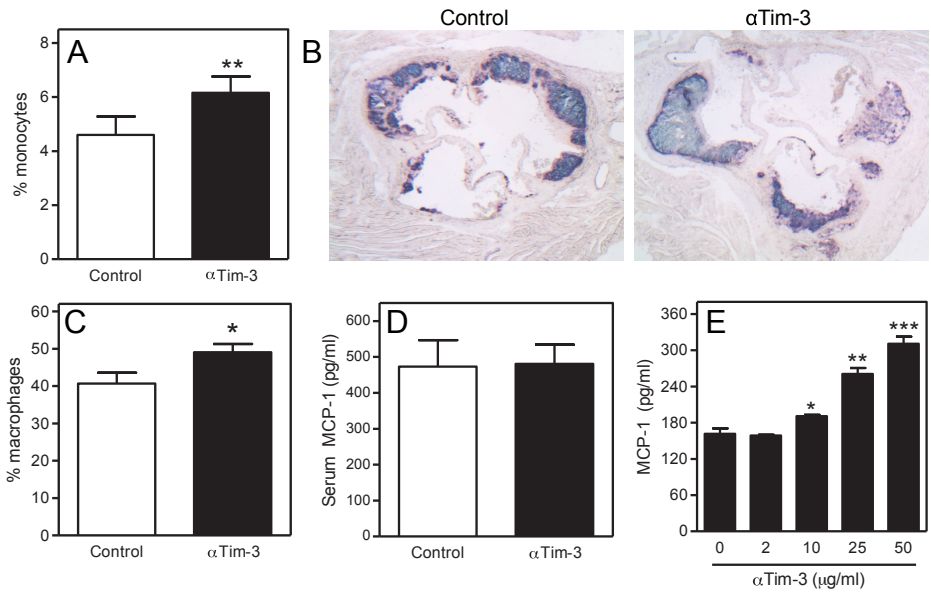


Figure 4. Increased circulating monocytes and lesional macrophages in anti-Tim-3-treated mice. At sacrifice, blood cells were isolated (n=5 per group) and stained for CD11b$^+$Ly6G$^-$ monocytes (A). Relative macrophage content was determined with a MOMA-2 staining (B) and quantified (C). MCP-1 concentrations were measured in the serum of control (n=11) and anti-Tim-3-treated mice (n=12) with ELISA (D). BM-derived macrophages were stimulated for 24 hours with 2.5 μg/mL oxLDL in the presence of various concentrations of anti-Tim-3. MCP-1 levels in the supernatant were determined with ELISA (E). *P<0.05, **P<0.01, ***P<0.001

TIM-3 blockade enhances the number of circulating monocytes and lesional macrophages

Monocytes play an important role in atherosclerosis development, since monocytes migrate into the sub-endothelial space where they can differentiate into macrophages and eventually become foam cells. Previously, Tim-3 blockade has been associated with increased monocyte and macrophage activation and induces the expansion of macrophages.[12] In our study, mice that received the Tim-3 antibody showed a 33% increase in circulating monocytes (anti-Tim3: 6.2±0.3% versus control: 4.6±0.6%, $P<0.01$, Figure 4A). In addition, lesional macrophage content was increased with 20% in anti-Tim-3-treated mice (49.0±2.3%) compared with control mice (40.7±2.9%, $P<0.05$, Figure 4B-C). An important key regulator of monocytes is the chemokine MCP-1, which enhances monocyte recruitment into the arterial wall.[19] As shown in Figure 4D, we did not observe differences in MCP-1 levels in serum of anti-Tim-3 and control-treated mice. However, oxLDL-stimulated macrophages exposed to anti-Tim-3 *in vitro* enhanced their secretion of MCP-1 in a dose-dependent manner (Figure 4E).

Anti-Tim-3 treatment reduces apoptosis of lymphocytes

Tim-3 expressed on macrophages and dendritic cells is also involved in phagocytosis of apoptotic cells by recognizing apoptotic cells through the FG loop in the IgV domain.[20] In addition, Tim-3 can induce cell death of T cells. As shown in Figure 5A, we did not observe differences in lesion necrotic core content of anti-Tim-3 mice (24.5±2.7%) and control mice (28.7±2.5%). In addition, the percentage of apoptotic cells within the lesions did not significantly differ between anti-Tim-3 mice (0.70±0.21%) and control mice (0.80±0.18%, Figure 5B). Interestingly, a lower percentage of splenic

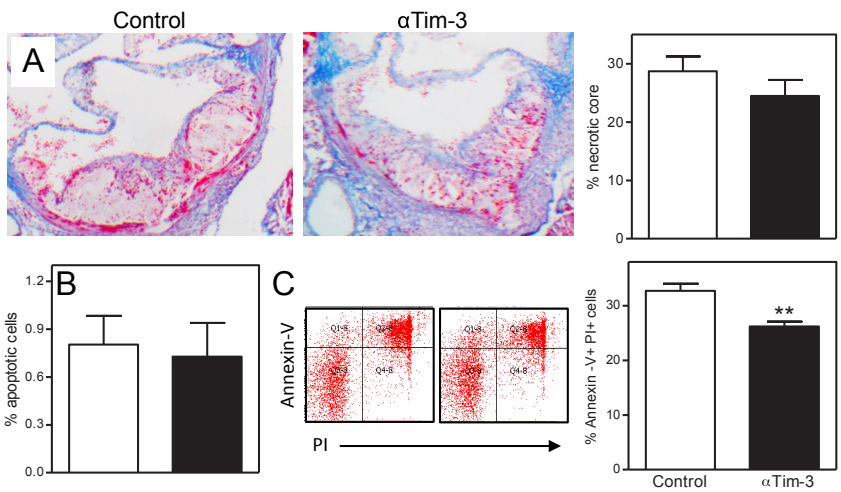


Figure 5. Necrotic areas within atherosclerotic lesions of anti-Tim-3 and control mice were determined (A). The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. Apoptosis was defined by TUNEL positive nuclei as percentage of total number of cells present in the atherosclerotic lesions (B). The percentage of apoptotic lymphocytes in the spleen was determined with an Annexin-V/PI staining and analyzed with flow cytometry (C). $^{**}P<0.01$

lymphocytes isolated from anti-Tim-3 mice (26.2±0.9%) underwent apoptosis than control splenocytes (32.7±1.3%, $P<0.01$, Figure 5C).

Reduced IL-10 producing regulatory T and B cells in anti-Tim-3-treated mice

Several studies showed that interactions of Tim-3 with galectin-9 are essential for the generation of Tregs.[13, 21] Tregs play an important role in the regulation of T cell-mediated immune responses through suppression of T cell proliferation and cytokine production. Therefore, increased Treg numbers may be beneficial for patients suffering from atherosclerosis. In accordance with previous findings, we observed an 18% reduction of circulating Tregs within the $CD4^+$ T cell population in anti-Tim-3-treated mice (8.8±0.2%) compared with control mice (10.8±0.6%, $P<0.05$, Figure 6A). Additionally, a 17% reduction of Tregs in the spleen was observed in anti-Tim-3-treated mice (5.8±0.3%) compared with control mice (7.0±0.4%, $P<0.05$, Figure 6A). Since Tregs control effector T cells and Tim-3 blockade reduces Tregs, we determined the percentage of effector T cells. As expected, we found significantly increased $CD4^+$ T cell numbers in anti-Tim-3 treated mice (25.1±1.4%) compared with control mice (21.4±0.6%, $P<0.05$, Figure 6B). Another regulatory cell type is the regulatory B cell (Breg), which is an IL-10 producing B cell that induces Treg differentiation and inhibits pro-inflammatory cytokine production.[22] We observed a 37% decrease of splenic Bregs ($CD5^+CD1d^{hi}CD21^+$ cells) in anti-Tim-3-treated mice (3.7±0.6%) compared with control mice (5.8±0.4%, $P<0.05$) and a 44% decrease in peritoneal Bregs ($CD5^+CD1d^{hi}$) in anti-Tim-3 treated mice (6.5±1.6%) compared with control mice (11.7±0.7%, $P<0.05$, Figure 6C). Additionally, a 55% decrease in IL-10 producing cells was measured in spleens of anti-Tim-3-treated mice (3.5±1.0%) compared with the control group (7.9±1.5%, $P<0.05$, Figure 6D).

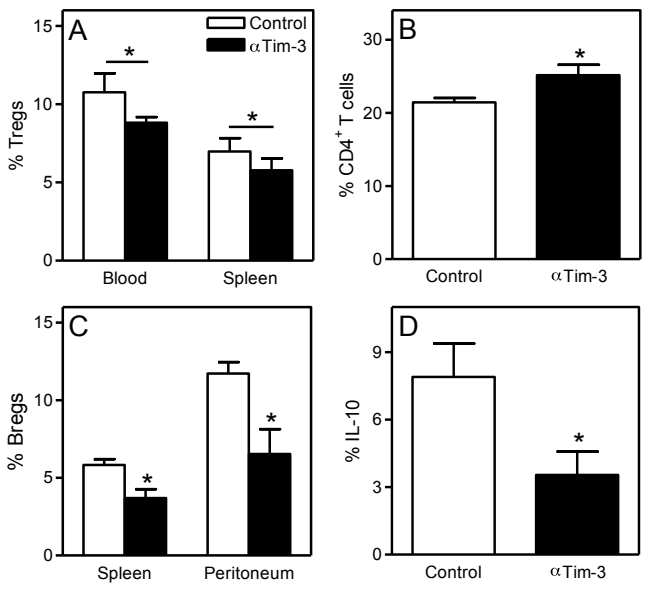


Figure 6. At sacrifice, blood and spleen cells were isolated and stained for $CD4^+CD25^+Foxp3^+$ regulatory T cells (A) and analyzed by flow cytometry (n=5 per group). $CD4^+$ T cells were determined in the blood (B). Spleen and peritoneal cells were stained for regulatory B cells, which were quantified as $CD5^+CD1d^{hi}CD21^+$ cells and $CD5^+CD1d^{hi}$ cells, respectively (C). The amount of IL-10 producing cells in the spleen was determined by flow cytometry (D). * $P<0.05$

Discussion

Acute cardiovascular syndromes are a major cause of death in Western society and are generally triggered by rupture of an atherosclerotic plaque. In atherosclerosis an imbalance between pro- and anti-inflammatory T cells exists, with increased numbers of the first. Restoration of this balance by modulation of costimulatory and coinhibitory molecules may provide a very promising strategy to prevent cardiovascular disease. The role of coinhibitory Tim-3 has been established in several autoimmune diseases, such as EAE[5], GVHD[14] and type 1 diabetes[13], however, the contribution of Tim-3 to atherosclerosis development remained to be elucidated.

In the present study, we show that TIM-3 is expressed within the atherosclerotic lesion and increases during Western-type diet feeding. Furthermore, we found that Tim-3 was expressed on large populations of monocytes, dendritic cells and NK cells, whereas only a few T cells, B cells and neutrophils expressed Tim-3 on their surface. Previously, Hou et al. showed that patients with atherosclerosis have increased Tim-3$^+$ NK cells compared with healthy controls.[16] In line with these findings, we observed that Western-type diet feeding increased the percentage of Tim-3$^+$ NK cells, but also the percentage of Tim-3$^+$ monocytes and Tim-3$^+$ DCs, cell types that largely contribute to the inflammatory process of atherosclerosis.

To investigate the contribution of Tim-3 to atherosclerosis development, we treated Western-type diet fed LDLr$^{-/-}$ mice with an anti-Tim-3 antibody for 8 weeks. Blockade of Tim-3 increased atherosclerosis development with 35% in the aortic root and with 50% in the aortic arch compared with control treatment, independent of plasma cholesterol levels. Whereas there was no difference in lesion stability, lesions of anti-Tim-3 treated mice contained significantly more macrophages than lesions of control mice. In agreement, circulating monocytes were enhanced in anti-Tim-3 mice, and since monocytes migrate into the arterial wall and differentiate into macrophages that can take up oxLDL, we believe that blockade of Tim-3 increases monocyte recruitment into the arterial wall and thus enhances the lesional macrophage content. To mimic foam cell responses in the lesion, we exposed oxLDL-loaded macrophages to anti-Tim-3 *in vitro* and showed that blockade of Tim-3 enhanced MCP-1 secretion, which may be responsible for increased monocyte infiltration and subsequent elevated lesional macrophages in anti-Tim-3-treated mice. This is in line with studies that show an essential role for Tim-3 in monocyte and macrophage function. Monney et al. appointed increased macrophage numbers and activation as the major cause of exacerbated EAE in mice treated with a blocking anti-Tim-3 antibody.[5] Frisancho-Kiss et al. showed that anti-Tim-3 treatment during the innate response to viral infection in BALB/c mice increases macrophages and their activation in the heart, resulting in increased inflammatory heart disease[15] and Zhang et al. found that Tim-3 regulated pro- and anti-inflammatory cytokine expression in human CD14$^+$ monocytes.[12]

Since Tim-3 signaling has previously been associated with apoptosis, we determined the percentage of apoptotic cells and necrotic core in lesions of anti-Tim-3-treated mice. In early lesions apoptosis of macrophages limits lesion cellularity and suppresses

lesion progression. However, in advanced stages it induces necrotic core formation, which makes lesions susceptible for rupture. Therefore it has to be taken into account that Tim-3 signaling could be beneficial in initial stages of atherosclerosis, but in later stages of atherosclerosis could promote plaque rupture. In our study, we do not observe differences in apoptosis or necrotic core content in the lesions of anti-Tim-3-treated mice and Tim-3 signaling also enhances Tregs, which have been shown to promote lesion stability in advanced stages of atherosclerosis.[23] More specifically, it has been reported that the Tim-3-galectin 9 interaction promotes apoptosis of T cells.[9] We did observe reduced apoptosis of lymphocytes in anti-Tim-3-treated mice, which in turn could be responsible for the increase in $CD4^+$ T cells.

Moreover, Tim-3 has also been implicated in regulating pro-inflammatory T cell responses by promoting regulatory T cell function. Tregs dampen the immune response in atherosclerosis by secreting anti-inflammatory cytokines, such as IL-10, and by directly inhibiting effector T cell proliferation. Seki et al. reported that Tim-3 activation by galectin-9 resulted in an induction of Tregs[21] and Frisancho-Kiss et al. showed that anti-Tim-3 administration reduces Treg populations in the heart during acute myocarditis.[15] In addition, blockade of the Tim-3 pathway accelerated type I diabetes in nonobese diabetic mice in part by dampening Treg function.[13] In accordance with these findings, we also observed reduced Treg levels in spleen and blood after anti-Tim-3 administration, which contributed to the exacerbation of atherosclerosis. Additionally, Bregs were significantly decreased with 37% in the spleen and with 44% in the peritoneum of anti-Tim-3-treated mice. The exact role of Bregs in atherosclerosis has not been clarified yet, however, Bregs secrete IL-10 and thereby inhibit secretion of pro-inflammatory cytokines and support Treg differentiation, which indicates a protective role for Bregs in atherosclerosis. The atheroprotective role of IL-10 has already been shown by von der Thüsen et al. who showed that IL-10 overexpression reduced atherosclerosis in $LDLr^{-/-}$ mice.[24] Furthermore, adoptive transfer of Bregs inhibited the initiation of EAE in Bl6 mice immunized with MOG peptides[25] and Mauri et al. demonstrated that a transfer of IL-10 producing B cells into DBA mice prevented rheumatoid arthritis development and ameliorated established arthritis.[26] Finally, the percentage of IL-10-producing splenocytes was decreased with 55% after anti-Tim-3 treatment, which is in line with decreased Tregs and Bregs in anti-Tim-3 mice, which exert their function in part via IL-10 secretion.

In the present study, we are the first to provide direct evidence that the Tim-3 pathway is involved in the development of atherosclerosis. We show that Tim-3 acts as a negative regulator of atherosclerosis, since blockade of Tim-3 augmented atherosclerotic lesion development and enhances immune responses by increasing circulating monocytes and lesional macrophages, and by decreasing IL-10 producing Tregs and Bregs. In the future, approaches to promote the Tim-3 pathway, such as treatment with agonistic Tim-3 antibodies or galectin-9, may represent novel therapeutic strategies to inhibit atherosclerotic lesion development and prevent cardiovascular diseases.

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Chapter 6

Agonistic anti-TIGIT treatment inhibits T cell responses in atherosclerosis without affecting lesion development

Amanda C. Foks
Ingrid A. Ran
Vanessa Frodermann
Peter J. van Santbrink
Ilze Bot
Johan Kuiper
Gijs H.M. van Puijvelde

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

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Abstract

Objective: Costimulatory and coinhibitory molecules are mainly expressed on T cells and antigen presenting cells (APCs) and strongly orchestrate adaptive immune responses. Whereas costimulatory molecules enhance immune responses, signaling via coinhibitory molecules dampens the immune system thereby showing great therapeutic potential to prevent cardiovascular diseases. Signaling via coinhibitory T cell immunoglobulin and ITIM domain (TIGIT) directly inhibits T cell activation and proliferation, and therefore represents a novel therapeutic candidate to specifically dampen pro-atherogenic T cell reactivity. In the present study, we used an agonistic anti-TIGIT antibody to determine the effect of excessive TIGIT-signaling on atherosclerosis.

Methods and Results: TIGIT was upregulated on $CD4^+$ T cells isolated from mice fed a Western-type diet in comparison with mice fed a chow diet. Agonistic anti-TIGIT suppressed T cell activation and proliferation both *in vitro* and *in vivo*. However, agonistic anti-TIGIT treatment of $LDLr^{-/-}$ mice fed a Western-type diet for 4 or 8 weeks did not affect atherosclerotic lesion development in comparison with PBS and control IgG treatment. Furthermore, elevated percentages of dendritic cells were observed in the blood and spleen of agonistic anti-TIGIT-treated mice. Additionally, these cells showed an increased activation status.

Conclusions: Despite the inhibition of T cell responses, agonistic anti-TIGIT treatment does not affect initial atherosclerosis development, possibly due to increased activity of dendritic cells.

Introduction

Atherosclerosis, a chronic autoimmune-like disease, results from imbalanced pro- and anti-inflammatory responses, which promotes infiltration of inflammatory cells in the vessel wall. This results in the formation of an atherosclerotic plaque and eventually causes plaque rupture. Immune responses are regulated by a network of costimulatory and coinhibitory molecules present on T cells and antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs). The immune system provides a large diversity of costimulatory and coinhibitory pathways and each pathway has its own unique effect on the fate of individual immune cells. Costimulatory signals can promote T cell survival, cell cycle progression and differentiation to effector and memory T cells, whereas coinhibitory molecules can terminate these processes directly or indirectly via for example the induction of regulatory T cells (Tregs).

A new-emerging complex network of costimulatory and coinhibitory molecules is formed by T cell immunoreceptor with Ig and ITIM domains (TIGIT, Vstm3, WUCAM), CD226 (DNAM-1), CD112 (PVRL2, nectin-2), and the poliovirus receptor (PVR, CD155). TIGIT is expressed on different subsets of T cells, including Tregs, activated $CD4^+$ T cells, $CD8^+$ T cells, and on NK cells and NKT cells. PVR is highly expressed on DCs, fibroblasts, endothelial cells and some tumor cells.[1, 2] Signaling through the TIGIT-PVR pathway can inhibit T cell responses in a cell-intrinsic manner by directly targeting the TCR signaling cascade as well as via the induction of tolerogenic DCs that produce increased levels of IL-10.[3-5] In addition, TIGIT engagement may modulate DC responses by influencing ERK activity.[5] TIGIT also interacts with CD112 present on APCs while PVR can bind to CD226 present on T cells. TIGIT and CD226 share common binding sites on PVR and CD112 and are therefore cross-competing for binding to PVR and CD112.[3] Several studies showed that CD226 is associated with costimulatory T cell signals, as it was capable to induce Th1 responses.[6-8] Since TIGIT and CD226 compete for PVR binding, it is also believed that TIGIT attenuates T cell responses by interference of the CD226-mediated costimulation.[3]

Interference in this pathway by using TIGIT deficient mice has been shown to aggravate EAE through elevated secretion of pro-inflammatory cytokines, such as IL-6, IL-17 and IFN-γ, and by increased T cell proliferation.[4] In line with this finding, Levin et al. showed that TIGIT overexpression reduces the development of EAE.[3] Furthermore, soluble TIGIT inhibits collagen-induced arthritis by dampening $CD4^+$ T cell responses and by interfering with CD226-mediated costimulation. Additionally, blocking TIGIT accelerated mortality in a mouse model of graft versus host disease.[3]

To date, the role of the coinhibitory TIGIT-PVR axis in atherosclerosis has not been explored. In the present study, we therefore treated $LDLr^{-/-}$ mice with an agonistic anti-TIGIT antibody to determine the effect of coinhibitory TIGIT on atherosclerosis.

Material and Methods

Animals

Female 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, Witham, Essex, UK). 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.

TIGIT expression on $CD4^+$ T cells under hypercholesterolemic conditions

Splenocytes were isolated from $LDLr^{-/-}$ mice fed a Western-type or chow diet. Single cell suspensions were obtained by squeezing the organs through a 70 µm cell strainer. Red blood cells were removed using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently, $CD4^+$ T cells (>95% purity) were isolated by using the BD IMag™ mouse CD4 T lymphocyte enrichment set according to the manufacturer's protocol (Beckton Dickinson, Mountain View, CA). After isolation, $CD4^+$ T cells were stained with a fluorescent antibody for CD4 and TIGIT (clone 1G9). FACS analysis was performed on a FACSCantoII (Beckton Dickinson, Mountain View, CA). Data were analyzed using FACSDiva software (Beckton Dickinson).

Functionality of the TIGIT agonist under hypercholesterolemic conditions

The agonistic anti-TIGIT antibody was kindly provided by Nicole Joller (Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School).[4] To investigate the effect of agonistic anti-TIGIT on splenocyte activation and proliferation under hypercholesterolemic conditions, splenocytes from Western-type diet fed mice (n=3) were cultured for 48 hours at 37°C in triplicate in a 96-wells round-bottom plate (2×10^5 cells/well, Greiner Bio-One) in RPMI 1640 supplemented with L-Glutamine, 100 U/mL streptomycin/penicillin and 10% FCS. Splenocytes were stimulated with αCD3 and αCD28 (2 µg/mL) in the presence of different concentrations of agonistic anti-TIGIT (0–30 µg/mL). Activated T cells ($CD4^+CD25^+$) were determined with FACS as described above. Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences, The Netherlands) 16 hours prior to cell lysis. 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 quintuplicate cultures with αCD3/αCD28 stimulation to quintuplicate cultures without stimulation. To detect IL-2 in the supernatant of splenocytes, an ELISA was performed according to manufacturer's protocol (eBioscience, Vienna). Absorbance was measured at 450 nm.

Atherosclerosis

Atherosclerosis was induced in $LDLr^{-/-}$ mice by feeding a Western-type diet for 4 and 8 weeks. Mice were treated i.p. with 100 µg agonistic anti-TIGIT antibody (n=9 and

n=12, respectively), 100 µg hamster IgG (Innovative Research, n=8 and n=12, respectively) or sterile PBS (n=9 and n=11, respectively) at day 0, 2, 4, 10, 17 and 24 after start of Western-type diet. At week 4 and week 8 mice were sacrificed and tissues were harvested after *in situ* perfusion using PBS. Tissues for histology were fixated in Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA).

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 and 4 after start of the 4 week experiment and at week 0, 2, 4, 6 and 8 after start of the 8 week experiment. The concentration of serum cholesterol was determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). 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. Lesion 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. Morphology was studied using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK).

Flow cytometry

At sacrifice, blood and splenocytes were isolated (n=5 per group). Single cell suspensions were obtained as described above. Cells were stained with fluorescent antibodies for CD11c, MHC II and CD40 to detect dendritic cells and to determine their activation status.

Spleen cell proliferation

At sacrifice, splenocytes (n=5 per group) were cultured for 72 hours in quintuplicate in a 96-wells round-bottom plate (2×10^5 cells/well, Greiner Bio-One) in RPMI 1640 supplemented with L-Glutamine, 100 U/mL streptomycin/penicillin and 10% FCS. As a positive control cells were stimulated with αCD3 and αCD28 (2 µg/mL). Proliferation was measured as described above.

Statistical analysis

All data are expressed as mean±SEM. A one-way ANOVA with post test was performed to compare normally distributed data between three groups of animals. Probability values of $P<0.05$ were considered significant.

Results

TIGIT is upregulated on CD4⁺ T cells from Western-type diet fed LDLr⁻/⁻ mice

The coinhibitory molecule TIGIT is mainly expressed on T cells and previous studies have shown that upon TCR stimulation the number of TIGIT expressing CD4⁺ T cells increases.[3-5] To investigate the effect of a high-fat diet on the surface expression of TIGIT on CD4⁺ T cells, splenocytes were isolated from chow diet fed mice (n=3) and Western-type (cholesterol-rich) diet fed mice (n=3) and cultured for 48 hours in the presence or absence of αCD3/αCD28 stimulation. As shown in Figure 1, Western-type diet feeding significantly enhanced the percentage of TIGIT⁺ cells within the unstimulated CD4⁺ T cell population (8.5±0.7%) in comparison with chow diet feeding (3.7±0.9%, $P<0.05$). In addition, we show that αCD3/αCD28 stimulation indeed increased the percentage of TIGIT⁺ cells within the CD4⁺ T cells in both chow (10.1±1.3%, $P<0.05$) and Western-type diet mice (16.4±2.3%, $P<0.05$).

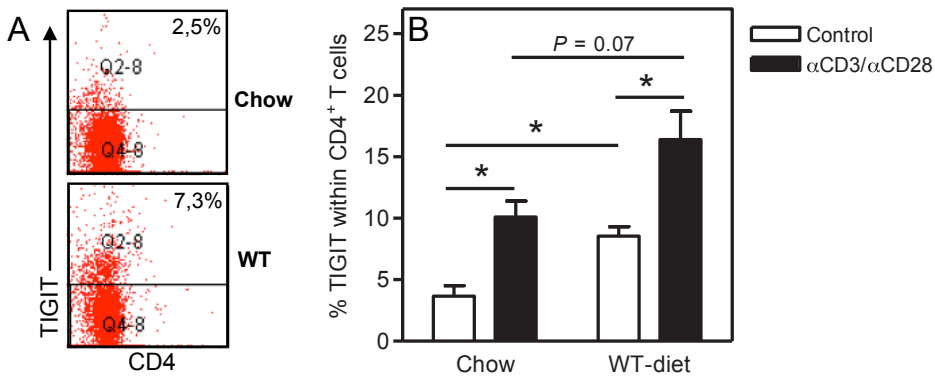


Figure 1. Representative FACS dot plots of TIGIT surface expression on CD4⁺ T cells isolated from LDLr⁻/⁻ mice fed a chow diet or a Western-type (WT) diet (A). Splenocytes from LDLr⁻/⁻ mice fed a chow diet (n=3) and Western-type diet (n=3) were cultured for 48 hours in the presence or absence of αCD3/αCD28. The percentage TIGIT expressing CD4⁺ T cells was determined with flow cytometry (B). * $P<0.05$

Agonistic anti-TIGIT inhibits the activation and proliferation of splenocytes

Since TIGIT can directly inhibit T cell proliferation, we determined *ex vivo* the capacity of the agonistic anti-TIGIT antibody to impair T cell reactivity in our atherosclerosis mouse model; LDLr⁻/⁻ mice fed a Western-type diet. We stimulated splenocytes from Western-type diet fed mice for 72 hours with αCD3/αCD28 in the presence or absence of different concentrations of agonistic anti-TIGIT. As shown in Figure 2A, agonistic anti-TIGIT decreased the percentage of activated T cells ($P<0.01$). Most importantly, splenocyte proliferation as measured by the amount of ^{3}H-thymidine incorporation (Figure 2B) and IL-2 secretion (Figure 2C), was strongly inhibited in a dose-dependent manner upon excessive TIGIT triggering.

Impaired T cell function in agonistic anti-TIGIT-treated mice

To study the effect of TIGIT triggering on T cells *in vivo*, LDLr⁻/⁻ mice (n=9) were

treated with an agonistic anti-TIGIT antibody at day 0, 2, 4, 10, 17 and 24, while being fed a Western-type diet for 4 weeks. As a control, mice were treated with Armenian hamster IgG (n=8) and PBS (n=9). To determine the proliferative capacity of T cells from agonistic anti-TIGIT-treated mice in comparison with PBS and hamster IgG-treated mice, splenocytes from all groups were cultured for 72 hours in the presence of αCD3/αCD28. A significant 45% decrease in splenocyte proliferation was observed in mice treated *in vivo* with agonistic anti-TIGIT (stimulation index of 20.8±1.8) compared with PBS mice (stimulation index of 37.4±0.8, $P<0.01$) and hamster IgG mice (stimulation index of 38.2±3.2, $P<0.001$, Figure 2D).

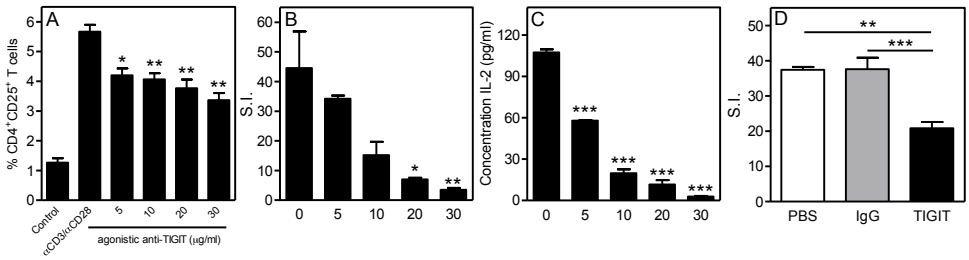


Figure 2. Splenocytes from Western-type diet fed mice (n=3) were cultured for 72 hours with αCD3/αCD28 in the presence or absence of agonistic anti-TIGIT (0-30 µg/mL). Activated T cells ($CD4^+CD25^+$) were determined with flow cytometry (A). Proliferation was assessed by the amount of ^3H-thymidine incorporation in dividing cells and is expressed as stimulation index (B) and by the amount of IL-2 produced by the splenocytes as determined with ELISA (C). Splenocytes of PBS, Armenian Hamster IgG and agonistic anti-TIGIT-treated mice (n=5 per group) were cultured in the presence of αCD3/αCD28 stimulation and proliferation was assessed by the amount of ^3H-thymidine incorporation expressed as stimulation index (D). * $P<0.05$, ** $P<0.01$, *** $P<0.001$

6

Agonistic anti-TIGIT treatment does not affect initial atherosclerosis development

To determine whether the TIGIT-mediated impairment of T cell function affects the development of early atherosclerosis (4 weeks of Western type diet), we measured atherosclerotic lesion sizes upon agonistic anti-TIGIT treatment. As shown in Figure 3A, no difference in atherosclerotic lesion size was observed after agonistic anti-TIGIT treatment (1.41±0.07x10^5 µm^2) in comparison with hamster IgG treatment (1.46±0.15x10^5 µm^2) or PBS (1.59±0.13x10^5 µm^2). During the experiment, agonistic anti-TIGIT treatment did not affect body weight and total plasma cholesterol levels (data not shown). Furthermore, collagen content did not significantly differ between the three groups (PBS: 7.7±0.9%, hamster IgG: 7.0±1.2% and TIGIT: 5.5±1.0%, Figure 3B). In addition, the percentage of macrophages in the lesions is comparable in all the groups (PBS: 50.3±4.2%, hamster IgG: 47.7±4.9% and agonistic anti-TIGIT: 53.2±3.5%, Figure 3C).

Increased percentages and activation of dendritic cells in agonistic anti-TIGIT-treated mice

Whereas agonistic anti-TIGIT treatment reduced T cell proliferation, dendritic cells

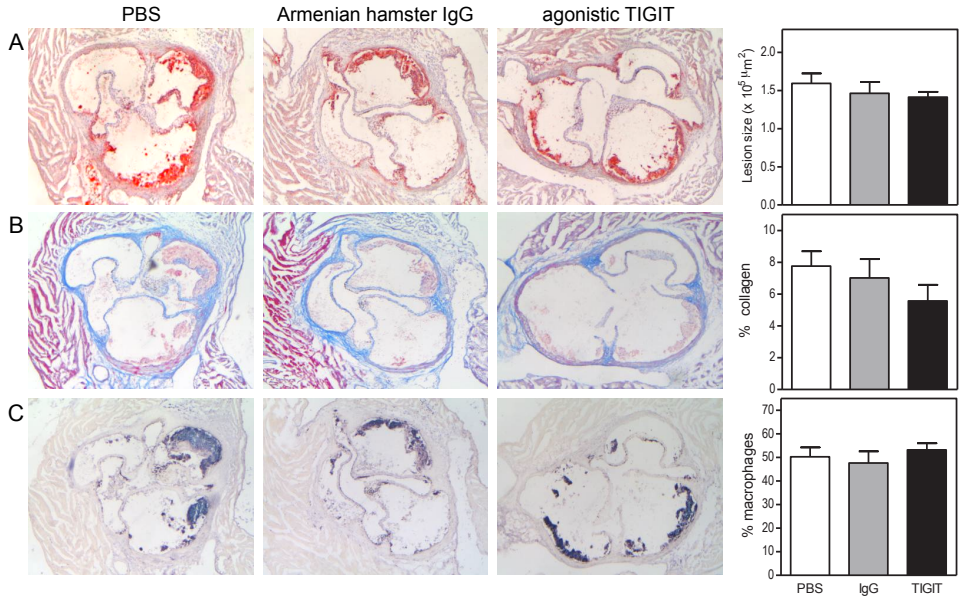


Figure 3. No difference in atherosclerotic lesion size between agonistic anti-TIGIT, Armenian Hamster IgG and PBS treated LDLr$^{-/-}$ mice fed a Western-type diet for 4 weeks. Representative cross-sections of lesion formation in the three valves area of the aortic root stained with Oil-Red-O and hematoxylin are shown and lesion size was determined (A). Sections of the aortic root were stained for collagen using Masson's trichrome staining. The percentage of collagen relative to the lesion size was determined (B). Furthermore, relative macrophage content was determined with a MOMA-2 staining (C).

which are also involved in the pathogenesis of atherosclerosis were elevated in these mice. As shown in Figure 4A, agonistic anti-TIGIT treatment significantly enhanced the percentage of dendritic cells (14.7±1.3%, $P<0.05$) in the circulation in comparison with hamster IgG and PBS treatment (10.7±0.4% and 10.8±0.4%, respectively). Similar results were observed in the spleen (Figure 4B), where agonistic anti-TIGIT treatment enhanced the percentage of dendritic cells (15.6±1.2%, $P<0.05$) in comparison with hamster IgG and PBS treatment (12.0±1.2 and 11.6±0.3%, respectively). Moreover, their activation status as measured with MHC II and CD40 is also elevated in agonistic anti-TIGIT-treated mice compared with both control groups.

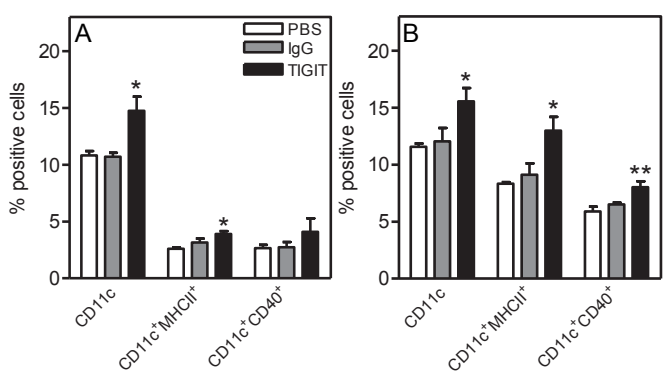


Figure 4. At sacrifice, blood (A) and spleen (B) cells were isolated and stained for dendritic cells and their activation status and analyzed by flow cytometry (n=5 per group). *$P<0.05$, ** $P<0.01$

Agonistic anti-TIGIT treatment does not affect atherosclerosis after 8 weeks of Western-type diet

We also determined atherosclerosis development after 8 weeks of Western-type diet feeding in combination with agonistic anti-TIGIT treatment. Lesion size in the agonistic anti-TIGIT-treated mice (n=12, 5.15±0.32x10^5 μm^2) was not affected compared with lesion size in the hamster IgG-treated mice (n=12, 5.12±0.26x10^5 μm^2). In fact, both groups of antibody-treated mice have 18% smaller atherosclerotic lesions compared with the PBS group (n=11, 6.28±0.44x10^5 μm^2), although this did not reach statistic significance (Figure 5A). Furthermore, no differences were observed in collagen content (PBS: 13.1±1.0%, hamster IgG: 13.3±2.0% and TIGIT agonist: 11.1±1.5%, Figure 5B) and macrophage content (PBS: 47.2±2.3%, IgG: 47.4±1.9% and TIGIT agonist: 47.9±2.6%, Figure 5C) of the atherosclerotic lesions.

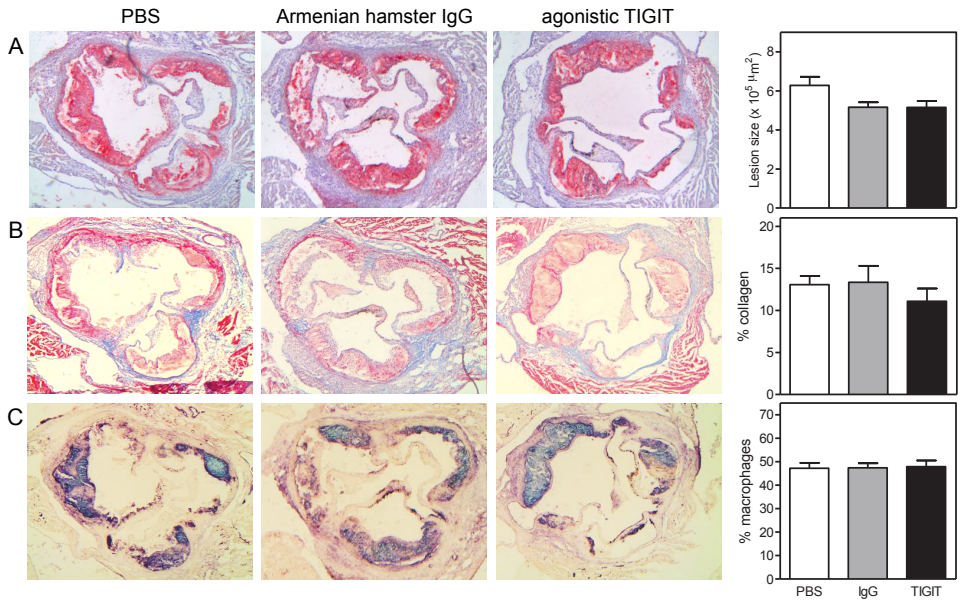


Figure 5. Agonistic anti-TIGIT treatment (n=12) and Armenian Hamster IgG treatment (n=12) reduces atherosclerosis development in LDL$r^{-/-}$ mice fed a Western-type diet for 8 weeks in comparison with PBS treatment (n=11). Representative cross-sections of lesion formation in the three valves area of the aortic root stained with Oil-Red-O and hematoxylin are shown and lesion size was determined (A). Sections of the aortic root were stained for collagen using Masson's trichrome staining. The percentage of collagen relative to the lesion size was determined (B). Furthermore, relative macrophage content was determined with a MOMA-2 staining and quantified (C).

Discussion

The TIGIT/CD226 pathway has been associated with several human autoimmune diseases[8] and studies in mice demonstrate that interference in this pathway may be an attractive approach to modulate autoimmune diseases.[3-5] In the present study we determined the role of TIGIT in atherosclerosis.

Previous studies have shown that TIGIT is mainly expressed on activated $CD4^+$ T

cells in both men and mice.[4, 9] We have previously shown that during the induction of atherosclerosis by feeding a Western-type diet, T cells are activated.[10] We now observe that TIGIT expression was upregulated on $CD4^+$ T cells in Western-type diet fed $LDLr^{-/-}$ mice in comparison with chow diet fed $LDLr^{-/-}$ mice. The expression of TIGIT was further enhanced after αCD3/αCD28 stimulation of isolated splenic T cells. This increase in TIGIT surface expression has been associated with a decrease in T cell proliferation in a number of studies[4, 9], while TIGIT deficiency remarkably increased T cell proliferation in lymph nodes and spleen upon immunization.[4] Since T cell activation is strongly correlated with the development of atherosclerosis[11-13], we aimed to diminish T cell responses by using an agonistic anti-TIGIT antibody which triggers TIGIT signaling. First we showed that exposure of splenocytes isolated from Western-type diet fed mice to agonistic anti-TIGIT *ex vivo* greatly inhibited T cell activation and proliferation, as measured by ^{3}H-thymidine incorporation and IL-2 secretion. In agreement with our *in vitro* data, $LDLr^{-/-}$ mice that received Western-type diet for 4 weeks and were treated with the agonistic anti-TIGIT antibody, show a 45% decrease in splenocyte proliferation in comparison with PBS and Hamster IgG-treated mice.

Subsequently, we investigated whether agonistic anti-TIGIT treatment can be beneficial for the development of atherosclerosis since TIGIT-mediated dampening of T cell responses has been associated with decreased susceptibility to several autoimmune diseases. Levin et al. showed that administration of soluble TIGIT inhibited the severity of collagen-induced arthritis by decreasing T cell infiltration in the paws and by reducing T cell proliferation.[5] Interestingly, both pro-inflammatory cytokines, such as IL-6, IL-17A and TNFα, and anti-inflammatory cytokines, such as IL-10, were reduced in soluble TIGIT-treated mice. Furthermore, TIGIT transgenic mice are protected against the development of EAE[5], whereas $TIGIT^{-/-}$ mice develop exacerbated EAE through elevated T cell proliferation and increased IL-6, IFN-γ, and IL-17 secretion.[4] In addition, adoptive transfer of TIGIT-deficient T cells accelerated GVHD in comparison with transfer of wild-type T cells.[5] Surprisingly, the significant effect of the TIGIT agonist on T cell responses did not affect the development of atherosclerosis after 4 and 8 weeks of Western-type diet feeding, as we observed no significant differences in atherosclerotic lesion sizes between PBS, hamster IgG and agonistic anti-TIGIT-treated mice. Furthermore, in both atherosclerosis studies we did not observe any differences in the collagen and macrophage content of these lesions. Interestingly, the beneficial effect of the TIGIT agonist on T cell activity was accompanied by an activating effect on DCs. Dendritic cells are potent antigen presenting cells and numerous studies have shown the importance of DCs in the development of atherosclerosis. The number of DCs increases with the progression of atherosclerosis in $ApoE^{-/-}$ mice[14, 15] and Wu et al. showed that $CD11c^{-/-}ApoE^{-/-}$ mice fed a Western-type diet have reduced atherosclerosis with a concomitant attenuation of lesional macrophages.[16] Additionally, Paulson et al. showed that CD11c-diphtheria toxin receptor (DTR) $LDLr^{-/-}$ mice fed a cholesterol-rich diet for 5-10 days have a 55% reduced intimal lipid area in comparison with non-depleted mice.[17] Therefore,

increased percentages and activation of dendritic cells in agonistic anti-TIGIT-treated mice can possibly counteract the diminished T cell activity in these mice and thereby neutralize the effect on atherosclerosis. This more pro-inflammatory phenotype of DCs in agonistic anti-TIGIT-treated mice may be caused by the agonistic antibody, which blocks the interaction between TIGIT and PVR expressed on DCs, normally resulting in a tolerogenic phenotype of DCs.

In conclusion, we showed that, although triggering of the TIGIT pathway decreases proliferation and activation of T cells both *in vitro* and *in vivo*, agonistic anti-TIGIT treatment does not affect atherosclerosis development. Future research should concentrate more on the role of TIGIT-PVR signaling, since the generation of tolerogenic DCs in combination with intrinsic T cell inhibition possibly does affect atherosclerosis.

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Chapter 7

Vaccination against Foxp3^{+} regulatory T cells aggravates atherosclerosis

Thomas van Es*
Gijs H.M. van Puijvelde*
Amanda C. Foks
Kim L.L. Habets
Ilze Bot
Eli Gilboa[#]
Theo J.C. van Berkel
Johan Kuiper

* Both authors contributed equally

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

[#] Department of Surgery, Duke Center for Translational Research, Durham, North Carolina

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Abstract

Objective: Regulatory T cells are crucial for immune homeostasis and an impaired regulatory T cell function results in many pathological conditions. Regulatory T cells have already been described to be protective in atherosclerosis. However the exact contribution of Foxp3 expressing natural regulatory T cells in atherosclerosis has not been elucidated yet.

Methods and Results: In this study we vaccinated LDL receptor deficient mice with dendritic cells, which are transfected with Foxp3 encoding mRNA, and studied the effect on initial atherosclerosis. Vaccination against Foxp3 resulted in a reduction of $Foxp3^+$ regulatory T cells in several organs and in an increase in initial atherosclerotic lesion formation. Furthermore we observed an increase in plaque cellularity and increased T cell proliferation in the Foxp3 vaccinated mice.

Conclusions: We further establish the protective role of Tregs in atherosclerosis. The results illustrate the important role for Foxp3 expressing regulatory T cells in atherosclerosis, thereby providing a potential opportunity for therapeutic intervention against this disease.

Introduction

Atherosclerosis is an autoimmune like disease, in which both innate and adaptive immune responses are involved.[1] T helper (Th) cells are crucial for an adequate immune response and can be divided in Th1 and Th2 cells. Several studies show that inflammatory processes in atherosclerosis are associated with a Th1-driven immune response (IFN-γ, IL-12), while the Th2 cells (IL-5 and IL-13) exert an anti-atherogenic role.[2, 3] It was postulated that an imbalance between Th1 and Th2 cells was, at least partially, responsible for the development of atherosclerotic lesions. However, more recently, IL-4, a Th2-cytokine, was found to be pro-atherogenic in early lesion formation.[4, 5] This finding, together with studies establishing an anti-atherogenic role for regulatory T cells (Tregs), suggested another mechanism of immune regulation in atherosclerosis, where T cells (both Th1 and Th2) are suppressed by regulatory T cells (Tregs).

Tregs are characterized by the expression of both CD4 and CD25 and are subdivided in adaptive Tregs and natural Tregs. Adaptive Tregs develop from naive T cells in the periphery and can produce IL-10 (Tr1 cells) and TGF-β (Th3 cells). Natural Tregs originate from the thymus as $CD4^+CD25^+$ cells and exert their suppressive function especially via cell-cell contact and membrane bound TGF-β and CTLA-4. Forkhead box protein P3 (Foxp3) is characteristically expressed in this subclass of Tregs and this transcription factor is necessary for the development of Tregs. Deficiency in Foxp3 leads to a lack of Tregs and severe auto-immune disorders.[6-9]

Recently, we showed that oral administration of atherosclerosis-related antigens (HSP60 and oxLDL) increases the number of Foxp3-expressing Tregs in several organs, which leads to a decrease in development of atherosclerotic lesions in $LDLr^{-/-}$ mice.[10, 11] These results are in line with studies on the role of Tregs in atherosclerosis after oral and nasal tolerance induction[12-14] but our studies specifically demonstrate the contribution of $Foxp3^+$ Tregs. Furthermore, a study by Mallat and colleagues showed that a transfer of Tregs reduced lesion formation in $ApoE^{-/-}$ mice[15], while others showed that treatment of $ApoE^{-/-}$ mice with a depleting CD25-specific antibody (PC61), results in an increase in lesion size.[16] Additionally, bone marrow transplantation of $CD80^{-/-}$ $CD86^{-/-}$ bone marrow into $LDLr^{-/-}$ mice results in a decrease in the number of Tregs and an increase in lesion size, again indicating an inverse relationship between the presence of Tregs and atherosclerotic lesion development.[16]

However, these studies do not directly demonstrate the role of Foxp3 expressing cells in atherosclerosis. To specifically establish the role of Foxp3-expressing Tregs, we targeted Foxp3 expressing cells using DCs electroporated with mRNA encoding for Foxp3 as described by Nair et al., which induces a cytotoxic T lymphocyte response against Foxp3.[17] In the present study, we show that vaccination against Foxp3 results in a reduction of $Foxp3^+$ Tregs and a subsequent increase in initial atherosclerotic lesion formation, thereby establishing a prominent role for $Foxp3^+$ Tregs in this process.

Material and Methods

Animals

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

Synthesis of Foxp3 and GFP mRNA

The pSP73-Spf/Foxp3/A64 construct was kindly provided by E. Gilboa (Duke University Medical Center, Durham, NC, USA).[17] As a control we created a pSP73-Spf/eGFP/A64 construct. The pSP73-Spf/Foxp3/A64 and pSP73-Spf/eGFP/A64 constructs were used as a DNA template in a T7 mMessage mMachine® (Ambion, Austin, TX) reaction to produce large amounts of capped Foxp3 and GFP *in vitro* transcribed mRNA. To eliminate excessive DNA, a TurboDNase® (Ambion, Austin, TX) treatment was performed. The Megaclear Kit® (Ambion, Austin, TX) was used for purifying mRNA from the *in vitro* transcription reactions. All reactions were performed according to the manufacturer's protocol.

Generation and assessment of the DC based vaccine

Bone marrow cells were isolated from the tibia and femora of C57BL/6J mice. Cells were pooled and cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) for 10 days in IMDM supplemented with 8% fetal calf serum (FCS, PAA), 100 U/mL streptomycin/penicillin (PAA), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μM β-mercaptoethanol. Hereafter, the DCs were harvested, washed, and resuspended in Opti-MEM (GIBCO, Grand Island, NY). The used DC culture medium was saved as conditioned media for later use. 5 x 10^6 DCs in 200 μl Opti-MEM were electroporated with either GFP or Foxp3 mRNA as described by Nair et al.[18] After electroporation the DCs were transferred to culture petridishes containing GM-CSF and a 1:1 combination of conditioned DC growth medium and fresh medium. Transfected DCs were incubated at 37°C, 5% CO_2 overnight, and subsequently were washed 2 times in PBS before vaccination. As a control for maturation of DCs, LPS (1 μg/mL) was added to one group of DCs for 24 hours. To assess the maturation profile, the electroporated DCs were stained against surface markers (CD80-FITC, CD86-PE and CD40-PE) and analyzed using FACS. To detect intracellular Foxp3 expression in the electroporated DCs, the cells were stained with Foxp3-APC. For intracellular staining of Foxp3, DCs were fixated and permeabilized overnight and subsequent stained against Foxp3. All antibodies were purchased from eBioscience (Belgium) and used for FACS analysis according the manufacturer's protocol. Total RNA was isolated from the electroporated DCs using the GTC method.[19] cDNA synthesis was performed using Revert

Aid™ M-MuZV Reverse Transcriptase (Fermentas Life Science). Quantitative Foxp3 gene expression (5'-GGAGCCGCAAGCTAAAAGC-3' and 5'-TGCCTTCGTGCCCACTGT-3') analysis was performed on a 7500 fast Real-Time PCR System (Applied Biosystem) using SYBR Green technology. Acidic ribosomal phosphoprotein PO (36B4; 5'-GGACCCGAGAAGACCTCCTT-3' and 5'-GCACATCACTCAGAATTTCAATGG-3') and hypoxanthine phosphoribosyl transferase (HPRT; 5'-TTGCTCGAGATGTCATGAAGGA-3' and 5'-AGCAGGTCAGCAAAGAACTTATAG-3') were used as reference genes.

Vaccination and the induction of atherosclerosis

Mice were injected with $5x10^5$ DCs in 100 µl per mouse (GFP n=14, Foxp3 n=13) subcutaneously at the base of the ear pinna at day 0. Mice of the control group (n=15) were injected with 100 µl PBS. After treatment, the mice were fed a Western-type diet (0.25% cholesterol and 15% cocoa butter) for 8 weeks to induce hypercholesterolemia and atherosclerosis. At sacrifice, tissues were harvested after *in situ* perfusion using PBS and subsequent perfusion with Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). Tissues were snap-frozen in nitrogen and stored at –80 °C until further use. During the experiment blood samples were obtained by tail vein bleeding at various time points. The concentration of serum cholesterol was determined using an enzymatic colorimetric procedure (Roche/Hitachi). Precipath (Roche/Hitachi, Mannheim, Germany) was used as an internal standard. Blood samples of week 5 were also used to determine the percentage of Tregs in blood using flow cytometry as described below.

Flow cytometry

At sacrifice, Peripheral Blood Mononuclear Cells (PBMCs) were isolated via orbital bleeding and erythrocytes were removed by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Spleens, heart lymph nodes (HLN) and mesenteric lymph nodes (MLN) were dissected from the mice (n=5/group) and a single cell suspension was obtained by passing the organs through a 70 µm cell strainer (Falcon, The Netherlands; n=5/group). Cells were stained with surface markers (0.25 µg antibody/300.000 cells) and subsequently analyzed by flow cytometric analysis. For the detection of $CD4^+CD25^+Foxp3^+$ T cells, the spleen, blood, MLN and HLN were stained with CD4-FITC and CD25-PE and subsequently intracellularly with Foxp3-APC. All antibodies were purchased from eBioscience (Immunosource, Belgium). All data were acquired on a FACSCalibur (Becton Dickinson, Mountain View, CA) and analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Spleen cell proliferation

Splenocytes (n=5/group) were cultured for 72 hours in triplicate in a 96-wells round-bottom plate (2×10^5 cells/well, Greiner Bio-One) in RPMI 1640 supplemented with L-Glutamine, 10% FCS and 100 U/mL streptomycin/penicillin. As a positive control cells were stimulated with Concanavalin A (2 µg/mL, Con A, Sigma Diagnostics, MO).

Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences, The Netherlands) for the last 16 hours. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The proliferation is expressed in disintegrations per minute (dpm).

Histological analysis

The heart was embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and cryosections (10 µm) of the aortic root containing the three aortic valves were made. Cryosections were routinely stained with Oil-Red-O and hematoxylin (Sigma Diagnostics, MO). Corresponding sections on separate slides were also stained for collagen, macrophages and fibroblasts using Masson's Trichrome staining (Sigma Diagnostics), MoMa-2 antibody (Research Diagnostics Inc.), and ER-TR7 antibody (AbD Serotec), respectively, according to manufacturer's protocols. For the cellularity assessment a hematoxylin staining was performed. The different histological stainings were quantified using a Leica DM-RE microscope and Leica Qwin Imaging software (Leica Ltd., Germany).

Statistical Analysis

All data are expressed as mean±SEM. The two-tailed student's T-test was used to compare individual groups of mice or cells. When indicated, a Mann-Whitney test was used to analyze not normally distributed data. The frequency of thickened cap structure was analyzed by a Fisher's exact test. P values of <0.05 were considered significant.

Results

A DC based vaccination strategy against Foxp3

To test whether a reduction in Foxp3-expressing Tregs affects atherosclerosis, a DC

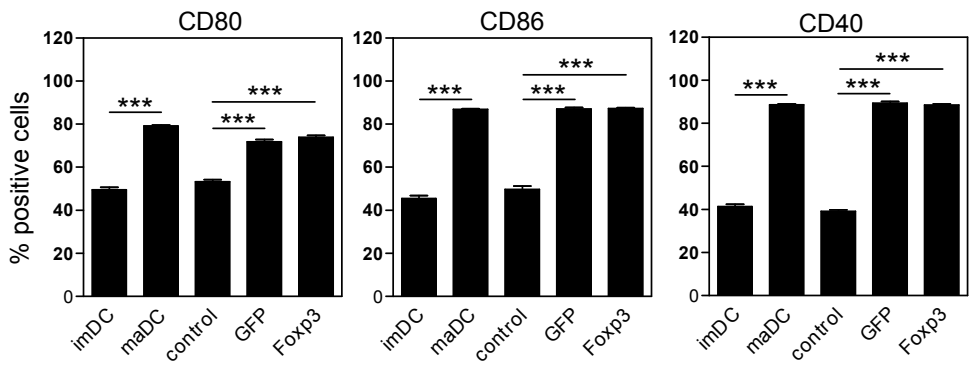


Figure 1. Expression of costimulatory molecules on electroporated DCs. After electroporation with either PBS (control, n=3), GFP mRNA (n=3) or Foxp3 mRNA (n=3) the DCs were cultured o/n. One group of DCs were stimulated with LPS as a positive control for maturation (mDCs). Unstimulated DCs (imDCs) were used as a negative control for maturation. DCs were subsequently stained for CD80, CD86 and CD40 and analyzed by FACS. ***$P<0.001$

based vaccination strategy was used in which DCs were transfected with mRNA encoding for Foxp3. A significant increase in the surface expression of the co-stimulatory molecules, CD80, CD86 and CD40 was observed in DCs that were electroporated with mRNA encoding Foxp3 or GFP, when compared with electroporation without mRNA (Control) and immature DCs (imDCs). The increase is comparable to the LPS induced maturation of DCs (mDCs) (Figure 1). After electroporation with mRNA encoding for Foxp3 a strong increase in Foxp3 expression in DCs was observed on mRNA level, which indicated a successful transfection of the DCs (Figure 2A). To determine the intracellular protein expression of Foxp3, an intracellular Foxp3 FACS staining was performed (Figure 2B and C). Foxp3 transfected DCs expressed 6.3-fold more intracellular Foxp3 compared to GFP transfected DC, which expressed no Foxp3.

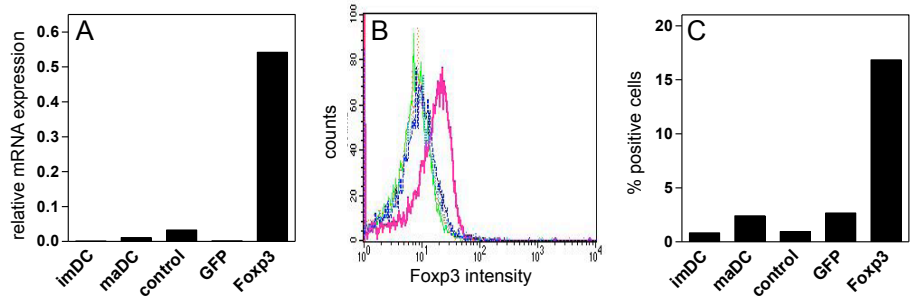


Figure 2. Assessment of Foxp3 specific mRNA and the subsequent expression in DCs. After electroporation of DCs with mRNA encoding for Foxp3 or GFP, total RNA was isolated and cDNA was synthesized. Subsequently the amount of mRNA was detected with Foxp3 specific qPCR primers (A). The expression is relative to HPRT and 36B4. Next we determined, with FACS analysis, the intracellular expression of Foxp3 in PBS (green), GFP (blue) and Foxp3 (pink) electroporated DCs. Additionally, electroporated DCs were cultured o/n and stained intracellularly for Foxp3 and analyzed by FACS. A representative histogram (B) and percentage of intracellular Foxp3 expression is depicted (C).

Vaccination against Foxp3 reduces the number of Tregs

To asses the efficacy of the Foxp3 vaccine, LDLr$^{-/-}$ mice were vaccinated with Foxp3 electroporated DCs to induce a cytotoxic immune response against Foxp3 as described by Nair et al.[17] As a control, mice were vaccinated with DCs electroporated with mRNA encoding for GFP. Before vaccination, the number of Tregs in blood in all groups of mice was equal, but five weeks after vaccination a 34% reduction in the number of Foxp3$^+$ Tregs in Foxp3 vaccinated mice was observed, compared to GFP vaccinated mice (Figure 3A; 0.773±0.032% versus 1.175±0.065%; P<0.001). Upon sacrifice, vaccination with Foxp3 transfected DCs (black bars; Figure 3B) resulted in a 27-30% decrease in Foxp3$^+$ Tregs in blood (0.67±0.045% versus 0.92±0.096%, P<0.05), spleen (2.88±0.24% versus 3.94±0.34%, P<0.05), MLN (3.59±0.10% versus 5.01±0.45%, P<0.05) and HLN (3.68±0.08% versus 5.50±0.46%, P<0.01), when compared to mice vaccinated with DCs electroporated with mRNA encoding for GFP (grey bars; Figure 3B). As a control for the DC vaccination, one group of mice was treated with PBS. We observed no differences in the numbers of Foxp3$^+$ Tregs in lymphoid organs and blood between mice treated with PBS and mice vaccinated with

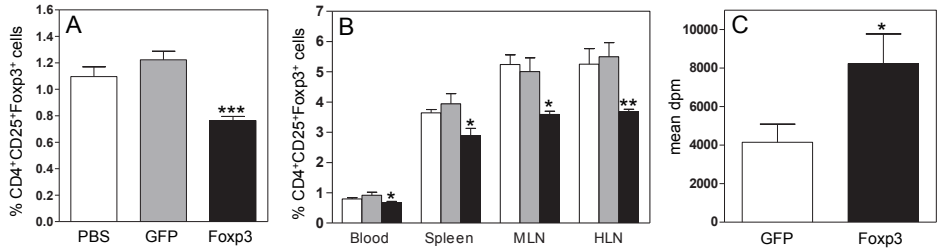


Figure 3. Effect of vaccination against Foxp3 on the percentage of Tregs in lymphoid organs and on spleen cell proliferation. LDLr$^{-/-}$ mice were vaccinated against GFP or Foxp3 using electroporated DCs. As a second control, one group of mice was vaccinated with PBS. Five weeks after vaccination blood was taken from the mice vaccinated with either PBS (A, n=5, white bar), GFP (n=5, grey bar) or Foxp3 (n=5, black bar) and was analyzed for CD4$^+$CD25$^+$Foxp3$^+$ cells using FACS analysis. Eight weeks after vaccination the mice vaccinated with either PBS (B, n=5, white bars), GFP (n=5, grey bars) or Foxp3 (n=5, black bars) were sacrificed. Blood, spleen, MLN and HLN, were isolated and the number of CD4$^+$CD25$^+$Foxp3$^+$ cells in these organs was determined. The effect of vaccination against Foxp3 on spleen cell proliferation was determined by culturing splenocytes isolated after 8 weeks *ex vivo* (C). Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The proliferation is expressed in disintegrations per minute (dpm). *P<0.05, **P<0.01, ***P<0.001

DCs electroporated with mRNA encoding for GFP (white bars; Figure 3A and B). Regulatory T cells have an inhibitory effect on T cell proliferation. Therefore we investigated whether a vaccination against Foxp3 and a subsequent reduction in the number of Tregs influences the proliferation of splenic T cells. We observed a significant 1.9-fold increase in spleen cell proliferation compared to the GFP vaccinated mice (Figure 3C: 4148.5±941.4 dpm versus 8230.5±1542.5 dpm P<0.05).

Vaccination against Foxp3 increased lesion formation in LDLr$^{-/-}$ mice

Eight weeks after vaccination and Western-type diet feeding the plaque size at the aortic root was analyzed. Representative slides of the aortic root of GFP and Foxp3 vaccinated mice are shown in Figure 4A and B, respectively. Mice vaccinated against Foxp3 showed a significant 34% increase in plaque size compared to the GFP vaccinated mice (Figure 4C; 538.932±46.043 μm^2 versus 382.865±29.044 μm^2,

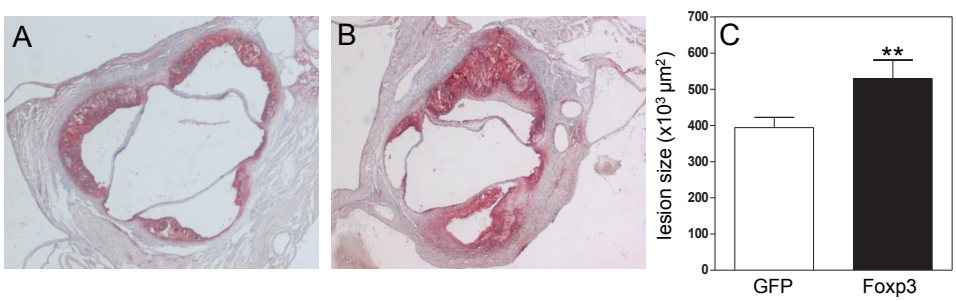


Figure 4. Effect of vaccination against Foxp3 on initial atherosclerotic lesion development. Eight weeks after vaccination and Western-type diet feeding the LDLr$^{-/-}$ mice were sacrificed and the hearts of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with Oil-Red-O and hematoxylin. The lesions were quantified and the plaque size was determined (C). **P<0.01

P<0.01). During the experiment, all mice developed hypercholesterolemia, however no significant differences in serum cholesterol levels and body weight (data not shown) were observed between the different groups of mice. No differences in plaque size were observed between PBS treated mice and mice vaccinated against GFP (data not shown).

Vaccination against Foxp3 results in increased plaque cellularity

To investigate whether there is a change in plaque composition in the enlarged initial plaques of the Foxp3 vaccinated mice the number of cells within the lesions were quantified. The plaques of Foxp3 vaccinated mice showed a 27% increase in cellularity compared to GFP vaccinated mice (Figure 5A-C; $3.84x10^{-3}\pm0.19x10^{-3}$ cells/μm^2 versus $2.81x10^{-3}\pm0.79x10^{-3}$ cells/μm^2, respectively; *P*<0.01). Subsequently we determined the plaque stability by measuring both the amount of collagen within the plaque and the fibrous cap thickness. The collagen content of the plaque was not significantly changed between the Foxp3 and GFP vaccinated mice (Figure 5D-F, 9.0±1.1% versus 6.5±1.9%, respectively).

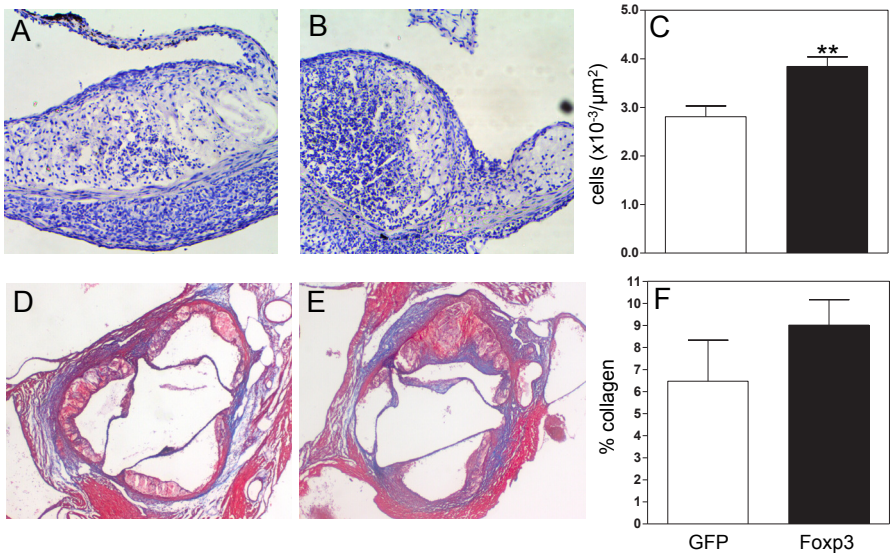
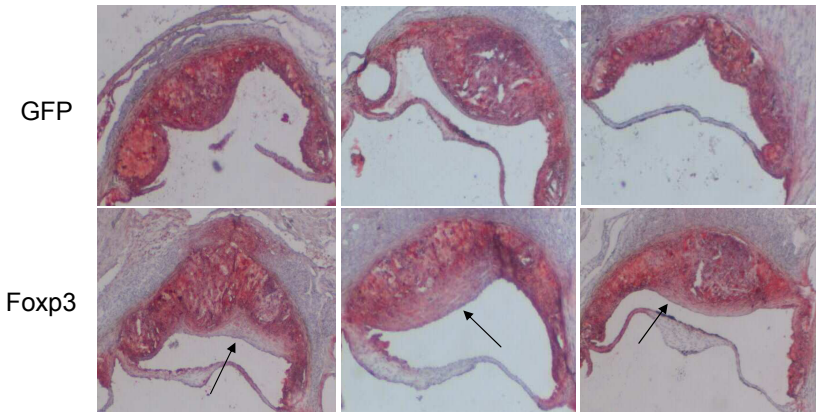


Figure 5. Effect of vaccination against Foxp3 on plaque composition. Eight weeks after vaccination and Western-type diet feeding the $LDLr^{-/-}$ mice were sacrificed and the hearts of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with hematoxylin to stain the nucleus of the cells within the lesion. The number of nuclei was quantified (C). Sections of the hearts of GFP treated (D) and Foxp3 treated (E) mice were also analyzed for collagen content using the Masson's Trichrome staining which stains collagen blue. The percentage of collagen relative to the lesion size was determined (F). ***P*<0.01

Additionally, we noticed that the lesions in 9 of the 13 Foxp3-vaccinated mice displayed significant characteristic changes in morphology. These lesions are characterized by a thickened fibrous cap-like structure (Figure 6, arrows). This cap however does not cover the entire plaque and seems to grow into the lesion. In addition, this cap-like

structure does not seem to provide a stable phenotype. When compared with GFP vaccinated mice in which 2 out of 14 mice showed these thickened structures, a significant difference is observed when a Fisher's exact test is performed (Figure 6, $P<0.01$). These thickened structures do not contain any macrophages or fibroblasts and overall there was no significant difference in cap thickness in the different groups.



	No thickened cap	Thickened cap
GFP	12	2
Foxp3	4	9

Figure 6. Thickened cap structure in Foxp3 vaccinated mice. Within the Foxp3 vaccinated group more thickened structures in the fibrous cap (arrows) were observed when compared to the GFP vaccinated mice. Frequency of thickened structure between GFP and Foxp3 vaccinated mice is depicted in the table. A Fisher's exact test was used ($P<0.01$).

Discussion

In this study we demonstrate that vaccination against Foxp3$^+$ Tregs aggravates atherosclerotic lesion formation, thereby directly establishing the protective role of Foxp3$^+$ Tregs in atherosclerosis. The beneficial role of Tregs in atherosclerosis is already indicated in a number of publications.[16, 20] We showed before that induction of Foxp3 positive Tregs via oral tolerance induction against HSP60 and oxLDL reduces atherosclerotic lesion formation.[10, 11] Furthermore, Ait-Oufella and colleagues demonstrated that a deficiency in costimulatory molecules promotes atherosclerosis because of a decrease in the number of Tregs and showed that a depletion of CD25$^+$ cells using anti-CD25 antibodies increased lesion formation.[16] However, the final proof for the involvement of Foxp3 expressing T cells in atherosclerosis has not been provided since CD25 is not exclusively expressed on Tregs, as it is also present on activated T cells[21], NK cells[22] and myeloid DCs.[23]

To specifically study the role of Foxp3 positive Tregs in atherosclerosis, we vaccinated atherosclerosis prone mice against Foxp3 using a DC vaccination strategy. DCs are

electroporated with mRNA encoding for Foxp3 and this approach, described by Nair et al, results in a cytotoxic T lymphocyte (CTL) response against Foxp3 and a subsequent depletion of Foxp3+ Tregs.[17] As a control, mRNA encoding for GFP was used to exclude that mRNA electroporation into DCs activates the DCs leading to immune modulatory effects. In all the experiments, the treatment with GFP electroporated DCs was compared with PBS treatment and no differences were observed between both control treatments. After electroporation of DCs with mRNA, either with GFP or with Foxp3, an activated phenotype of DCs was observed. This was not observed when DCs were electroporated without mRNA. It is known that single stranded RNA (ssRNA), such as mRNA, is a natural ligand for Toll like receptors 7 and 8,[24, 25] which may explain the activated phenotype of the DCs after electroporation with mRNA.

We successfully demonstrated that the DCs were transfected with the mRNA coding for Foxp3 via qPCR and FACS analysis. Foxp3 is a nuclear product and is not expressed on the cell surface. However, we use a truncated Foxp3 in which the nuclear localization sequence is removed. This results in relatively high concentration of cytosolic Foxp3 protein and therefore may result in the cross-presentation of Foxp3-peptides on MHC class I. Furthermore, mRNA may act as a natural agonist of TLR7/8 and thereby even further enhance cross-presentation, which is already described by other groups.[24-27] This may lead to the presentation of Foxp3 peptides to CD8+ T cells, which subsequently target Foxp3 expressing cells.

Our present data show a significant reduction in Foxp3+ Tregs in blood 5 weeks after vaccination. A similar reduction in Foxp3+ Tregs was observed within the blood, HLN, MLN and spleen, 8 weeks after vaccination, indicating a systemic reduction in Foxp3+ Tregs and a persistent effect of the vaccination. As shown before by Nair et al.[17] this reduction in Tregs is caused by the induction of a robust Foxp3-specific cytotoxic T lymphocyte response. Additionally, we observed an increased spleen cell proliferation in the Foxp3 vaccinated mice compared with GFP vaccinated mice which is indicative for a reduced number of Tregs since these cells suppress effector T cells. The reduction in Foxp3+ Tregs resulted in a 34% increase in initial lesion size. The increase in atherosclerosis is not related to a change in total cholesterol serum levels since there is no significant difference in cholesterol levels between the different groups.

Besides an increase in lesion size, vaccination against Foxp3 also induced a 30% increase in cellularity of the initial lesions. The increased cellularity may indicate an increase in inflammation within the lesion. This may be caused by an increase in proliferation of inflammatory cells or by an increase in influx of inflammatory cells, which in normal conditions would be under the control of Tregs. The increase in cellularity is not caused by an accumulation of macrophages or fibroblasts.

In addition to plaque cellularity, we analyzed the plaque stability but no differences in both the collagen content and the fibrous cap thickness were observed between GFP and Foxp3 vaccinated mice. In spite of the fact that the fibrous cap thickness was not changed, we observed significant more thickened fibrous cap-like structures in the Foxp3 vaccinated mice. These thickened structures, which also seem to grow into

the lesion, do not contain macrophages and fibroblasts and do not cover the entire plaque. The exact composition and function of these structures remains therefore unclear and needs further investigation, especially because these cap-like structures do not look like a stable fibrous cap.

The results in this study are in line with the observations that the induction of Tregs exerts an atheroprotective effect. The increase in lesion size is comparable with the decrease of initial lesion size upon the induction of Tregs via oral administration of oxLDL (30.0%) or HSP60 (27.5%).[10, 11] In conclusion, we confirmed the protective role of Foxp3$^+$ Tregs in atherosclerosis by vaccinating LDLr$^{-/-}$ mice using a DC based Foxp3 vaccination strategy. The results illustrate an important role for Foxp3$^+$ Tregs in atherosclerosis, thereby providing a potential opportunity for therapeutic intervention against atherosclerosis.

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Chapter 8

Differential effects of regulatory T cells on the initiation and regression of atherosclerosis

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

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

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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.

Conclusions: 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 the atherosclerotic lesions.

Introduction

Atherosclerosis is considered 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 shown 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 is 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 EAE 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 of the 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, Witham, Essex, UK). 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, Abington, UK) in sterile PBS and incubated at 37°C for 30 minutes before injecting intraperitoneally (i.p.).

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 i.p. with the IL-2 complex (n=11) or with sterile PBS as a control (n=11). Initially, mice were treated i.p. 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 i.p. 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. Pittsburg, USA). 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, Mannheim, Germany). 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. Lesion 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, Cambridge, UK).

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/200.000 cells). For intracellular staining of Foxp3, cells were fixated and permeabilized overnight and subsequently stained against Foxp3 according to manufacturer's protocol (eBioscience, San Diego, CA). 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 70 µm cell strainer. Red blood cells were removed as described above. 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 (Beckton Dickinson, Mountain View, CA). Data were analyzed using FACSDiva software (Beckton Dickinson).

Spleen cell proliferation

The splenocytes (n=5 per group) were cultured for 48 hours in triplicate in a 96-wells round-bottom plate (3×10^5 cells/well, Greiner Bio-One) in RPMI 1640 supplemented with L-Glutamine, 100 U/mL streptomycin/penicillin and 10% FCS. As a positive control cells were stimulated with αCD3 and αCD28 (2 µg/mL). Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences, The Netherlands) 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 αCD3/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 (Utrecht, The Netherlands). 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 αCD3 and αCD28 (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, Belgium).

Real-time PCR assays

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, CA) using SYBR green technology. The following primer pairs were used: 5'-TCTTACTGACTGGCATGAGGATCA-3' and 5'-GTCCGCAGCTCTAGGAGCAT-3' for IL-10 and 5'-AGGGCTACCATGCCAACTTCT-3' and 5'-GCAAGGACCTTGCTGTACTGTGT-3' for TGF-β. The following primers were used as endogenous references: 5'-GGACCCGAGAAGACCTCCTT-3' and 5'-GCACATCACTCAGAATTTCAATGG-3' for acidic ribosomal phosphoprotein PO (36B4) and 5'-TTGCTCGAGATGTCATGAAGGA-3' and 5'-AGCAGGTCAGCAAAGAACTTATAG-3' for hypoxanthine phosphoribosyltransferase (HPRT).

Statistical analysis

All data are expressed as mean±SEM. An unpaired two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Probability values of $P<0.05$ were considered significant.

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 (control) 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,B). The Tregs expanded 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. 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 control whether the Treg expansion induced by the IL-2 complex is still functional under hyperlipidemic, pro-inflammatory circumstances, LDLr$^{-/-}$ mice were fed a Western-type diet for 8 weeks. Two weeks after initiation of the experiment, mice were i.p. 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).

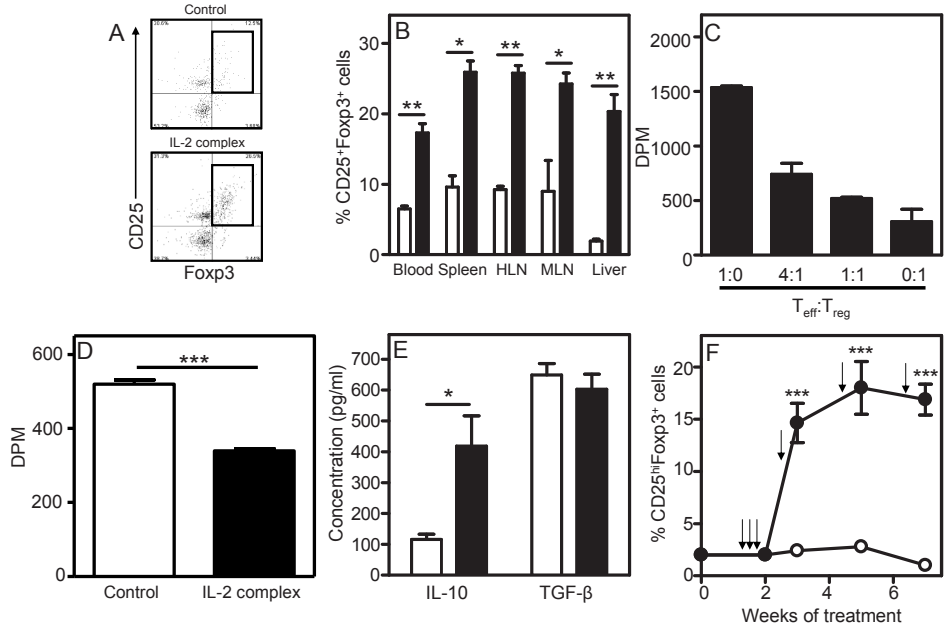


Figure 1. IL-2 complex induces persistent high levels of Tregs in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were injected 3 times with an IL-2 complex (n=3, black bars) or PBS as a control (n=3, open bars). Mice were sacrificed 5 days after initiation of the experiment. Blood, spleen, mediastinal lymph nodes 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 (A,B). A suppression assay was performed to determine the suppressive capacity of the expanded Tregs by measuring the proliferation of splenocytes (C, n=3). Data are shown as the mean disintegration per minute (dpm) of triplicate cultures. 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 (D). 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 (E, control: open bars, IL-2 complex: black bars). 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 week 2, 3, 5 and 7 using flow cytometry (F, n=5 per group). * P<0.05, ** P<0.01, *** P<0.001

IL-2 complex administration reduces the development of atherosclerosis

Since a limited increase in Tregs already affects the 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 ($1.73\pm0.13\times10^5$ μm^2) in comparison with control mice ($2.84\pm0.30\times10^5$ μm^2, P<0.01, Figure 2A,B). No difference in lesion stability, as determined by Masson's Trichrome staining,

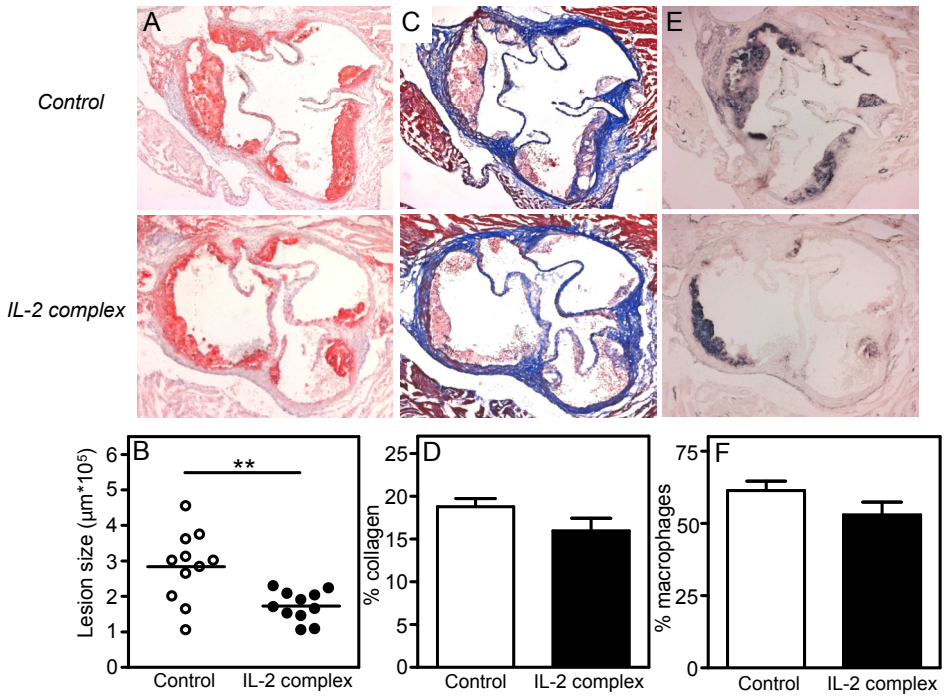


Figure 2. Expansion of Tregs reduces lesion formation. LDLr$^{-/-}$ mice were fed a Western-type diet for 8 weeks and were treated i.p. with the IL-2 complex (n=11) or PBS as a control (n=11). Representative cross-sections of lesion formation in the three valves area of the aortic root stained with Oil-Red-O and hematoxylin are shown (A) and lesion size was determined (B). Corresponding sections on separate slides were stained for collagen using Masson's trichrome staining (C). The percentage of collagen relative to the lesion size was determined (D). Furthermore, relative macrophage content was determined with a MOMA-2 staining and quantified (E,F). ** P<0.01

was observed between IL-2 complex treated mice (16.0±1.5%) and control treated mice (18.8±0.9%) (Figure 2C,D). Furthermore, no difference in macrophage content was observed (control: 61.4±3.3% and IL-2 complex: 53.0±4.4%, Figure 2E,F). 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.

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 (Figure 3D, 1.7±0.2% vs. 3.6±0.4%, P<0.01) and reduced GATA-3 expression (16.1±0.6% vs. 19.8±1.3%, P<0.05) in the $CD4^+$ T cell population of the spleen, compared to the control group. Accordingly, reduced $CD4^+IFN\text{-}\gamma^+$ T cells were observed (Figure 3E, 6.1±0.5% vs. 9.4±1.0%, P<0.05). Interestingly, the percentage of $IL\text{-}4^+$ 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).

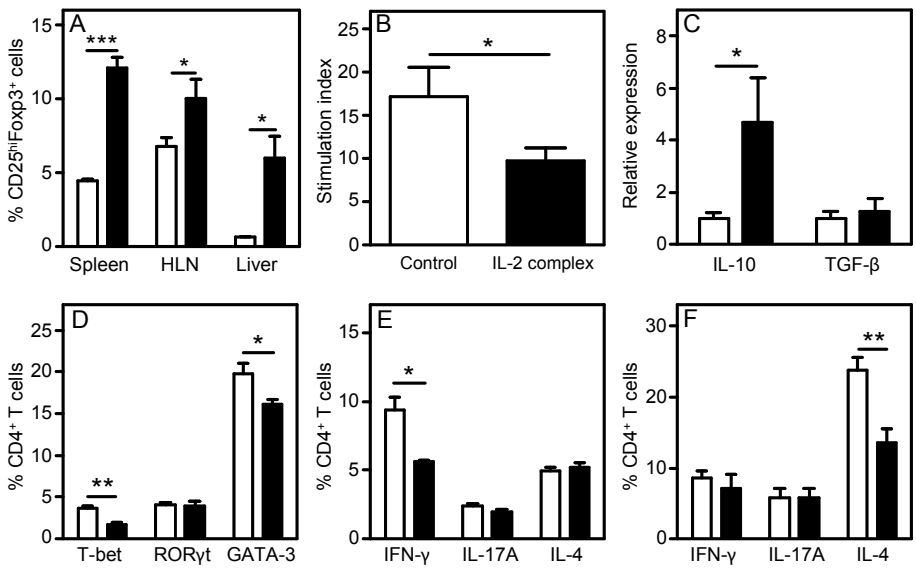


Figure 3. Effect of the IL-2 complex on the percentage and functionality of the Tregs. Eight weeks after induction of atherosclerosis mice were sacrificed. Spleen, HLN and liver cells were isolated and stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry (n=5 per group, A). The effect of boosting Tregs with the IL-2 complex on spleen cell proliferation was determined by culturing splenocytes (n=5 per group) in the presence or absence of CD3/CD28 stimulation (B). Proliferation was assessed by the amount of ^3H-thymidine incorporation in dividing cells. The proliferation is expressed as stimulation index. mRNA levels of IL-10 and TGF-β in the spleen were determined with RT-PCR (C). Spleen cells were stained for CD4 and the transcription factors T-bet (Th1), RORγt (Th17) and GATA-3 (Th2, D). In addition, cytokine production in the spleen (E) and blood (F) 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 a treatment with the IL-2 complex. 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, 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 and at sacrifice *ex vivo* effector T cell proliferation was suppressed (data not shown). In addition, highly elevated Treg levels were observed in blood, spleen and HLN at sacrifice (Figure 4C).

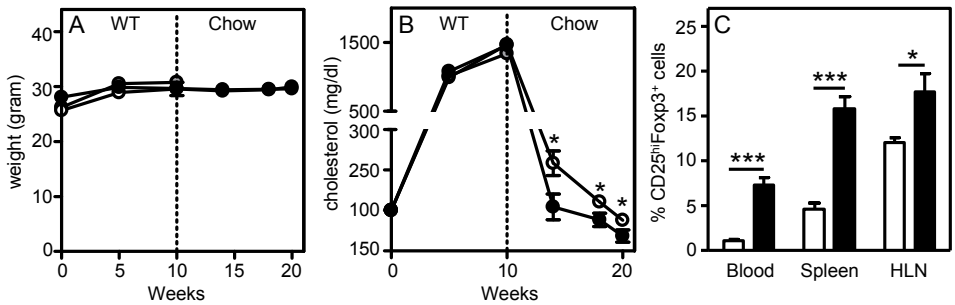


Figure 4. Effect of IL-2 complex on weight, cholesterol levels and Tregs during regression of atherosclerosis. LDLr$^{-/-}$ mice received 10 weeks Western-type diet and were subsequently put on chow diet for 10 weeks and treated with the IL-2 complex (n=13, black circles) or PBS (n=14, open circles). A baseline group was sacrificed after 10 weeks of Western-type diet (n=11, grey circles). During the experiment, mice were weighed (A) and blood was taken by tail vein bleeding. Total cholesterol concentration was determined within the serum (B). To check whether the IL-2 complex expanded the Tregs, 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, C). * 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±0.25×10^5 μm^2 and IL-2 complex: 3.10±0.25×10^5 μm^2) when compared with the baseline group (2.74±0.10×10^5 μm^2, Figure 5A,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 and macrophage content of the lesions was determined (Figure 5B and 5D-E). Lesions from IL-2 complex treated mice (38.3±2.3% collagen) showed a substantially increased stability compared to control treated mice (31.6±1.8% collagen, P<0.05).

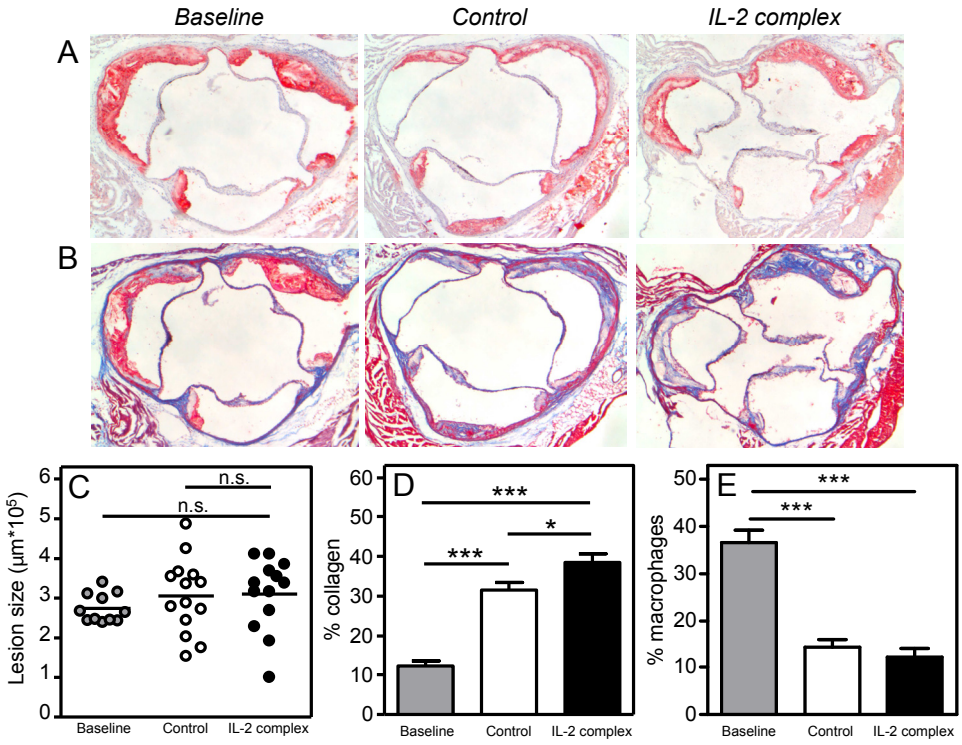


Figure 5. IL-2 complex treatment stabilizes atherosclerotic lesions during regression. LDLr$^{-/-}$ mice received Western-type diet for 10 weeks and were subsequently put on chow diet for 10 weeks and simultaneously treated with the IL-2 complex (n=13) or PBS (n=14). A baseline group was sacrificed after 10 weeks of Western-type diet (n=11). Sections of the aortic root were stained with Oil-Red-O and hematoxylin (A) and subsequently lesion size was determined (C). Corresponding sections on separate slides were also stained for collagen using Masson's trichrome staining (B). The percentage of collagen relative to the lesion size was determined (D). Furthermore, relative macrophage content was determined with a MOMA-2 staining and quantified (E). * P<0.05, *** P<0.001

Additionally, both groups showed very significantly increased collagen content (P<0.001) compared to baseline mice (12.2±1.2%). 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 significantly 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 treated mice (86.4±10.6 cells/section) compared to control treated mice (40.3±6.3 cells/section, P<0.01, Figure 6).

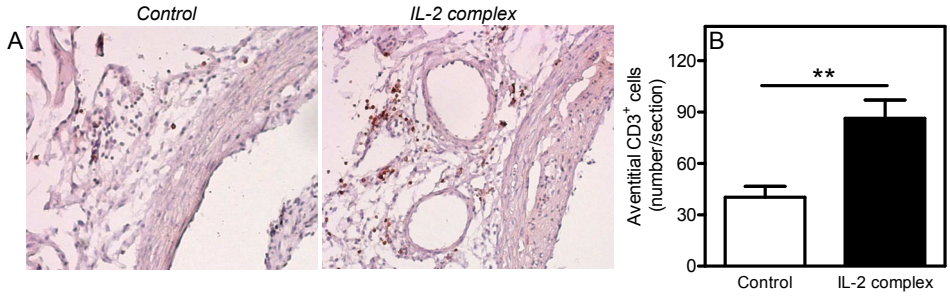


Figure 6. Increased adventitial CD3$^+$ T cell infiltration in IL-2 complex treated mice. LDLr$^{-/-}$ mice received 10 weeks Western-type diet and were subsequently put on chow diet for 10 weeks and simultaneously treated with the IL-2 complex (n=13) or PBS (n=14). Sections of the aortic root were stained for CD3 (red) to determine the number of infiltrating T cells (A-B). $^{**}P<0.01$

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 2-3 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 2 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 6 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 high 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 2 weeks after treatment, while the present technique enabled us to maintain the enhanced numbers of Tregs for more than 6 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 focuses on preventing the initiation and to a minor extent on 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 in 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 and Western-type diet 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 found 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 cell expansion and function and limits 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 complex 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.

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Chapter 9

Regulation of atherosclerosis by CD11b^{+}Gr-1^{+} myeloid-derived suppressor cells

Amanda C. Foks*
Gijs H.M. van Puijvelde*
Vanessa Frodermann
Thomas van der Heijden
Louis Boon[#]
Ilze Bot
Johan Kuiper

* Both authors contributed equally

Division of Biopharmaceutics, Leiden University, Leiden, The Netherlands

[#] Bioceros BV, Utrecht, The Netherlands

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Abstract

Objective: Restoration of immune homeostasis in atherosclerosis represents the ultimate goal of an immune-based therapy. Myeloid-derived suppressor cells (MDSCs) are a population of immature myeloid cells that potently suppress immune responses in various pathological settings, via multiple mechanisms, including inhibition of T cell responses. They express the myeloid markers CD11b and Gr-1 and can be subdivided in monocytic-MDSCs, expressing high levels of Ly6C, or granulocytic-MDSCs, which are Ly6G positive. In the present study, we determined the role of MDSCs in atherosclerosis by an adoptive transfer of $CD11b^{+}Gr\text{-}1^{+}$ cells into $LDLr^{-/-}$ mice fed a Western-type diet.

Methods and Results: We isolated $CD11b^{+}Gr\text{-}1^{+}$ cells from $LDLr^{-/-}$ mice fed a Western-type diet for 2 weeks with magnetic beads and found that they strongly suppressed αCD3/CD28-induced splenocyte proliferation in an IFN-γ and iNOS-dependent manner. Subsequently, we adoptively transferred $6x10^{6}$ MDSCs every 10 days into $LDLr^{-/-}$ mice fed a Western-type diet for 6 weeks, which resulted in a 35% reduction in atherosclerotic lesion formation in the aortic root. MDSC treatment reduced splenic Th1 and Th17 cells with 50% and diminished B cells, in particular circulating B2 cells, and concomitantly impaired their proliferative capacity.

Conclusions: Our data prove that MDSCs could represent a novel cell-based immune-therapy to dampen pro-atherogenic immune responses and thereby reduce atherosclerosis.

Introduction

Myeloid-derived suppressor cells (MDSCs) form a heterogeneous population of cells that consists of early myeloid progenitor cells and immature myeloid cells, which strongly suppress immune responses during cancer and other diseases. MDSCs express the myeloid cell markers CD11b and Gr-1 and are found in spleen and bone marrow.[1] In healthy individuals, MDSCs differentiate into neutrophils, macrophages or dendritic cells. However, under pathological conditions, this differentiation is partially blocked, which results in the expansion of MDSCs that accumulate in various lymphoid and non-lymphoid tissues. Two subtypes of MDSCs have been identified based on the expression of surface markers; granulocytic-MDSCs, which are CD11b^{+}Ly6G^{+}Ly6C^{low}, and monocytic-MDSCs, which are CD11b^{+}Ly6G^{-}Ly6C^{hi}.[2]

MDSCs particularly suppress T cell function via an increased expression of immune suppressive factors, such as inducible nitric oxide synthase (iNOS) and arginase 1 (arg-1)[3], and via increased production of nitric oxide (NO) and reactive oxygen species (ROS).[4] iNOS expression in MDSCs is driven by Th1 signals[5], such as IFN-γ and LPS, whereas arg-1 expression is driven by Th2 cytokines, such as IL-4, IL-10 and IL-13.[6] Both iNOS and arg-1 compete for their common substrate L-arginine. iNOS consumes L-arginine to NO, which renders T cells non-responsive to IL-2.[7] In contrast, arg-1 converts L-arginine to urea and polyamines, which reduces the availability of L-arginine and subsequently impairs T cell function by loss of CD3ζ expression.[8] Both arg-1 and iNOS can generate ROS, which inhibits T cell function via nitration of the T cell receptor, thereby impairing the T cell-antigen-MHC interaction.[3] Moreover, MDSCs can also promote *de novo* development of Foxp3, which results in elevated levels of regulatory T cells (Tregs) that efficiently suppress effector T cells.[9] Besides regulating adaptive immune responses, MDSCs can also stimulate macrophages to a more anti-inflammatory phenotype, producing more IL-10 and less IL-12.[10]

MDSCs have been extensively investigated in cancer but their effect on the course of other diseases has only recently been appreciated. MDSCs suppresses inflammation in obese mice[11] and in an EAE mouse model[12] and prevented type 1 diabetes[13] and graft versus host disease.[14] However, so far studies that describe the role of MDSCs in the development of atherosclerosis are lacking. Atherosclerosis is considered a chronic autoimmune-like disease with an underlying imbalance between pro-inflammatory and anti-inflammatory processes.[15, 16] Restoration of this balance by induction of Tregs that suppress effector T cells has proven to be of therapeutic potential in the treatment of atherosclerosis.[17-19] Since MDSCs can suppress effector T cells, MDSCs may represent a novel therapeutic tool to regulate pro-inflammatory immune responses in atherosclerosis.

To investigate whether and how MDSCs contribute to the development of atherosclerosis, we isolated bone marrow-derived CD11b^{+}Gr-1^{+} cells from LDLr$^{-/-}$ mice fed a Western-type diet for 2 weeks and adoptively transferred them into LDLr$^{-/-}$ mice fed a Western-type diet for 6 weeks.

Material and methods

Animals

Female 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 Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). 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.

Isolation of CD11b^{+}Gr-1^{+} cells

Bone marrow cells were isolated from front and hind limbs of LDLr$^{-/-}$ mice fed a Western-type diet for 2 weeks. CD11b^{+}Gr-1^{+} cells (MDSCs) were isolated by a negative depletion as described by Hasenberg et al.[20] An antibody cocktail containing biotin-labeled antibodies against CD5, CD45R, CD49b, CD117, F4/80 and TER119 (eBioscience, Vienna) was added to the cells for 10 minutes at 4°C. After centrifugation, the cells were resuspended in 100 µl MACS buffer and 15 µl of Anti-Biotin-Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10^7 cells. Cells were incubated for 15 minutes at 4°C. Subsequently, the cells were washed with MACS buffer and CD11b^{+}Gr-1^{+} cells were collected using an LS MACS Column (Miltenyi Biotec). Purity of the isolated CD11b^{+}Gr-1^{+} cells (>95 %) was determined with FACS. FACS analysis was performed on a FACSCantoII (Beckton Dickinson, Mountain View, CA). Data were analyzed using FACSDiva software (Beckton Dickinson).

Suppressive capacity of CD11b^{+}Gr-1^{+} cells

The suppressive capacity of CD11b^{+}Gr-1^{+} cells was determined by co-culture with splenocytes. 2×10^5 splenocytes were plated per well of a 96-well plate with or without titrated amounts of isolated CD11b^{+}Gr-1^{+} cells from LDLr$^{-/-}$ mice fed a Western-type diet for 2 weeks (n=3). Cells were activated with αCD3 (1 µg/mL) and αCD28 (0.5 µg/mL) for 72 hours and pulsed with ^{3}H-thymidine (0.5 µCi/well, Perkin Elmer, The Netherlands) 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 the mean counts per minute (cpm) of triplicate cultures. To determine which suppressive factors are responsible for the MDSC-mediated splenocyte suppression, we co-cultured CD11b^{+}Gr-1^{+} cells from LDLr$^{-/-}$ mice in a 1:1 ratio with splenocytes. Cells were activated with αCD3 (1 µg/mL) and αCD28 (0.5 µg/mL) in the presence or absence of several compounds: L-NMMA (0.5 mM, Cayman Chemicals), superoxide dismutase (SOD) (200 U/mL, Sigma-Aldrich), COX-2 inhibitor II (100 nM, Calbiochem), NorNOHA (0.5 mM, Calbiochem) and anti-IFN-γ (10 µg/mL, provided by Louis Boon). Proliferation was measured as described above.

Initiation of atherosclerosis

Atherosclerosis was induced in LDLr$^{-/-}$ mice by feeding a Western-type diet for 6

weeks. During these 6 weeks, mice received every 10 days an i.v. injection of 6×10^{6} MDSCs (n=12) or PBS (n=12). After 6 weeks, mice were sacrificed and tissues were harvested after *in situ* perfusion using PBS. Tissues were stored in Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA) or snap 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, 3 and 6 after start of the atherosclerosis experiment. The concentrations of serum cholesterol were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). 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. Lesion 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. Morphology was studied using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK).

Flow cytometry

At sacrifice, blood and spleen cells were isolated (n=5 per group) and a single cell suspensions were obtained by squeezing the organs through a 70 µm cell strainer. Red blood cells were removed from blood and splenocytes using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Different immune cells were analyzed with flow cytometry: T cells ($CD4^{+}$ and $CD8^{+}$), naive T cells ($CD4^{+}CD44^{low}CD62L^{+}$), effector T cells ($CD4^{+}CD62L^{low}$), B cells ($CD19^{+}$), B2 cells ($CD19^{+}IgM^{low}IgD^{+}$), Th1 cells ($CD4^{+}$T-bet^{+}), Th2 cells ($CD4^{+}$GATA-3^{+}), Th17 cells ($CD4^{+}$RORγt^{+}) and Tregs ($CD4^{+}CD25^{+}Foxp3^{+}$). To detect Th1/Th2/Th17/Treg cells, cells were fixed and permeabilized according to manufacturer's protocol (eBioscience, Vienna). Subsequently, the cells were stained for T-bet, GATA-3, RORγt or Foxp3. All antibodies were purchased from eBioscience (Vienna) and Beckton Dickinson (Mountain View, CA). FACS analysis was performed as described above.

Spleen cell proliferation

At sacrifice, splenocytes (n=5 per group) were cultured in the presence or absence of αCD3 (1 µg/mL) and αCD28 (0.5 µg/mL) for 72 hours in quintuplicate in a 96-wells round-bottom plate (2×10^{5} cells/well) in RPMI 1640 supplemented with L-Glutamine,

100 U/mL streptomycin/penicillin and 10% FCS. Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences, The Netherlands) 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 αCD3/CD28 stimulation to triplicate cultures without stimulation.

Serum antibody detection

IgM, IgG1, IgG2a and IgG2b levels against oxLDL were detected in serum using Abs recognizing mouse IgM, IgG1, IgG2a and IgG2b and HRP-labeled goat anti-rat Ig (BD Pharmingen). OxLDL (5 µg/mL) was dissolved in $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and was coated o/n onto a flat-bottom 96-well high binding plate (Corning, NY). Serum samples were 1:1 diluted in PBS and absorbance was detected at 450 nm.

Statistical analysis

All data are expressed as mean±SEM. An unpaired two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Probability values of $P<0.05$ were considered significant.

Results

MDSCs suppress splenocyte proliferation in an iNOS and IFN-γ dependent manner

First, we isolated MDSCs from the bone marrow of $LDLr^{-/-}$ mice fed a Western-type diet for 2 weeks by an immunomagnetic negative enrichment of $CD11b^{+}Gr\text{-}1^{+}$ cells (purity>95%, Figure 1A). To determine whether these high-fat diet-associated MDSCs exert a suppressive function, we performed a suppression assay with titrated amounts of MDSCs in co-culture with splenocytes isolated from Western-type diet fed $LDLr^{-/-}$ mice in the presence of αCD3/CD28. As shown in Figure 1B, MDSCs very effectively suppressed splenocyte proliferation up to 93% when cultured 1:1 with splenocytes (1339±108 cpm versus splenocytes only: 18314±1686 cpm, $P<0.001$). To check whether this MDSC-mediated inhibition of splenocyte proliferation is due to enhanced apoptosis of splenocytes, we determined the percentage of $AnnexinV^{+}PI^{+}$ cells with flow cytometry. As shown in Figure 1C, MDSCs did not induce apoptosis of splenocytes, as no differences were observed in the percentage of apoptotic cells.

To determine the mechanism by which the high-fat diet-associated MDSCs suppress T cells, we added Nor-NOHA, L-NMMA, SOD and a COX-2 inhibitor to the co-cultures of MDSCs and splenocytes, to suppress the action of arg-1, iNOS, ROS and prostaglandins, respectively. Whereas addition of Nor-NOHA, SOD and a COX-2 inhibitor did not affect the suppressive function of MDSCs (97%, 99% and 93% suppression, respectively, $P<0.001$), the iNOS inhibitor L-NMMA completely abrogated the suppressive capacity of the MDSCs (Figure 1D). Since iNOS expression is promoted by IFN-γ, we also added anti-IFN-γ to the co-cultures. Similar to L-NMMA,

anti-IFN-γ inhibited MDSC-mediated suppression and resulted in a 30% (P=0.14) reduction of splenocyte proliferation.

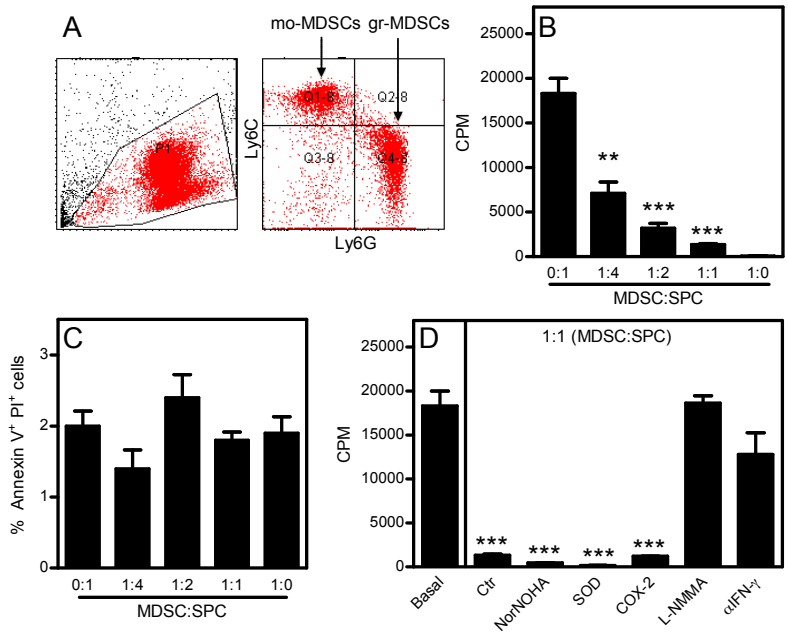


Figure 1. MDSCs (CD11b^{+}Gr-1^{+} cells) were isolated from LDLr$^{-/-}$ mice fed a Western-type diet for 2 weeks and purity was determined with flow cytometry (A). A suppression assay was performed to determine the suppressive capacity of MDSCs by measuring the αCD3/CD28 induced proliferation of splenocytes (B). Data are shown as the mean counts per minute (cpm) of triplicate cultures. The percentage of apoptotic splenocytes was determined with an Annexin-V/PI staining and analyzed with flow cytometry (C). To test the mechanism of MDSC-mediated splenocyte proliferation, a suppression assay was performed in the presence of several inhibitors: Nor-NOHA (0.5 mM, arg-1 inhibitor), SOD (200 U/mL, ROS inhibitor), COX-2 inhibitor (100 nM), L-NMMA (0.5 mM, iNOS inhibitor) and anti-IFN-γ (10 μg/mL) (D). Data are shown as the mean counts per minute (cpm) of triplicate cultures. **P<0.01, *** P<0.001

Fate of adoptively transferred MSDC

To obtain information on the half-life of MDSCs and their distribution profile, we injected MDSCs i.v. in Western-type diet fed LDLr$^{-/-}$ mice and sacrificed them 3 and 9 days after injection. As shown in Figure 2A, increased numbers of MDSCs are still identified in the flow cytometer scatter plots up to 9 days after the injection. Three days after the injection we analyzed the percentage of MDSCs (CD11b^{+}Gr-1^{+} cells) with flow cytometry (Figure 2B) and observed a 3-fold increase in MDSCs in the circulation (MDSCs: 30.6±4.8% vs. control: 10.5±1.6%, P<0.05) and spleen (MDSCs: 4.1±0.1% vs. control: 1.4±0.2%, P<0.001) of MDSC-treated mice. Moreover, after 9 days MDSC levels were still elevated in the circulation (MDSCs: 25.6±2.6% vs. control: 13.1±0.2%, P<0.05) and spleen (MDSCs: 6.4±0.1% vs. control: 2.5±0.6%, P<0.05) of MDSC-treated mice (Figure 2B). Increased numbers of MDSCs were not found in mediastinal lymph nodes near the heart 3 and 9 days after injection.

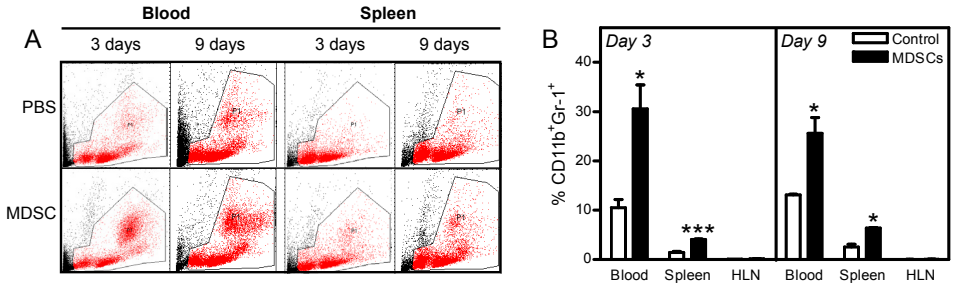


Figure 2. LDLr$^{-/-}$ mice fed a Western-type diet were injected i.v. with 6x10^6 MDSCs. The presence of MDSCs in blood, spleen and HLN 3 and 9 days after injection was determined with flow cytometry. Representative dotplots of the Forward and Side Scatter of blood and spleen at 3 and 9 days are shown (A). Percentages of CD11b$^+$Gr-1$^+$ cells in the blood, spleen and HLN are determined (B). *P<0.05, ***P<0.001

Adoptive transfer of MDSCs reduces the development of atherosclerosis

Based on the ability of the MDSCs to inhibit T cell responses, we investigated the effect of adoptively transferred MDSCs on atherosclerosis development. LDLr$^{-/-}$ mice were fed a Western-type diet for 6 weeks and received four i.v. injections with 6x10^6 MDSCs. The first injection was given at the start of the experiment and thereafter mice received every ten days an i.v. injection with 6x10^6 MDSCs. The MDSCs were isolated from bone marrow of LDLr$^{-/-}$ mice that had been fed a Western-type diet for

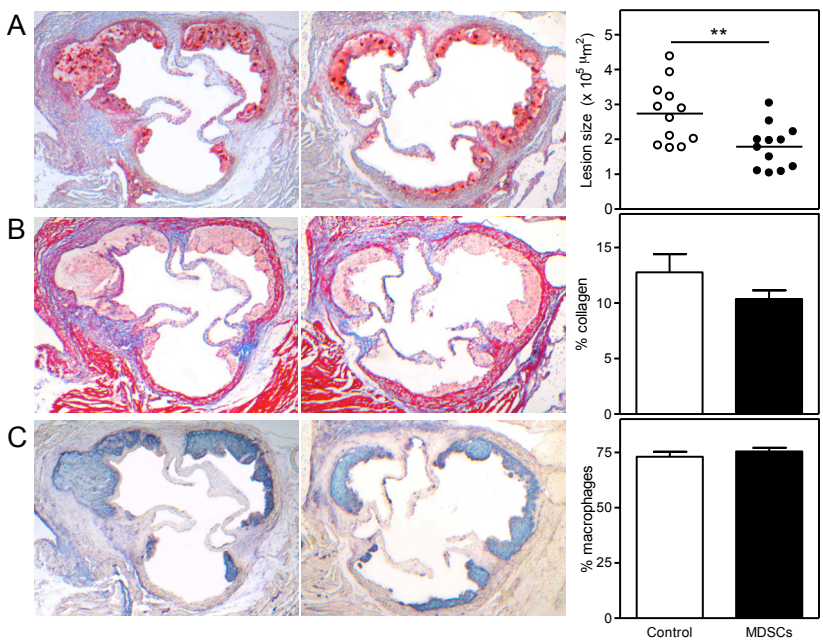


Figure 3. Adoptive transfer of MDSCs reduces atherosclerosis development compared with PBS treatment in LDLr$^{-/-}$ mice (n=12 per group) fed a Western-type diet for 6 weeks. Representative cross-sections of lesion formation in the aortic valve area stained with Oil-Red-O and hematoxylin are shown and lesion size was determined (A). Sections of the aortic root were stained for collagen using Masson's Trichrome staining. The percentage of collagen relative to the lesion size was determined (B). Relative macrophage content was determined with a MOMA-2 staining (C). **P<0.01

2 weeks. During the experiment no differences in body weight and total plasma cholesterol levels were observed (data not shown). We observed a significant 35% reduction in aortic root lesion size of MDSC-treated mice ($1.79\pm0.18\times10^5$ μm^2) in comparison with control mice ($2.74\pm0.25\times10^5$ μm^2, $P<0.01$, Figure 3A). No difference in lesion stability as determined by Masson's Trichrome staining was observed between MDSC-treated mice (10.4±0.8%) and control mice (12.8±1.6%, Figure 3B). Furthermore, no difference in macrophage content was observed (control: 73.0±2.2% and MDSC: 75.4±1.6%, Figure 3C).

Increased naive T cells and reduced Th1 and Th17 cells upon MDSC treatment

Since MDSCs suppress T cells, we determined the effect of MDSC treatment on T cell subsets. At sacrifice, there were no differences in the percentages of total CD4$^+$ T cells and total CD8$^+$ T cells in the blood and spleen of MDSC-treated mice and control mice (Figure 4A). However, we did observe a different balance between naive and effector CD4$^+$ T cells in the spleen of MDSC-treated mice compared with control mice (Figure 4B). Whereas naive CD4$^+$ T cells were increased in MDSC-treated mice (9.6±0.4%) in comparison with control mice (7.7±0.2%, $P<0.01$), effector CD4$^+$ T cells were decreased (MDSC: 8.1±0.4% vs. control: 9.6±0.5%, $P<0.05$). To evaluate whether adoptive transfer of MDSCs can affect specific T helper cell subsets, splenocytes were stained for the transcription factors T-bet, GATA-3, RORγt and Foxp3, which control the differentiation of Th0 cells into Th1, Th2, Th17 cells and Tregs, respectively. Flow cytometry analysis showed that MDSC-treated mice had significantly reduced T-bet expression (6.4±0.4% vs. 12.9±0.3% in control mice, $P<0.001$) and reduced RORγt expression (2.5±0.1% vs. 5.1±0.2% in control mice, $P<0.001$) in the CD4$^+$ T cell

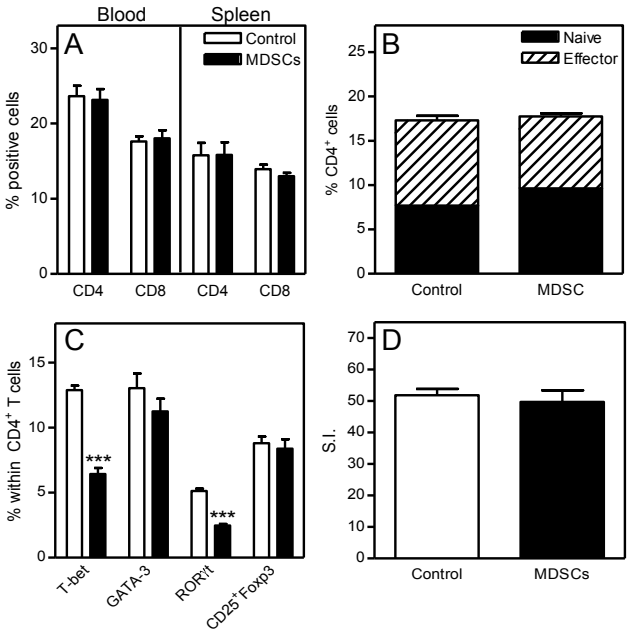


Figure 4. At sacrifice, blood and spleen cells were isolated (n=5 per group) and percentages of CD4$^+$ and CD8$^+$ T cells were determined with flow cytometry (A). In addition, CD4$^+$CD62L^{low} effector T cells and CD4$^+$CD44^{low}62L^{high} naive T cells were determined in the spleen (B). Splenic T cell subsets were determined by staining for CD4 and the transcription factors T-bet (Th1), GATA-3 (Th2), RORγt (Th17) and Foxp3 (CD25$^+$ Tregs) (C). The effect of MDSC administration on spleen cell proliferation was determined by culturing splenocytes (n=5 per group) in the presence or absence of CD3/CD28 stimulation (D). Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells. The proliferation is expressed as stimulation index. ***$P<0.001$

population of the spleen compared with control mice (Figure 4C). In contrast, Th2 and Treg responses remained unchanged following adoptive transfer of MDSCs. Although adoptive transfer of MDSCs decreased the pool of splenic effector $CD4^+$ T cells and more specifically, decreased Th1 and Th17 cell subsets, we did not observe any difference in splenocyte proliferation of MDSC-treated mice (stimulation index of 49.7±3.6) in comparison with control mice (stimulation index of 51.8±2.0) after stimulation with αCD3/CD28 (Figure 4D).

Reduced B2 cells in MDSC-treated mice

Adoptive transfer of MDSCs also affected B cell responses since MDSC-treated mice had reduced circulating $CD19^+$ B cells (23.7±1.4% vs. 30.3±2.3% in control mice, $P<0.05$, Figure 5A). More particular, we observed a 30% decrease in circulating B2 cells in MDSC-treated mice (15.2±1.0%) in comparison with control mice (21.6±2.1%, $P<0.05$, Figure 5B). Furthermore, we found that splenic B cells of MDSC-treated mice proliferated less vigorously (29.5±1.4% $CD19^+Ki\text{-}67^+$ cells) than B cells of control mice (35.9±1.2% $CD19^+Ki\text{-}67^+$ cells, $P<0.05$, Figure 5C). We also determined oxLDL-specific antibodies in serum (Figure 5D) but did not find any difference in MDSC-treated mice in comparison with control mice.

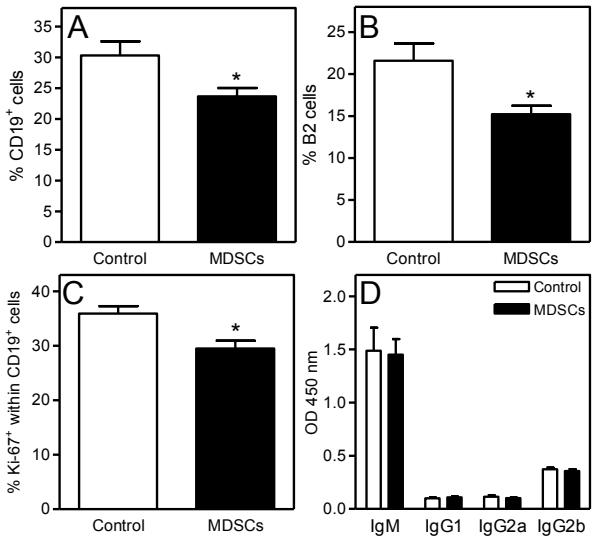


Figure 5. At sacrifice, blood cells were isolated (n=5 per group) and the percentage of $CD19^+$ B cells was determined with flow cytometry (A). B2 cells were analyzed as the percentage of $CD19^+IgM^{low}IgD^{high}$ cells (B). Splenocytes were cultured for 72 hours and the proliferation of B cells was determined by the percentage of Ki-67^+ cells within $CD19^+$ cells (C). oxLDL-specific IgM, IgG1, IgG2a and IgG2b production was detected in serum of control and MDSC-treated mice (n=6 per group) with ELISA (D). *$P<0.05$

Discussion

In this study, we propose a novel cellular therapy using MDSCs to inhibit atherosclerosis development. MDSCs expand in the bone marrow upon disease as a protective negative feedback mechanism and migrate into the periphery to eventually home in inflammatory tissues where they are powerful suppressors of immune responses. In contrast to cancer where expanded MDSCs aggravate the disease by the suppression of cytotoxic T cells, MDSC activity is highly appreciated in autoimmune diseases

where the unwanted activation of the immune system needs to be suppressed. Although accumulating evidence implicates MDSCs as potent suppressors of several autoimmune diseases[11, 12, 14, 21, 22], the role of MDSCs in atherosclerosis remains to be elucidated.

We isolated bone marrow-derived MDSCs (CD11b^{+}Gr-1^{+}) from LDLr$^{-/-}$ mice in which the immune system was boosted by feeding a high-fat diet for 2 weeks.[15] These 'high-fat diet-associated MDSCs' potently suppressed T cell proliferation *in vitro* without inducing T cell apoptosis. MDSCs have been described to suppress T cell responses via several factors such as arg-1, iNOS, ROS and NO. Our data show an important role for iNOS produced NO and IFN-γ in high-fat diet MDSC-mediated T cell suppression as the blockade of these factors with L-NMMA and anti-IFN-γ abrogated the suppressive capacity of the MDSCs. iNOS and IFN-γ are strongly associated with each other, since activated Th1 cells produce IFN-γ, which induces iNOS expression in MDSCs.[9, 23] Our findings on atherosclerosis associated MDSCs are in line with studies that have shown that blocking IFN-γ abolishes MDSC-mediated T cell suppression.[2, 24] In LDLr$^{-/-}$ mice fed a high-fat diet, a Th1-biased environment may contribute to this IFN-γ/iNOS-dependent mechanism of MDSC-mediated T cell suppression. Inhibition of arg-1, ROS or prostaglandin synthesis did not affect the suppressive function of MDSCs derived from LDLr$^{-/-}$ mice fed a high-fat diet for 2 weeks. Moreover, preliminary data showed a differential expression of arg-1 and STAT1 in MDSCs isolated from chow fed mice in comparison with high-fat diet fed mice. Arg-1 was significantly lower expressed in high-fat diet MDSCs, whereas STAT1, the main regulator activated by IFN-γ signaling, was significantly higher (*unpublished data*). More research will be performed to characterize the MDSC phenotype under hypercholesterolemic conditions.

Adoptive transfer of 6x10^{6} MDSCs into LDLr$^{-/-}$ mice fed a high-fat diet for 6 weeks ameliorated atherosclerosis with 35%. No differences were observed in collagen and macrophage content of the lesions. This reduction in atherosclerosis formation was associated with increased percentages of naive T cells and decreased percentages of effector T cell in the spleens of mice that received MDSCs. More specifically, MDSC-treated mice showed a 50% reduction in splenic Th1 and Th17 cells. Th1 cells produce pro-inflammatory cytokines such as IFN-γ and TNFα and LDLr$^{-/-}$ mice deficient in T-bet[25] or ApoE$^{-/-}$ mice deficient in IFN-γ[26], show attenuated atherosclerosis. In line with our findings, Highfill et al. showed that bone marrow MDSCs cultured *in vitro* with IL-13 to enhance their suppressive capacity, reduced T cell alloresponses and GVHD lethality via dimished effector T cells and strongly reduced IFN-γ producing CD4^{+} and CD8^{+} T cells.[14] Zhu et al. showed that MDSCs isolated from the spleen of mice with EAE suppressed the production of several cytokines by CD4^{+} T cells, including IFN-γ and IL-17.[27] Additionally, iNOS and arg-1 deplete the microenvironment of T cells from L-arginine, which results in an inhibition of proliferation and a decreased production of IFN-γ.[14]

The role of Th17 cells in atherosclerosis is controversial but mainly considered pro-atherogenic since the expression of IL-17 and RORγt is correlated to plaque size and

exogenous IL-17 promotes the formation of atherosclerotic lesions in ApoE$^{-/-}$ mice.[28] Moreover, a deficiency in IL-17R or blockade of IL-17 by using neutralizing antibodies[28, 29] or by use of adenovirus-produced soluble IL17-RA[30] reduces atherosclerosis. In contrast to our findings, Yi et al. discovered that MDSCs can drive the differentiation of Th17 cells under Th17-polarizing conditions (IL-6/TGF-β) and thereby contribute to the pathogenesis of EAE.[31] However, they show this is an arg-1 dependent mechanism, while our high-fat diet-MDSCs do not seem to suppress via this pathway. Moreover, in contrast to Yi et al. several other studies indicate a protective role for MDSCs in EAE.[12, 27]

Despite the reduction in effector T cells in MDSC-treated mice, splenocyte proliferation was unaffected in MDSC-treated mice in comparison with control mice. Possibly, MDSCs suppress antigen-specific T cells as showed previously by Nagaraj et al.[1] and the CD3/CD28 activation we used is possibly too aspecific to identify the effect of MDSCs on specific T cells involved in the pathogenesis of atherosclerosis. Additionally, we did not observe increased percentages of Tregs upon MDSC treatment, which is also described as one of the mechanisms through which MDSCs suppress T cells.[9, 13]

Besides hindering T cell reactivity, we showed that MDSC-treated mice also have reduced B cell percentages, in particular B2 cells, possibly as a consequence of impaired proliferative capacity. B2 cells comprise the majority of circulating B cells and reside in lymphoid tissues. In atherosclerosis, B2 cells are considered pro-atherogenic since anti-CD20-mediated depletion of B2 cells ameliorates atherosclerosis and a B2 cell transfer into ApoE$^{-/-}$ mice aggravates atherosclerosis.[32, 33] In line with our findings, Green et al. showed that MDSCs, especially monocytic-MDSCs, can inhibit B cell proliferation.[21]

Although we isolated CD11b^{+}Gr-1^{+} MDSCs from high-fat diet fed LDLr$^{-/-}$ mice and adoptively transferred both monocytic and granulocytic MDSC subsets, it seems that monocytic-MDSCs are most likely responsible for the observed inhibition of atherosclerosis. Previous studies have shown that monocytic-MDSCs are driven by Th1 signals[5], such as IFN-γ and LPS, and therefore suppress immune cells in an iNOS-dependent manner, which is in line with our findings. Furthermore, it has been well documented that monocytic-MDSCs exhibit more potent suppressive activity than granulocytic-MDSCs[2, 34, 35] but the relative potency of monocytic and granulocytic MDSCs to provoke immune suppression in atherosclerosis will be an area of future investigation. Culturing MDSCs with Nor-NOHA did not interfere with their inhibitory capacity, but it remains possible that our high-fat diet-induced MDSCs also act via arg-1, since the *in vitro* microenvironment of splenocytes isolated from high-fat diet LDLr$^{-/-}$ mice differs from the *in vivo* microenvironment in the atherosclerotic lesion. Arg-1 can be induced by IL-4, IL-10 and IL-13, cytokines that are differentially regulated during atherosclerosis development. Therefore, it is possible that MDSCs exhibit distinct biological activities depending on the microenvironment in the different pathological stages of atherosclerosis. Future atherosclerosis experiments including adoptive transfer of MDSCs isolated from bone marrow of Arg-1$^{-/-}$ and iNOS$^{-/-}$ mice

and characterization of the MDSC phenotype under hypercholesterolemic conditions will provide more insight.

Interestingly, a number of atherosclerosis studies refer to CD11b^{+}Ly6G^{-}Ly6C^{hi} cells as 'inflammatory monocytes' and to CD11b^{+}Ly6G^{+}Ly6C^{low} as neutrophils, but these cell populations may also contain MDSCs as they are also positive for CD11b and Ly6G or Ly6C. In other experimental models for diseases such as cancer and EAE, MDSCs expand in the bone marrow and are found to accumulate in blood and spleen. During atherosclerotic lesion development, monocyte and neutrophil accumulation also occurs and correlates strongly to the lesion size.[36, 37] However, it remains to be determined whether these monocytic and neutrophilic populations also contain MDSCs. To clarify this issue, a suppression assay and phenotypic characterization of CD11b^{+}Gr-1^{+} cells isolated from high-fat diet fed LDLr$^{-/-}$ mice in different stages of atherosclerosis should be performed.

Although further studies are required to fully comprehend the role of MDSCs in cardiovascular diseases, our study defines a specific population of immature immune cells that regulates T- and B cells in atherosclerosis.

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Chapter 10

Summary and Perspectives

Summary

Acute cardiovascular syndromes are a major cause of death in Western society and are generally triggered by rupture of an atherosclerotic plaque.[1, 2] Current treatment of atherosclerosis involves lipid lowering using statins, beta blockers, anti-thrombotic drugs, and life style advice. Even though improvements in treatment have led to a reduction in atherosclerosis-associated deaths and have led to an improved quality of life of patients, present treatment is inadequate to halt progression of cardiovascular disease with respect to plaque size (degree of occlusion) or to reverse existing plaques, and the number of people diagnosed with atherosclerosis still remains high. This indicates an urgent need for new therapeutic strategies to inhibit atherosclerosis and to prevent cardiovascular complications and acute syndromes.

Besides lipid accumulation, inflammation is considered a key process in atherosclerotic plaque development and in the pathogenesis of plaque rupture.[3, 4] Antigen presenting cells, such as dendritic cells and macrophages, play an important role in the inflammatory process within atherosclerotic plaques and are responsible for presentation of atherosclerosis-related antigens, such as oxLDL and HSP60, resulting in the attraction and activation of T cells.[5, 6] Upon activation T cells produce large amounts of pro-atherogenic cytokines that contribute to both the growth and destabilization of lesions, which can result in rupture of the lesion leading to thrombus formation and cardiovascular complications. T cells can be divided into several T cell subsets that can either be pro-inflammatory/pro-atherogenic, such as Th1 and Th2 cells, or anti-inflammatory/anti-atherogenic, such as regulatory T cells.

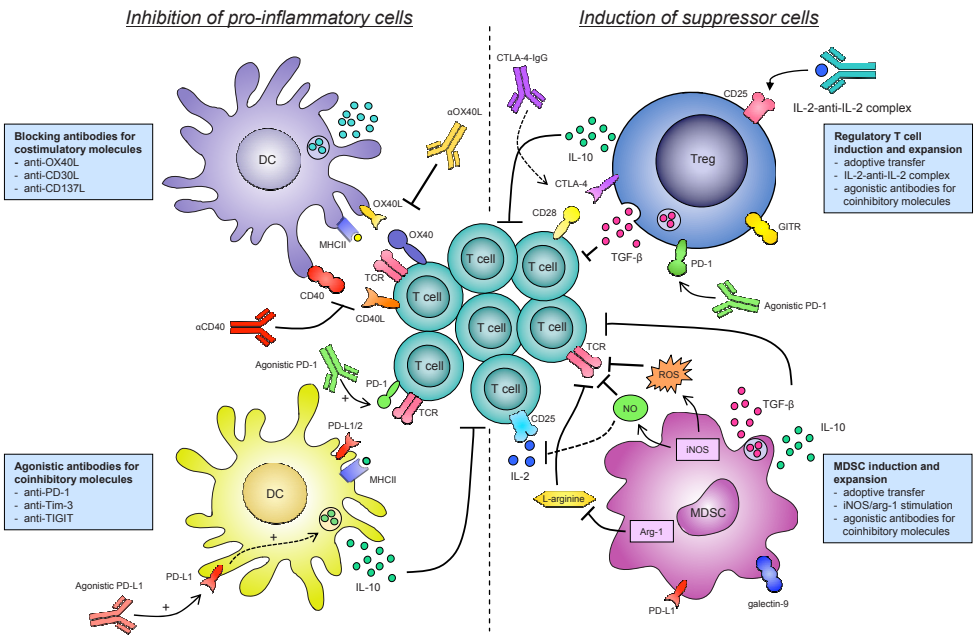


Figure 1. Pro-atherogenic T cells can be inhibited by blocking antibodies for costimulatory molecules or by agonistic antibodies for coinhibitory molecules and through the induction of suppressor cells, such as Tregs and MDSCs. Examples of possible treatments are provided in the blue boxes.

An imbalance between pro- and anti-inflammatory cells exists in atherosclerosis, with increased numbers of the first. Therefore, restoration of this balance by (1) inhibition of pro-inflammatory responses or by (2) inducing suppressor cells has great therapeutic potential to prevent cardiovascular disease (Figure 1). In this thesis, several therapeutic strategies to restore the balance of pro- and anti-inflammatory immune responses in atherosclerosis were investigated. In **Chapter 3-6**, modulation of costimulatory and coinhibitory pathways, a network of ligands present on antigen presenting cells that bind to their corresponding receptors on T cells and can either promote or inhibit immune cell function, was evaluated. In **Chapter 7-9**, the protective role of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) was studied.

Modulation of costimulatory and coinhibitory pathways as a treatment of atherosclerosis

The immune system provides a large diversity of costimulatory and coinhibitory pathways and each pathway has its own unique effect on the fate of individual immune cells. Costimulatory signals can promote T cell survival, cell cycle progression and differentiation of naive T cells to effector and memory T cells, whereas coinhibitory molecules can terminate these processes directly or indirectly via the induction of Tregs. In this thesis we performed several studies that addressed the role of several costimulatory and coinhibitory pathways in atherosclerosis.

The costimulatory pathway formed by OX40 and OX40L is involved in the proliferation and survival of T cells, particularly Th2 cells, drives isotype switching of B cells, and is associated with cardiovascular disease incidence. In **Chapter 3**, we investigated the effect of OX40-OX40L interference on the regression of atherosclerosis in LDLr$^{-/-}$ mice by a combined anti-inflammatory (10 week treatment with anti-OX40L) and lipid-lowering strategy (switching to chow diet after 10 weeks of Western-type diet). Lipid lowering alone increased lesion stability without reducing lesion size, while additional anti-OX40L treatment also induced lesion regression. Treatment with anti-OX40L reduced circulating OX40-expressing CD4^{+} T cells and adventitial T cells.

In line with previous findings, interruption of the OX40-OX40L pathway reduced the Th2 response, as shown by decreases in GATA-3, IL-4 and IL-10 expression.[7] Notably, we demonstrate that the production of another typical Th2 cytokine, IL-5, was increased in B1 cells and T cells. B1 cells are dependent on IL-5 and enhance the secretion of atheroprotective natural oxLDL-specific IgM antibodies.[8] Anti-OX40L-treated mice had increased numbers of B1 cells and increased oxLDL-specific IgM titers. T cells that produce IL-5, independently of IL-4, can be induced by IL-33, which previously has been shown to be protective in atherosclerosis by inducing IL-5 and anti-oxLDL-specific IgM antibody formation[9], and by inhibiting foam cell formation.[10] We observed increased IL-33 expression in the spleen of anti-OX40L-treated mice and showed that IL-33 production is dose-dependently increased by anti-OX40L treatment of DCs and macrophages exposed to oxLDL. The increase in the atheroprotective factors IL-

33, IL-5 and oxLDL-specific IgM in anti-OX40L-treated mice likely contributed to the observed regression of atherosclerosis.

Another pathway through which OX40-OX40L blockade may facilitate lesion regression is via reduced IgE levels and subsequent reduced mast cell numbers and activation. IL-4 induces isotype switching of B cells from IgM- to IgE- and IgG-producing cells. Previously, we showed that anti-OX40L treatment reduced IgG1 levels[7] and we now show that interruption of OX40-OX40L treatment also induced a strong reduction in serum IgE. Activated mast cells are found in the adventitia of vulnerable and ruptured lesions of patients suffering from myocardial infarction [11, 12], and mast cell numbers correlate with the incidence of plaque rupture and erosion.[11] Our lab has previously shown that mast cells also play a crucial role in plaque progression and destabilization *in vivo*.[13] In addition, enhanced IgE levels were observed in patients with unstable angina pectoris and in dyslipidemia[14, 15] and recently, Wang et al. showed that IgE promotes atherosclerosis in ApoE$^{-/-}$ mice.[16] In line with these findings, we suggest that the reduced IgE levels due to the anti-OX40L treatment contribute to the observed lesion regression.

An overview of the mechanism of anti-OX40L-mediated regression of atherosclerosis is shown in Figure 2. Finally, it must be noted that interruption of the OX40-OX40L pathway did not induce full regression of lesions. Further research into modulating immune responses to induce regression must be explored and, in combination with lipid lowering, may hold the key to therapies for cardiovascular patients with well-established lesions.

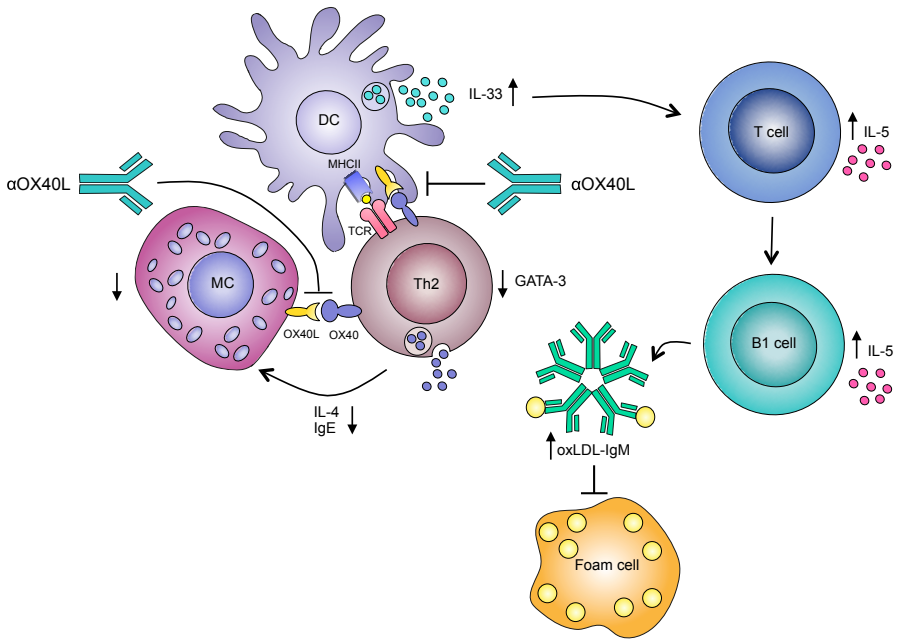


Figure 2. Schematic overview of the mechanism through which OX40-OX40L interruption can reduce atherosclerosis. Anti-OX40L treatment induces IL-33, IL-5 and oxLDL-specific IgM and reduces IL-4 and IgE levels, resulting in reduced mast cells numbers and activation.

Similar to OX40 and OX40L, CD30 and CD30L are members of the TNF(R) superfamily and are involved in the activation and proliferation of T and B cells. Whereas the CD30-CD30L pathway has been implicated in various autoimmune diseases, such as asthma[17], GVHD[18] and type I diabetes[19], no studies describe a role for the CD30-CD30L axis in atherosclerosis. In **Chapter 4** we therefore treated Western-type diet fed LDLr$^{-/-}$ mice with an anti-CD30L antibody for 8 weeks, which resulted in a reduction in atherosclerotic lesion formation in the aortic root by 35%. This reduction in atherosclerosis coincided with reduced adventitial T cell numbers, reduced percentages of CD4^{+} T cells in the spleen and lymph nodes and with strongly reduced splenocyte proliferation. In particular, CD4^{+} T cells isolated from anti-CD30L-treated mice proliferated less vigorously after αCD3/CD28 stimulation than CD4^{+} T cells from control mice, whereas their adhesion and migration capacity remained unaffected. Although signaling via CD30-CD30L may also affect humoral responses and mast cell activity, we did not detect a significant difference in immunoglobulin production and mast cell activity in anti-CD30L-treated mice compared with control mice. We conclude that the CD30-CD30L pathway solely exerts its function via inhibition of T cell responses in atherosclerosis, which identifies anti-CD30L treatment as a novel therapeutic modality in the inhibition of atherosclerotic lesion development and the prevention of acute cardiovascular syndromes.

In **Chapter 5**, we studied the role of T cell immunoglobulin and mucin domain 3 in atherosclerosis. Tim-3 is a coinhibitory type I transmembrane protein, which affects the function of several immune cells involved in atherosclerosis, such as monocytes, macrophages, effector T cells and Tregs. It has been reported that patients with atherosclerosis have increased Tim-3^{+} NK cells compared with healthy controls.[20] In line with these findings, we observed that Western-type diet feeding increased the percentage of Tim-3^{+} NK cells in blood of LDLr$^{-/-}$ mice, but also increased the percentage of Tim-3^{+} monocytes and DCs, cell types that largely contribute to the inflammatory process of atherosclerosis. When we treated Western-type diet fed LDLr$^{-/-}$ mice with an anti-Tim-3 antibody for 8 weeks, atherosclerotic plaque formation was increased with 35% in the aortic root and with 50% in the aortic arch. Although lesion stability did not differ between anti-Tim-3-treated mice and control mice, lesions of anti-Tim-3-treated mice contained significantly more macrophages than lesions of control mice. This might be the consequence of an increased influx of monocytes into the arterial wall, since we observed increased circulating monocytes in anti-Tim-3-treated mice. In addition, oxLDL-loaded macrophages treated with anti-Tim-3 secreted higher levels of MCP-1 *in vitro*, which might suggest that MCP-1 secreted by foam cells in the atherosclerotic lesions of anti-Tim-3-treated mice attracts monocytes to the inflamed arterial wall. These data are in line with studies by Monney and Frisancho-Kiss et al. who show that anti-Tim-3 treatment increases macrophage numbers and activation in mouse models of EAE[21] and inflammatory heart disease.[22] Additionally, we showed that anti-Tim-3 administration increased CD4^{+} T cells and reduced percentages of IL-10 producing Tregs and Bregs. Tim-3 has previously been associated with Tregs, as

blocking Tim-3 enhances type 1 diabetes in NOD-mice and prevents the generation of immunological tolerance in a transplantation model by dampening the function of Tregs.[23] To conclude, inducing Tim-3 signaling could provide a novel approach to inhibit pro-atherogenic immune responses.

To evaluate the contribution of another coinhibitory molecule, T cell immunoreceptor with Ig and ITIM domains (TIGIT), to atherosclerosis, we used an agonistic anti-TIGIT antibody as described in **Chapter 6**. Signaling via TIGIT directly inhibits T cell activation and proliferation through downregulation of the T cell receptor[24, 25] but can also induce IL-10 producing tolerogenic DCs upon binding to the poliovirus receptor (PVR).[25, 26] Several studies have shown that TIGIT is essential for T cell function in mice and humans and is mainly expressed on activated $CD4^+$ T cells.[25, 27] In line with these findings, we observed that TIGIT was upregulated on $CD4^+$ T cells from Western-type diet fed $LDLr^{-/-}$ mice in comparison with chow diet fed $LDLr^{-/-}$ mice and was further enhanced after αCD3/CD28 stimulation. Furthermore, we showed that agonistic anti-TIGIT greatly inhibited T cell activation and proliferation. This TIGIT-mediated downregulation of T cell responses inhibited several diseases such as EAE, collagen-induced arthritis and GVHD.[24, 25] Surprisingly, we observed that treatment of $LDLr^{-/-}$ mice fed a Western-type diet for 4 or 8 weeks with agonistic anti-TIGIT did not reduce atherosclerosis development and did not affect lesion composition. Possibly, the reduced T cell function was counteracted by enhanced activity of dendritic cells, which were elevated and expressed higher levels of MHC II and CD40 in agonistic anti-TIGIT-treated mice. Since TIGIT normally binds to PVR expressed on DCs to induce a tolerogenic phenotype, and agonistic anti-TIGIT blocks TIGIT-PVR signaling, this may explain a more pro-inflammatory phenotype of DCs in agonistic anti-TIGIT-treated mice. Despite agonistic anti-TIGIT treatment did not affect atherosclerosis, the TIGIT-PVR pathway could still be of interest to modulate pro-inflammatory immune responses in atherosclerosis and other autoimmune diseases.

Cellular targets of immune regulation to treat atherosclerosis

Another approach to regulate pathogenic immune responses in atherosclerosis is to promote suppressor cells, such as Tregs and myeloid-derived suppressor cells (MDSCs). Tregs play an important role in the regulation of T cell-mediated immune responses through suppression of T cell proliferation and cytokine production. Impaired Treg function has been associated with the pathogenesis of numerous diseases and in atherosclerosis, an imbalance between pro-inflammatory/pro-atherogenic cells (Th1/Th2) and Tregs exists.[28, 29] Therefore, increased Treg numbers may be beneficial for patients suffering from atherosclerosis. The role and therapeutic potential 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[30] while a depletion of $CD4^+CD25^+$ T cells aggravates lesion development.[30] Furthermore, van Puijvelde et al. showed that induction of antigen-specific Tregs via oral tolerance induction against oxLDL and HSP60 inhibits the initiation and progression of

atherosclerosis.[31, 32]

Tregs originate from the thymus and are characterized by the expression of the surface molecules CD4 and CD25, and expression of the transcription factor Forkhead box protein 3 (Foxp3).[33] Tregs may also acquire Foxp3 after oral tolerance induction. To study the effect of the elimination of $Foxp3^+$ Tregs in atherosclerosis, we used a novel vaccination strategy directed against Foxp3 expressing cells. The effect of Foxp3 specific elimination on initial and advanced atherosclerosis is described in **Chapter 7**. A dendritic cell based vaccine was used to provoke a cytotoxic T cell response against Foxp3 expressing cells. During the time course of the experiment we observed significant less $Foxp3^+$ Tregs in the circulation and lymphoid tissues of Foxp3 vaccinated mice. Furthermore, vaccination against Foxp3 aggravated atherosclerotic plaque formation in both initial and advanced atherosclerosis. This increase in lesion size was associated with increased cellularity, which may result from impaired inhibition of pathogenic T cells within the plaque upon depletion of Treg cells. Additionally, splenocytes from Foxp3 vaccinated mice proliferated more vigorously than splenocytes from control mice, which is indicative for a reduced number of Tregs since these cells suppress effector T cells. The results from this study further established the role of $Foxp3^+$ Tregs in atherosclerosis and are in line with the results of Ait-Oufella et al.[30]

Tregs have been shown to depend on IL-2 for optimal growth and survival.[34-37] Recently, it is shown that repeated injections of an IL-2 complex consisting of recombinant IL-2 and a specific anti-IL-2 monoclonal antibody expands Tregs[38], which very potently induces resistance to EAE and suppressed graft rejections[39], type I diabetes[40], murine-asthma[41] and myasthenia gravis.[42] The protective role of Tregs in atherosclerosis has been extensively investigated, however, only a modest increase in Treg numbers was achieved in the order of 1.5- to 2-fold, mostly for 2-3 weeks. In **Chapter 8** we therefore determined the effect of a vast IL-2 complex-mediated expansion of Tregs on the initiation and regression of well-established lesions. Administration of the IL-2 complex resulted in a 10-fold increase in Tregs in blood of $LDLr^{-/-}$ mice, which potently suppressed effector T cells and reduced initial atherosclerosis with 39%. Furthermore, we observed an increase in gene expression of IL-10 in the spleen and increased IL-10 secretion by Tregs, which suggests that IL-2 complex expanded Tregs exert their suppressive capacity via IL-10. These data are in line with previous reports in which IL-2 complex expanded Tregs suppressed airway inflammation[41] and EAE in an IL-10 dependent manner.[39] The increase in IL-10 in splenocytes of IL-2 complex treated mice may at least partially be responsible for the decrease in lesion size since IL-10 is protective in atherosclerosis.[43-45]

We also determined the role of IL-2 complex expanded Tregs in a more clinically relevant regression model of atherosclerosis, as most of the cardiovascular patients already have well-established lesions. Despite high Treg levels, 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.

Additionally, a 60% increase of adventitial $CD3^+$ T cells within lesions of IL-2 complex treated mice was observed, which most likely are the IL-2 complex-expanded Tregs. These data suggest a differential role for Tregs in different stages of atherosclerosis. Where Tregs inhibit lesion development during initial stages of atherosclerosis, they are important in the stabilization of well-established lesions during regression.

Besides Tregs, MDSCs also strongly suppress T cell responses. MDSCs are a population of early myeloid progenitor cells and immature myeloid cells that expand in the bone marrow under various pathological conditions and accumulate in lymphoid and non-lymphoid tissues where they serve a negative feedback function.[46] In contrast to cancer where expanded MDSCs aggravate the disease by the suppression of cytotoxic T cells, MDSC activity is highly appreciated in autoimmune diseases where the unwanted activation of the immune system needs to be suppressed. Although accumulating evidence implicates MDSCs as potent suppressors of several autoimmune diseases[47-51], the role of MDSCs in atherosclerosis is unknown.

In **Chapter 9** we isolated bone marrow-derived $CD11b^+Gr\text{-}1^+$ cells (MDSCs) from $LDLr^{-/-}$ mice in which the immune system was boosted by feeding a high-fat diet for 2 weeks.[28] These high-fat diet-associated MDSCs potently suppressed *in vitro* T cell proliferation in an IFN-γ/iNOS-dependent manner without inducing T cell apoptosis. Adoptive transfer of MDSCs into $LDLr^{-/-}$ mice fed a high-fat diet for 6 weeks ameliorated atherosclerosis with 35%. No differences were observed in collagen and macrophage content of the lesions. This reduction in atherosclerosis formation was associated with increased percentages of naive T cells and decreased percentages of effector T cells in the spleens of mice that received MDSCs. More specifically, MDSC-treated mice showed a 50% reduction in splenic Th1 and Th17 cells, subsets that are generally considered pro-inflammatory in atherosclerosis.[52-54] Additionally, adoptive transfer of MDSCs reduced B cell percentages, in particular pro-atherogenic B2 cells, possibly as a consequence of impaired proliferative capacity.

In conclusion, our study describes a novel cellular therapy using MDSCs to inhibit atherosclerosis. However, MDSCs can be subdivided in monocytic-MDSCs, induced by Th1 signals and expressing iNOS, or granulocytic-MDSCs, induced by Th2 signals and expressing arg-1, and whereas we adoptively transferred both monocytic and granulocytic MDSC subsets, it seems that monocytic-MDSCs are most likely responsible for the observed inhibition of atherosclerosis. In the Perspectives section, future approaches are described to investigate the relative potency of monocytic and granulocytic MDSCs to provoke immune suppression in atherosclerosis.

Perspectives

In this thesis, several mechanisms to regulate pathogenic immune responses in atherosclerosis have been studied. T cells play a major role in the pathogenesis of atherosclerosis by promoting inflammation and destabilizing advanced lesions. T cells are regulated by a network of costimulatory and coinhibitory molecules and by several suppressor cells such as Tregs and MDSCs. These pathways of immune regulation form potent candidates for an immunotherapy of atherosclerosis. Novel therapeutic strategies to treat atherosclerosis are needed since death from cardiovascular diseases continues to increase worldwide, despite the use of statins, anti-thrombotic drugs, and anti-hypertensive treatment.[55]

Modulation of costimulatory and coinhibitory pathways

In cardiovascular disease, modulation of costimulatory and coinhibitory molecules can be a powerful tool to target specific stages of atherosclerosis or specific cell types involved in the pathogenesis of atherosclerosis. A highly relevant feature of costimulatory and coinhibitory pathways is that they individually have a unique effect on the behaviour of specific immune cells and thus on the outcome of disease. As shown in **Chapter 3** and **4**, interference of OX40-OX40L and CD30-CD30L signaling both reduce atherosclerosis via different pathways; anti-OX40L specifically targets Th2 responses and mast cell activity, whereas anti-CD30L limits all $CD4^+$ T cell responses without affecting a specific T cell subset or other immune cells. In addition, several costimulatory and coinhibitory molecules are involved in the induction and function of Tregs. This enables the development of a treatment that particularly targets different subsets of T cells. Ultimately, it would be ideal to modulate antigen-specific pro-atherogenic T cells with blocking costimulatory antibodies and agonistic coinhibitory antibodies without affecting all T cells and other immune cells to limit any adverse effects on the immune system. Although several candidates of atherosclerosis specific antigens have been investigated, such as oxLDL, HSP60 and ApoB100, to date the exclusively atherosclerosis-associated antigen is not identified yet, which makes it difficult to specifically target the pro-atherogenic T cells. However, some costimulatory molecules, such as OX40, are virtually absent on naive T cells but are upregulated on activated T cells. Targeting these costimulatory molecules with blocking antibodies could specifically eliminate the pathogenic T cells without causing any side effects. Furthermore, the ligand of OX40, OX40L, is expressed on endothelial cells, which upon blockade can also reduce the attraction of $OX40^+$ T cells to the site of inflammation.

Blocking and agonistic antibodies for costimulatory and coinhibitory molecules have already been extensively explored in cancer and allograft rejections. Blocking antibodies for CTLA-4 and PD-1 to boost T cell responses are approved for treatment of patients with several types of cancer.[56] In contrast to cancer where T cell activity is highly appreciated, the unwanted activation of the immune system needs to be suppressed in atherosclerosis. Therefore, whereas in cancer for example a blocking

PD-1 antibody to promote T cell activity is beneficial, in atherosclerosis an agonistic PD-1 antibody is needed to suppress T cells. CTLA-4-Ig has already been established as an effective treatment for human autoimmune diseases including rheumatoid arthritis[57] and psoriasis.[58] At present, one clinical trial has been completed using anti-OX40L in the prevention of allergen-induced airway obstruction in adults with mild asthma.[59] However, no study results are reported yet.

Although many antibodies against costimulatory and coinhibitory molecules have been approved and are used in clinical settings, caution is needed when translating animal experiments to the clinic, as a Phase I clinical trial with an agonistic monoclonal anti-CD28 antibody induced a strong cytokine storm (IFN-γ, TNFα, IL-2) several hours after drug infusion, which caused multiorgan failure in six human volunteers who ended up on the intensive care unit.[60] Moreover, blocking costimulatory pathways and stimulating coinhibitory pathways may enable opportunistic infections to emerge. However, treatment can be adjusted in a way that patients will only receive blocking antibodies for costimulatory molecules or agonistic antibodies for coinhibitory molecules temporarily until the lesion is stabilized.

No clinical trials investigating antibodies for costimulatory and coinhibitory molecules in cardiovascular disease have been started yet. In fact, only recently the first clinical trial involving interference of inflammatory pathways to reduce major cardiovascular events in persons with pre-existing coronary artery disease was launched.[61, 62] This CANTOS trial is a large-scaled study in which over 17.000 subjects will be included to test three different doses of Canakinumab, a humanized monoclonal antibody specific for IL-1β, compared with placebo. Canakinumab is already approved in other autoimmune diseases where IL-1β plays a major role, such as Muckle-Wells syndrome and familial cold autoinflammatory syndrome.[63] This study will provide the first evidence whether interference in specific inflammatory pathways can reduce clinical events in cardiovascular patients and will possibly initiate numerous clinical trials focused on modulating immune responses in atherosclerosis.

Interestingly, some anti-tumor therapy studies have indicated that treatment with only a single costimulatory agonist, in addition to existing cytostatic therapy or cancer-antigen vaccination, is not effective or induces adverse immunological events. The co-administration of a second agonist or another factor that stimulates T cell function is necessary to achieve a greater anti-tumor reactivity. For example, a clinical trial with an anti-CTLA-4 antibody (MDX-010) in conjunction with anti-cancer antigen vaccination resulted in regression of cancer but unfortunately also induced severe autoimmune diseases in melanoma patients.[64] Kocak et al. showed that a combination therapy in mice with pre-existing tumors with anti-CTLA-4 and anti-4-1BB enhances anti-tumor immunity without any adverse effects on the immune system.[65] The mechanism through which the combination of anti-CTLA-4 and anti-41BB reduce each other's side effects is not fully explained but it is shown that they synergistically enhance the suppressive capacity of regulatory T cells. Currently, a phase I clinical

trial is carried out in which anti-CTLA-4 (Ipilimumab) is combined with anti-PD-1 (BMS-936558) to treat melanoma patients.[66]
It may be very likely that a combinatorial therapy may also be very effective in atherosclerosis. For example, previous studies reported a synergistic effect of OX40L and CD30L on T cell responses. Blocking CD30 together with OX40 signaling prevented lethal X-linked CD4 T cell-dependent Th1- and Th2-driven autoimmune disease in mice lacking regulatory T cells[67] and affected effector and memory T cell formation and function.[68] Moreover, a combined blockade of costimulatory signals, e.g. anti-OX40L or anti-CD30L, with activation of coinhibitory signals, e.g. PD-1 or Tim-3 agonists, could be explored to suppress for example pro-atherogenic T cells while stimulating athero-protective Tregs. More research should be performed to identify the most relevant combinations of blocking and agonistic antibodies for costimulatory and coinhibitory molecules respectively, which could be used as an immunotherapy to inhibit atherosclerosis.

Costimulatory and coinhibitory molecules are also essential for the establishment and maintenance of immunological tolerance via the induction of tolerogenic DCs and Tregs. A frequently used method to induce tolerance is by oral immunization. In atherosclerosis, oral tolerance induction to oxLDL[31], HSP60[32], β2-glycoprotein I[69] and ApoB100 peptide[70] has been shown to suppress atherosclerosis. Van Puijvelde et al. showed that oral tolerance induction against oxLDL and HSP60 increased Tregs and their CTLA-4 expression.[31, 32] Possibly, oral tolerance induction against auto-antigens, such as oxLDL, can be combined with blocking antibodies against costimulatory molecules or agonists for coinhibitory molecules to achieve T cell non-responsiveness against these auto-antigens and to promote the induction of antigen-specific Tregs.

Treg-based cell therapy

As shown in **Chapter 7** and **8**, Tregs are also efficient regulators of pathogenic immune responses and the usage of Tregs as a therapeutic agent shows great potential in the treatment of atherosclerosis. In several diseases the number or function of Tregs is decreased and restoring the balance between Tregs and pro-inflammatory cells may be beneficial. Therefore, a lot of research is nowadays focussed on the development of $Foxp3^+$ regulatory T cells (Figure 3). One possible treatment strategy is an adoptive transfer of Tregs. This procedure will require an enormous quantity of Tregs and can be achieved by isolation of $Foxp3^+$ T cells from the blood of a patient and subsequent *ex vivo* expansion to obtain large numbers for therapy.[71, 72] Two Phase I clinical trials have tested the ability of *ex vivo* expanded Tregs to prevent GVHD after allogeneic bone marrow transplantation.[73, 74] This Treg cell-based therapy proved to be safe and reduced GVHD. Moreover, new clinical trials are starting in which the safety and effectiveness of Treg cell-based therapy is tested in individuals with autoimmune diseases such as type 1 diabetes[75] and in organ transplantation patients.[76]
Alternatively, Tregs can be expanded *in vivo*. This can be achieved by targeting some

costimulatory and coinhibitory molecules as described previously but also with an IL-2 complex consisting of recombinant IL-2 and anti-IL-2. Besides effectively reducing atherosclerosis (**Chapter 8**), this IL-2 complex very potently induced resistance to EAE and suppressed graft rejections[39], type I diabetes[40], murine-asthma[41] and myasthenia gravis[42] in mouse studies. Future research should reveal whether administration of this IL-2 complex would also be beneficial in patients with cardiovascular disease.

Although current experimental treatments and clinical trials are based on the expansion of aspecific Tregs, it may be of great interest to induce antigen-specific Tregs. Previously, oral tolerance induction against oxLDL and HSP60 inhibited atherosclerosis development via the induction of antigen-specific Tregs[31, 32] Possibly, oral tolerance induction can be combined with an IL-2 complex treatment to first induce antigen-specific Tregs and thereafter expand these Tregs.

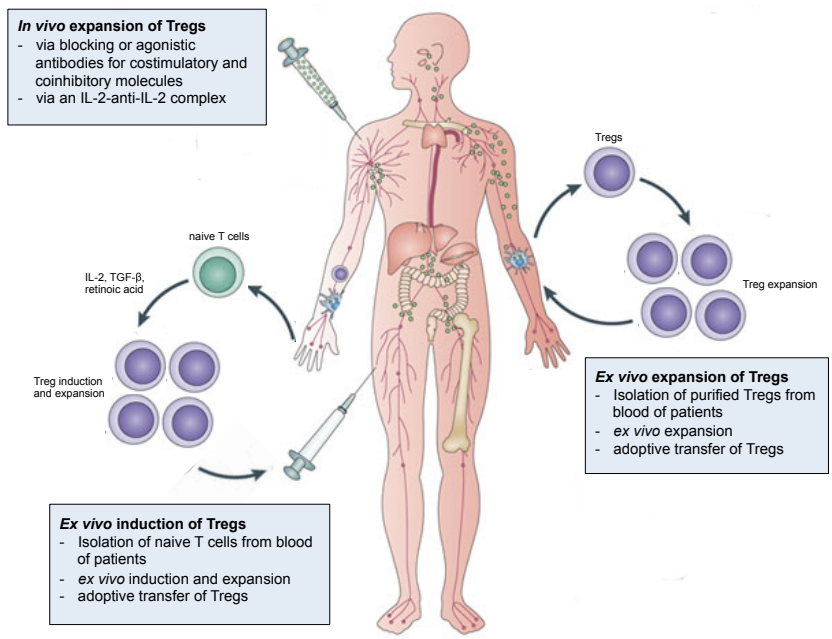


Figure 3. Schematic overview of different approaches to target Tregs for immunotherapy.

MDSC-based cell therapy

In atherosclerosis, elevated numbers of $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ cells (inflammatory monocytes) and $CD11b^{+}Ly6G^{+}Ly6C^{low}$ cells (neutrophils) are considered pro-inflammatory and correlate to lesion size.[36, 37] However, in the cancer field cells with the exact same phenotype exert an anti-inflammatory function and are called monocytic-MDSCs and granulocytic-MDSCs, respectively. MDSCs are known to expand in the bone marrow of diseased individuals and migrate into several lymphoid and non-lymphoid tissues. Elevated levels of circulating MDSCs are found in patients with cancer, multiple sclerosis and rheumatoid arthritis.[77-79] Further research is needed to

investigate whether MDSCs also accumulate in the blood of patients suffering from cardiovascular disease and to what extent these cells overlap with the inflammatory monocytes and neutrophils.

In **Chapter 9** we show that adoptive transfer of MDSCs (mo-MDSCs and gr-MDSCs) regulated T and B cell responses in an experimental model of atherosclerosis and inhibited lesion development. This shows the therapeutic potential of MDSCs as a novel immune-therapy to treat cardiovascular disease and opens an exciting new area of investigation. To fully characterize and comprehend the role of MDSCs in atherosclerosis multiple experiments should be performed. To determine the underlying mechanism of MDSC-mediated suppression, MDSCs can be isolated from Arg-$1^{-/-}$ and $iNOS^{-/-}$ mice, which lack functional gr-MDSCs and mo-MDSCs, respectively. To further determine the relative potency of mo-MDSCs and gr-MDSCs to inhibit atherosclerosis, each subset can be sorted with FACS or with magnetic bead labeling and subsequently adoptively transferred into Western-type diet fed $LDLr^{-/-}$ mice. Furthermore, since MDSCs respond to their microenvironment it is possible that they exhibit distinct biological activities depending on the microenvironment in the different pathological stages of atherosclerosis. It has also been shown that MDSC subpopulations from blood and tumors can differ in their capacity to mediate T cell suppression.[80] This raises the question whether MDSCs are present in lesions and whether MDSCs in lesions will have a different phenotype and suppression capacity in comparison with MDSCs present in the bone marrow and possibly in other sites.

Collectively, before MDSCs are considered as an innovative immunotherapeutic strategy to prevent atherosclerosis in cardiovascular patients, more research is required.

In conclusion, the research described in this thesis provided novel approaches to dampen the immune response in atherosclerosis. However, further characterization of these potential new drug targets and cellular therapies are necessary before they can be applied in clinical research.

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Nederlandse samenvatting

Hart- en vaatziekten

Hart- en vaatziekten zijn doodsoorzaak nummer 1 in de westerse wereld. In 2008 waren hart- en vaatziekten verantwoordelijk voor 30% van alle sterfgevallen, wat neerkomt op ongeveer 17.3 miljoen sterfgevallen wereldwijd. In Nederland overleden in 2010 meer vrouwen (30%) dan mannen (28%) aan de gevolgen van hart- en vaatziekten. Hart- en vaatziekten manifesteren zich vaak als een hartinfarct of beroerte en worden voornamelijk veroorzaakt door slagaderverkalking, ook wel atherosclerose genoemd. Atherosclerose ontwikkelt zich al tijdens de adolescentie en uit zich als een verdikking van de vaatwand. Deze verdikking is het gevolg van een chronische ontsteking in het bloedvat die samengaat met een plaatselijke ophoping van vetten. Het eerste stadium van atherosclerose, de 'fatty streak' is asymptomatisch. Pas als de verdikking uitgroeit tot een bloedvatvernauwende atherosclerotische plaque leidt dit tot klinische symptomen. De groei van een 'fatty streak' tot een onstabiele atherosclerotische plaque wordt beïnvloed door zowel erfelijke factoren, zoals dyslipidemie, een hoge bloeddruk, hoge cholesterol waardes en type 1 diabetes, als risicofactoren, zoals een vetrijk dieet, overgewicht, roken en te weinig lichaamsbeweging.

Behandeling van hart- en vaatziekten

Patiënten met acute klinische symptomen ondergaan vaak chirurgische ingrepen, zoals ballondilatatie (dotteren), soms in combinatie met plaatsing van een metalen buisje in het verkalkte bloedvat (stent) of een open hartoperatie waarbij een omleiding (bypass) langs vernauwde of afgesloten bloedvaten van de kransslagader wordt aangelegd om de bloeddoorstroming weer te herstellen. Verdere behandeling van hart- en vaatziekten berust op het verlagen van de risicofactoren met behulp van medicatie en aanpassingen in de levensstijl van de patiënten. Veel voorgeschreven medicatie bestaat uit bloeddrukverlagende medicijnen en statines. Statines remmen de productie van cholesterol in de lever, waardoor het cholesterolgehalte in het bloed verlaagd wordt. Daarnaast kunnen statines ook een ontstekingsremmende werking hebben. Alhoewel statines het risico op hart- en vaatziekten met 30% verminderen, hebben ze weinig effect op al bestaande atherosclerotische plaques. Dit is juist zo belangrijk aangezien patiënten met hart- en vaatklachten al een vergevorderde vorm van atherosclerose hebben. Het ontwikkelen van nieuwe behandelmethoden is noodzakelijk om de verdere progressie van atherosclerose te remmen en cardiovasculaire complicaties te voorkomen.

Atherosclerose; een chronische auto-immuunziekte

Het ontstaan van atherosclerose begint met een beschadiging van endotheelcellen die een laagje vormen aan de binnenkant van de vaatwanden. Deze beschadiging wordt meestal veroorzaakt door een turbulente bloedstroom, een verhoogde bloeddruk, verhoogd cholesterol, roken en andere risicofactoren zoals hierboven beschreven. De beschadigde endotheellaag is nu doorlaatbaar geworden voor vetten en ontstekingscellen die zich kunnen ophopen in de vaatwand. Cholesterol is een van de belangrijkste vetten in het menselijk lichaam. Het bevindt zich in alle celmembranen, zorgt voor de elasticiteit van de huid en is een essentiële bouwsteen voor hormonen en galzuren. Cholesterol kan onderscheiden worden in 'goed' en 'slecht' cholesterol. Het 'slechte' cholesterol, ofwel lage dichtheids lipoproteinen (LDL), hoopt zich op in de vaatwand en ondergaat daar verschillende modificaties, zoals oxidatie of aggregatie. Deze modificaties zorgen ervoor dat LDL niet meer herkend wordt als 'lichaamseigen' en het immuunsysteem komt in actie om dit vreemde molecuul op te ruimen. Verschillende soorten ontstekingscellen van het immuunsysteem worden vervolgens aangetrokken naar de vaatwand. Allereerst hechten monocyten zich aan de beschadigde endotheellaag en migreren de vaatwand in. Eenmaal in de vaatwand veranderen deze monocyten in macrofagen die het daar opgehoopte gemodificeerde LDL kunnen opnemen. Overmatige opname van het cholesterol leidt tot de vorming van zogenaamde 'foamcellen'. Deze foamcellen vormen een verdikking aan de binnenkant van de vaatwand, ook wel een 'fatty streak' genoemd: het beginstadium van de atherosclerotische plaque. Na opname van gemodificeerd LDL, produceren macrofagen allerlei ontstekingsmediatoren, zoals cytokines en chemokines, die andere ontstekingscellen zoals monocyten en T cellen aantrekken naar de plaque. Daarnaast behoren macrofagen ook tot 'antigen presenterende cellen' (APCs) wat inhoudt dat ze ook stukjes (antigenen) van het gemodificeerde LDL aan andere ontstekingscellen zoals T cellen kunnen presenteren. De T cellen die dit antigen herkennen worden geactiveerd en beginnen ook allerlei cytokines te produceren die vervolgens nog meer ontstekingscellen richting de plaque sturen. Dit leidt tot een chronische auto-immuun reactie waarbij er dus een immunologische reactie is ontstaan tegen een lichaamseigen antigen (gemodificeerd LDL). Naast gemodificeerd LDL zijn er nog meer antigenen die atherosclerose kunnen veroorzaken, zoals heat shock eiwitten die grote structurele overeenkomsten hebben met bacteriële moleculen en dus als lichaamsvreemd herkend worden. Naast een toename in het aantal ontstekingscellen in de plaque, migreren gladde spiercellen vanuit de wand naar de endotheellaag om daar samen met bindweefsel en collageen een beschermende 'cap' te vormen. Deze cap houdt de ontstekingscellen en vetdeposities op hun plaats en verhindert dat deze weer loskomen in de circulatie. Echter, gedurende de verdere ontwikkeling van atherosclerose kan er een scheur ontstaan in de cap waardoor de inhoud van de plaque in zijn geheel aan het bloed wordt blootgesteld. Dit leidt tot bloedstolling en de vorming van een bloedprop die het bloedvat of bij loslaten van de bloedprop een bloedvat elders compleet blokkeert zodat achterliggend orgaan onthouden wordt van

alle voedingsstoffen en zuurstofrijk bloed met bijvoorbeeld een hart- of herseninfarct als gevolg.

Regulatie van het immuunsysteem als behandeling van atherosclerose
Aangezien het immuunsysteem een grote rol speelt in de pathogenese van atherosclerose, kan regulatie van het immuunsysteem een veelbelovende therapie vormen om hart- en vaatziekten te voorkomen. Zoals hierboven beschreven zijn T cellen nauw betrokken bij het ontstaan van een chronische ontstekingsreactie. Er zijn echter verschillende soorten T cellen die ieder hun eigen functie hebben en na activatie hun eigen set ontstekingseiwitten (cytokines) produceren. In atherosclerose worden vooral T helper 1 (Th1) cellen geactiveerd die grote hoeveelheden van het cytokine IFN-γ uitscheiden, dat atherosclerose verergert. Deze pro-inflammatoire Th1 cellen kunnen geremd worden door een anti-inflammatoire T cel: de regulatoire T cel (Treg). Deze Tregs produceren de atherosclerose-remmende cytokines IL-10 en TGF-β en kunnen direct Th1 cellen remmen via cel-cel contact. In atherosclerose is er echter een verstoorde balans tussen de pro-inflammatoire, 'slechte', T cellen en de anti-inflammatoire, 'goede' T cellen, waarbij de balans verschoven is naar de pro-inflammatoire T cellen. Recent onderzoek richt zich daarom op het herstellen van deze balans door (1) pro-inflammatoire responsen te remmen of (2) het aantal anti-inflammatoire cellen te laten toenemen. Beide methoden van immuunregulatie maken deel uit van het in dit proefschrift beschreven onderzoek.

Modulatie van costimulatoire en coinhibitoire moleculen op ontstekingscellen
Voor de optimale activatie van T cellen zijn 3 signalen nodig: (1) de presentatie van een antigen op MHC moleculen van een antigen presenterende cel aan de T cel receptor aanwezig op het oppervlak van een T cel, (2) interactie tussen costimulatoire of coinhibitoire moleculen op antigen presenterende cellen en T cellen en (3) cytokines in de omgeving die onder andere kunnen beïnvloeden wat voor type, pro- of anti-inflammatoire, T cel er ontstaat. Signaal 1 kan zonder signaal 2 geen T cel activeren, wat signaal 2 erg belangrijk maakt. Costimulatoire en coinhibitoire moleculen komen voor in paren: een ligand (L), en een receptor waaraan het ligand kan binden. Bij sommige paren bevindt het ligand zich op de antigen presenterende cel en de receptor zich op de T cel, terwijl bij andere paren de receptor op de antigen presenterende cel zit en het desbetreffende ligand op de T cel. Wanneer een costimulatoir ligand bindt aan de bijbehorende receptor worden ontstekingscellen (in combinatie met signaal 1) geactiveerd, maar wanneer een coinhibitoir ligand aan zijn receptor bindt worden ontstekingscellen juist geremd. Er zijn veel verschillende costimulatoire en coinhibitoire paren met elk hun eigen unieke effect op verscheidene ontstekingscellen. Zo kunnen bijvoorbeeld OX40-OX40L en CD40-CD40L beiden atherosclerose verergeren, maar werken allebei via andere routes: binding van OX40 met OX40L zorgt ervoor dat T cellen veranderen in Th2 cellen, terwijl binding van CD40 met CD40L T cellen richting een Th1 fenotype stuurt. In **Hoofdstuk 2** wordt een overzicht gegeven van alle

costimulatoire en coinhibitoire moleculen en hun rol in atherosclerose.
Aangezien het remmen van pro-inflammatoire ontstekingscellen een veelbelovende therapie is om hart- en vaatziekten te behandelen, kan modulatie van signaal 2 door (1) het blokkeren van costimulatoire paren of (2) het stimuleren van coinhibitoire paren, een nieuwe basis voor immunotherapie vormen. In **Hoofdstuk 3-6** zijn met behulp van blokkerende en stimulerende antilichamen verschillende costimulatoire en coinhibitoire signaleringsroutes bestudeerd in muizen met atherosclerose. Normaliter zijn muizen ongevoelig voor de ontwikkeling van hart- en vaatziekten, maar muizen die deficiënt zijn in de LDL receptor ($LDLr^{-/-}$ muizen) hebben verhoogde gehaltes van LDL cholesterol in het bloed. Wanneer deze $LDLr^{-/-}$ muizen een vetrijk dieet ontvangen ontwikkelen ze "spontaan" atherosclerose.
In **Hoofdstuk 3** is de rol van de costimulatoire OX40-OX40L route in combinatie met een verlaging van lipiden in het dieet bestudeerd op het verminderen (regressie) van al bestaande atherosclerotische plaques. OX40 en OX40L hebben invloed op de activatie en functie van verschillende ontstekingscellen die nauw betrokken zijn bij de ontwikkeling van atherosclerose, zoals T en B cellen en mestcellen. Om de rol van OX40-OX40L in de regressie van atherosclerose te bestuderen, hebben $LDLr^{-/-}$ muizen eerst 10 weken een vetrijk dieet gekregen om atherosclerose te ontwikkelen. Vervolgens zijn ze op een vetarm dieet geplaatst om het cholesterol verlagende effect van statines na te bootsen en zijn ze behandeld met een blokkerend OX40L antilichaam om het immuunsysteem te reguleren. Na 10 weken vetarm dieet en de anti-OX40L behandeling is de atherosclerose ontwikkeling geanalyseerd in het drie-kleppen gebied van de aorta en in de aortaboog. Alleen het veranderen van een vetrijk dieet naar een vetarm dieet heeft geen invloed op de hoeveelheid atherosclerose: hoewel de plaques stabieler zijn, groeien de plaques zelfs nog in geringe mate door. Behandeling met anti-OX40L in combinatie met het vetarme dieet induceert echter wel regressie van atherosclerose. Dit komt enerzijds door een vermindering in het pro-atherogene IL-4, verlaging van IgE serum niveaus en mestcel activatie, en anderzijds door een toename in de productie van het beschermende IL-5 en IgM antilichaam productie door B cellen. Deze beschermende IgM antilichamen zijn gericht tegen geoxideerd LDL wat resulteert in verminderde atherosclerose.
In **Hoofdstuk 4** wordt de rol van een andere costimulatoire signaleringsroute gevormd door CD30 en CD30L in atherosclerose beschreven. Net zoals OX40 en OX40L zijn CD30 en CD30L betrokken bij de activatie en proliferatie van T en B cellen. Een eerdere studie heeft aangetoond dat CD30 positieve macrofagen aanwezig zijn in gescheurde atherosclerotische plaques van patiënten met hart- en vaatziekten, maar de exacte rol van CD30-CD30L aan atherosclerose ontwikkeling was onbekend. Daarom hebben we $LDLr^{-/-}$ muizen 8 weken een vetrijk dieet gegeven en zijn ze twee keer per week behandeld met een antilichaam tegen CD30L. Anti-CD30L behandelde muizen ontwikkelen 35% minder atherosclerose, onafhankelijk van cholesterol niveaus in het bloed en de samenstelling van de plaque. Deze afname in atherosclerose gaat gepaard met een verminderde hoeveelheid, activatie en proliferatie van $CD4^+$ T cellen

in de milt en daarnaast is er een 31% afname in $CD3^+$ T cellen in de adventitia van anti-CD30L behandelde muizen. Anti-CD30L behandeling heeft geen effect op de functie van andere ontstekingscellen zoals B cellen en mestcellen in $LDLr^{-/-}$ muizen op een vetrijk dieet. Samenvattend hebben we vastgesteld dat blokkade van de costimulatoire signaleringsroute CD30-CD30L specifiek via de remming van $CD4^+$ T cellen atherosclerose remt.

In **Hoofdstuk 5** bestuderen we de rol van Tim-3 in atherosclerose. Tim-3 behoort tot de familie van T cel immunoglobuline en mucine domein (Tim) eiwitten en is coinhibitoir. Tim-3 komt tot expressie op verschillende onstekingscellen zoals NK cellen, monocyten, macrofagen en T cellen en kan na binding van zijn ligand, galectin-9, celdood induceren. Daarnaast hebben eerdere studies beschreven dat signaaltransductie via Tim-3 regulatoire T cellen kan induceren. Allereerst hebben we vastgesteld dat Tim-3 tot expressie komt in de plaque en toeneemt naarmate muizen langer een vetrijk dieet gegeten hebben. Het percentage $Tim\text{-}3^+$ natural killer cellen, monocyten en dendritische cellen (DCs) is ook verhoogd in $LDLr^{-/-}$ muizen op een vetrijk dieet in vergelijking met muizen op een vetarm dieet. Vervolgens hebben we $LDLr^{-/-}$ muizen op een vetrijk dieet behandeld met een selectief antilichaam tegen Tim-3 gedurende 8 weken. Anti-Tim-3 behandeling leidt tot een toename in atherosclerose van 35% in het drie-kleppen gebied van de aorta en van 50% in de aortaboog. Deze verergering van atherosclerose wordt veroorzaakt door een toename in circulerende monocyten en in de plaque zijn 20% meer macrofagen aanwezig. Anti-Tim-3 behandeling leidt ook tot een toename in $CD4^+$ T cellen en een vermindering van regulatoire cellen zoals Tregs en regulatoire B cellen (Bregs). Hieruit kunnen we concluderen dat Tim-3 als een negatieve regulator van atherosclerose werkt.

In **Hoofdstuk 6** is de bijdrage van een ander recent ontdekt coinhibitoir paar gevormd door TIGIT en PVR, aan de ontwikkeling van atherosclerose bepaald. Signalering via TIGIT, een T cel immunoreceptor met immunoglobuline, en PVR, de poliovirus receptor, remt T cellen zowel direct door het down-reguleren van de T cel receptor, als indirect via de inductie van beschermende IL-10 producerende DCs. Het stimuleren van TIGIT met behulp van een agonist *in vitro* zorgt voor een sterke remming van de activatie en proliferatie van T cellen. Vervolgens hebben we $LDLr^{-/-}$ muizen op een vetrijk dieet behandeld met deze TIGIT agonist gedurende 4 en 8 weken. De muizen behandeld met de TIGIT agonist hebben minder T cellen en deze T cellen tonen een verminderde proliferatie. Ondanks dit sterke effect op T cellen heeft een behandeling van $LDLr^{-/-}$ muizen met deze TIGIT agonist nauwelijks effect op atherosclerose. Dit komt mogelijk door een verhoogde activiteit van DCs in TIGIT agonist behandelde muizen.

Immuunregulatie door suppressor cellen

Een andere manier om de balans tussen goede en slechte ontstekingscellen te herstellen is door het aantal goede ontstekingscellen te laten toenemen. Deze goede ontstekingscellen kunnen de slechte ontstekingscellen onderdrukken en zo

atherosclerose remmen. In **Hoofdstuk 7-9** hebben we gekeken naar de rol van twee soorten goede ontstekingscellen: regulatoire T cellen en 'myeloid-derived suppressor cellen'.

Zoals eerder beschreven verminderen Tregs ontsteking en kunnen daardoor beschermend zijn in auto-immuunziekten, zoals atherosclerose. Allereerst is in **Hoofdstuk 7** het effect van de blokkering van Tregs bestudeerd om de rol van Tregs in atherosclerose verder te bevestigen. In dit hoofdstuk wordt een vaccinatiemethode beschreven waarbij dendritische cellen gebruikt zijn om een immuunrespons op te wekken tegen cellen die Foxp3 tot expressie brengen. Foxp3 is een eiwit dat voornamelijk gemaakt wordt door Tregs. Door cellen die Foxp3 tot expressie brengen te vernietigen kan de bijdrage van Tregs in atherosclerose bestudeerd worden. We zien in deze studie dat de vaccinatie leidt tot minder Tregs in de muis. Dit heeft tot gevolg dat de atherosclerotische plaque toeneemt in grootte. Hetzelfde is ook waargenomen in muizen met een vergevorderde atherosclerotische plaque. Dit geeft aan dat Tregs zowel bij beginnende als gevorderde atherosclerose betrokken zijn.

Vervolgens zijn in **Hoofdstuk 8** Tregs gestimuleerd met behulp van een IL-2-anti-IL-2 complex en hebben we gekeken naar de rol van Tregs in de ontwikkeling en regressie van atherosclerose. Tregs worden gekenmerkt door de hoge expressie van CD25 op hun celoppervlak. Een complex van IL-2 en anti-IL-2 kan binden aan CD25 en zorgt ervoor dat er wel 10 keer zoveel Tregs aanwezig zijn in het bloed en in verschillende organen. Dit resulteert in een 39% reductie van de atherosclerotische plaque tijdens een vroeg stadium van atherosclerose, terwijl in een regressie model van atherosclerose de enorme expansie van Tregs geen invloed heeft op plaque grootte, maar wel de stabiliteit van de plaque sterk doet toenemen.

Naast Tregs zijn myeloid-derived suppressor cellen (MDSCs) ook goede kandidaten om heel gericht ontsteking te remmen. MDSCs vormen een populatie 'onrijpe' macrofagen, dendritische cellen en granulocyten die verhindert zijn om van beenmerg precursoren te veranderen tot rijpe ontstekingscellen. MDSCs kunnen geïdentificeerd worden door de expressie van CD11b en Gr-1 en zijn vooral beschreven in kanker, waar de aanwezigheid van MDSCs zorgt voor de verhindering van T cel reacties wat resulteert in progressie van de ziekte. In atherosclerose is een remming van T cellen juist gewenst, maar de rol van MDSCs in atherosclerose is tot op heden niet onderzocht.

In **Hoofdstuk 9** hebben wij daarom allereerst met behulp van magnetische opzuivering $CD11b^{+}Gr\text{-}1^{+}$ cellen geïsoleerd uit het beenmerg van $LDLr^{-/-}$ muizen die 2 weken op een vetrijk dieet hebben gestaan. We hebben aangetoond dat de geïsoleerde cellen daadwerkelijk MDSCs zijn en ze heel sterk T cellen kunnen remmen. Deze remming van T cellen door de MDSCs is afhankelijk van IFN-γ en iNOS, twee factoren die ervoor zorgen dat de voedingsstoffen voor T cellen weggenomen worden. Om te kijken of MDSCs beschermend zijn in atherosclerose, hebben we elke 10 dagen intraveneus MDSCs toegediend aan $LDLr^{-/-}$ muizen, terwijl zij gedurende 6 weken een vetrijk dieet

ontvingen. Muizen die MDSCs toegediend hebben gekregen ontwikkelen 35% minder atherosclerose in vergelijking met controle muizen. Deze muizen hadden aanzienlijk meer naïve T cellen en veel minder pro-atherogene Th1 en Th17 cellen. Daarnaast zijn er ook minder circulerende B cellen, met name pro-atherogene B2 cellen, in MDSC-behandelde muizen.
Toekomstige studies zijn nodig om de exacte rol van MDSCs in atherosclerose te beschrijven. Er zijn namelijk verschillende subtypes van MDSCs, en ieder subtype kan weer een uniek effect hebben op atherosclerose. Daarnaast zou het erg interessant zijn om de aanwezigheid en expansie van MDSCs in muismodellen van atherosclerose en patiënten met hart- en vaatziekten in kaart te brengen.

Samenvattend zijn in dit proefschrift een aantal studies beschreven die gericht zijn op het vinden van nieuwe targets om de balans tussen pro- en anti-inflammatoire ontstekingscellen in atherosclerose te herstellen. Aan de ene kant heeft het remmen van ontstekingscellen door costimulatoire en coinhibitoire eiwitten te beïnvloeden geleid tot een sterke afname in de grootte van atherosclerotische plaques. Aangezien er ontzettend veel verschillende costimulatoire en coinhibitoire eiwitten zijn en ze allemaal hun eigen functie hebben, kan modulatie van deze eiwitten een veelbelovende therapie zijn om hart- en vaatziekten te behandelen. Op dit moment zijn er al een aantal antilichamen voor costimulatoire en coinhibitoire moleculen goedgekeurd voor de behandeling van verschillende soorten kanker en zijn er klinische trials gaande met betrekking tot de rol van deze antilichamen in ziekten zoals astma. Deze kunnen waarschijnlijk op korte termijn ook toegepast worden op het gebied van hart- en vaatziekten.
Aan de andere kant is gebleken dat het gebruik van regulatoire T cellen en myeloid-derived suppressor cellen ook veelbelovende immunotherapiën kunnen vormen om atherosclerose te remmen. Vermeerdering of toediening van deze cellen in muismodellen voor atherosclerose leidt tot een sterke afname in plaque formatie. Vooral in het geval van myeloid-derived suppressor cellen, maar ook voor regulatoire T cellen dient er in de toekomst nog veel onderzoek verricht te worden naar de bruikbaarheid, veiligheid en effectiviteit van een cellulaire therapie voor patiënten met hart- en vaatziekten.
Al met al hebben de in dit proefschrift beschreven studies geleid tot meer en nieuwe inzichten in de immunologische processen van atherosclerose en deze kunnen mogelijk leiden tot nieuwe klinische toepassingen zodat hart- en vaatziekten patiënten in de toekomst beter en eerder behandeld kunnen worden.

Curriculum Vitae

Amanda Foks werd geboren op 9 juli 1985 in Voorburg. In juni 2004 werd het VWO diploma behaald aan het Sint Maartens College te Voorburg. In datzelfde jaar werd begonnen met de studie Biofarmaceutische Wetenschappen aan de Universiteit Leiden. Het propaedeutisch examen werd in september 2005 gehaald en de Bachelor of Science werd in september 2007 behaald. Van september 2007 tot en met juli 2008 werd in het kader van de masterstage onderzoek verricht binnen de vakgroep Biofarmacie van het Leiden Academic Centre for Drug Research onder begeleiding van Dr. G.H.M van Puijvelde, Dr. T. van Es en Prof.dr. J. Kuiper. Gedurende die stage werd onderzoek gedaan naar de rol van Foxp3$^+$ regulatoire T cellen in atherosclerose wat afgesloten werd met een verslag getiteld: "Reduction of Tregs aggravates atherosclerosis". Van september 2008 tot en met maart 2009 werd een tweede stage gevolgd bij de vakgroep Reumatologie op het Leids Universitair Medisch Centrum onder leiding van Dr. J. Wang en Prof.dr. R.E.M. Toes. Dit onderzoek was getiteld "*In vitro* induction of Foxp3$^+$ T cells from DBA CD4$^+$ T cells". De Master of Science werd in juni 2009 gehaald (*cum laude*).

Van juni 2009 tot juni 2013 was zij als promovendus werkzaam bij de vakgroep Biorfarmacie van het Leiden Academic Centre for Drug Research onder leiding van Dr. G.H.M. van Puijvelde en Prof.dr. J. Kuiper. Dit onderzoek maakte deel uit van een door de Nederlandse Hartstichting gefinancieerd project. Per 1 augustus 2013 is zij aangesteld als post-doctoraal onderzoeker binnen de vakgroep Pathologie van het Brigham and Women's Hospital, Harvard Medical School in Boston onder leiding van Prof.dr. A. Lichtman.

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