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Title: Novel immune cell-based therapies for atherosclerosis
Issue Date: 2015-05-27
General Introduction
Chapter 1

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

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

1. Atherosclerosis
Observations of atherosclerosis date back as far as Aristoteles, who observed that arteries of young individuals were straight and open vessels, while arteries of older individuals were tortuous and narrowed vessels\(^11\). Initially the process of arterial stiffening was seen as a result of aging and only in the 19\(^{th}\) century was the process acknowledged as pathogenic. Early work by Nikolai Anichkov determined the role of cholesterol in the development of atherosclerosis\(^12\). Soon after, Virchow\(^13\), von Rokitansky\(^13\), Hodgson\(^14\), and Hope\(^15\) proposed a role for inflammation in atherosclerosis. Virchow incorporated the inflammatory characteristic of the disease into its name “Endarteritis deformans s. nodosa” and thereby changed the perception of the disease. However, this term sharply divided the fatty degenerative aspect from the inflammatory proliferative aspect and soon after the term “atherosclerosis” was proposed by Marchand\(^16\). This incorporates both the “atheromatous” (degenerative, gruel-like) and “sclerotic” (hardening) aspect of the disease and has been used since to describe the disease process. Today it has become widely accepted that atherosclerosis is a chronic inflammatory autoimmune-like disease of the large and medium-sized arteries. It already starts in early adolescence\(^17\) and can either remain asymptomatic throughout an entire lifetime or can result in acute complications, such as myocardial infarction (MI). Interestingly, by means of \(^{14}\)C content of lesions (which was released into the atmosphere during nuclear weapons tests in the 1950s-60s) it
was shown that remodeling of human atherosclerotic lesions is a very slow process. The biological age of components was found to vary from 6.5 years in the cap regions, to 10 years in the core, and 13 years in the shoulder region\textsuperscript{18}. This minimal turnover already indicates the difficulty of modulating the atherosclerotic disease process. Endothelial dysfunction, vascular inflammation, and the accumulation of lipids and fibrous elements within the vessel wall are all characteristic for atherosclerosis. Eventually this will result in lesion formation, vascular remodeling, abnormal blood flow, stenosis, and possibly thrombus/emboli formation.

1.1 Initial Atherosclerotic Lesions

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

LDL can passively diffuse through the disturbed EC layer and is retained in the intima by interactions with proteoglycans\textsuperscript{20}. Trapped LDL can then undergo modifications, e.g. lipolysis, proteolysis, and oxidation. Evidence from animal models suggests that oxidation is a crucial step in the conversion of LDL into an atherogenic particle. Oxidation is likely facilitated by lipoxygenases, myeloperoxidases, inducible nitric oxide synthase (iNOS) and NADPH oxidases that are found within lesions\textsuperscript{21}. Oxidized LDL (oxLDL) leads to the activation of ECs, which increase their expression of adhesion molecules, cytokines, and growth factors (e.g. macrophage colony-stimulating factor; M-CSF)\textsuperscript{22}. These changes in the vascular environment result in an activation of resident lymphocytes, dendritic cells (DCs), macrophages, and smooth muscle cells (SMCs). Moreover, an enhanced recruitment and transmigration of leukocytes is mediated by three main chemokine receptor/chemokine pairs: CC chemokine receptor (CCR) 2/ CC chemokine ligand (CCL) 2, CX\textsubscript{3}C-C-chemokine receptor 1 (CX\textsubscript{3}CR1)/CX\textsubscript{3}CL1 and CCR5/CCL5\textsuperscript{23,24}. This culminates in a chronic low-grade inflammation of the vessel wall\textsuperscript{25}. Recruited monocytes are exposed to growth factors within the vessel wall, which stimulate their differentiation to macrophages\textsuperscript{26}. Moreover, M-CSF enhances the expression of scavenger receptors in macrophages, which recognize oxidation-specific epitopes and thereby enable uptake of cell debris and oxLDL\textsuperscript{27}. By accumulating cholesterol esters, macrophages transform into lipid-rich foam cells, so called due to cytoplasmic lipid droplets giving them a ‘foamy’ appearance under electron microscopy. These cells are one of the most characteristic features of the atherosclerotic lesion. Eventually cholesterol accumulation induces cytotoxicity, resulting in foam cell apoptosis. The lipid-rich remnants of foam cells contribute to formation of a necrotic core in the lesion\textsuperscript{28}. At this stage the lesions are referred to as fatty streaks (Figure 1A), which in humans can usually be found...
in the aorta as early as the first decade of life\textsuperscript{29}. In addition, lesional macrophages, as well as DCs, express Toll-like receptors (TLRs), which enable them to recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). TLR stimulation results in macrophage polarization and DC maturation, determined by upregulation of co-stimulatory molecules and release of pro-inflammatory mediators\textsuperscript{30,31}. Released cytokines and chemokines promote further inflammation and recruitment of more leukocytes – either from the luminal side or via the adventitia through vasa vasorum\textsuperscript{32,33}. Moreover, while only DCs can activate naïve T cells, both macrophages and DCs can present antigens and directly shape adaptive T cell responses.

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

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

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

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

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

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

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

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

LDLr−/− mice have much lower total cholesterol levels (175-225 mg/dL) on a normal chow diet than ApoE−/− mice. Additionally, they need to be fed a high cholesterol (Western-type) diet to induce lesion development. Lesions develop slower than those
in ApoE-/- mice and are rich in macrophages, which resembles more the slow process observed in humans. However, lesions are less severe and show less complexity.

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

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

HSPCs reside within the bone marrow and function to maintain hematopoietic homeostasis. They have been extensively used to restore bone marrow function in cancer patients after treatment with cytotoxic drugs and/or whole body irradiation. Recently phase I clinical trials have established a positive effect of HSPCs on cardiac repair upon MI, as shown by improved left ventricular function. In atherosclerosis, HSPC proliferation and mobilization might underlie the observed monocytosis. Studies by the Tall laboratory found that cholesterol trapped inside HSPCs increases their proliferation. In addition, it has been recognized that chronic psychosocial stress is a risk factor for atherosclerosis. Indeed, a study by the Nahrendorf laboratory has shown that chronic stress in mice as well as humans (medical residents) results in mobilization of leukocytes. In mice it was shown that chronic stress and thus increased sympathetic nervous system activity induces HSPC proliferation and thereby increases monocyte and neutrophil numbers, which promotes atherosclerosis. Acute tissue injury after MI was also shown to induce proliferation and mobilization of HSPCs. HSPC recruitment was found to increase circulating monocyte numbers, resulting
in accelerated atherosclerotic lesion progression\textsuperscript{77}. The effect was to a large degree dependent on sympathetic nervous system signaling indicating that acute anxiety and pain might affect HSPC mobilization from the bone marrow. These initial studies point towards a role of known risks factors for atherosclerosis, such as a high-fat diet and stress, in increasing HSPC production and output by the bone marrow, resulting in monocytosis.

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

### 2.2 Monocytes

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

The population of circulating murine monocytes (CD11b\textsuperscript{+}CD115\textsuperscript{+}F4/80\textsuperscript{int-lo}Ly-6G) consists of two main subsets differentiated by their expression of Ly-6C. Ly-6C\textsuperscript{hi} monocytes share properties with human classical CD14\textsuperscript{+}CD16\textsuperscript{−} monocytes based on gene expression profiles. They are pro-inflammatory and preferentially recruited to inflamed tissues, while Ly-6C\textsuperscript{low} monocytes share several properties with human non-classical CD14\textsuperscript{dim}CD16\textsuperscript{+} monocytes and are considered to patrol the vasculature and home to non-inflamed tissues\textsuperscript{97,98}. Ly-6C\textsuperscript{hi} monocytes are predominant during hypercholesterolemia and give rise to macrophages in the lesion\textsuperscript{90}. They express high
levels of CCR2, CCR5, CCR1, and low levels of CX3CR1, CD62L, and rely on CCL2/CCR2 for egression from the bone marrow\textsuperscript{99}. Ly-6C\textsuperscript{low} monocytes on the other hand express high levels of CX3CR1 and low levels of CCR2\textsuperscript{100}. Ly-6C\textsuperscript{hi} monocytes have been found to be able to convert to Ly-6C\textsuperscript{low} monocytes showing plasticity at the monocyte level\textsuperscript{97}. Chemokine deficiencies as well as monocyte deficiencies of chemokines receptors reduce lesional monocyte infiltration and lesion sizes in murine models of atherosclerosis.

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

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

CX3CL1 (also known as fractalkine) has been shown to be crucially involved in the recruitment of monocytes to atherosclerotic lesions in ApoE\textsuperscript{-/-} mice\textsuperscript{112}. Both deficiency of CX3CL1\textsuperscript{113} and its receptor CX3CR1\textsuperscript{114,115} reduces atherosclerosis in ApoE\textsuperscript{-/-} mice. Interestingly, also in humans single nucleotide polymorphisms (SNPs) of CX3CR1 have been associated with cardiovascular disease\textsuperscript{116-119}. CX3CR1 signaling plays an additional role, besides CCR2, in the recruitment of monocytes to lesions. CX3CR1\textsuperscript{-/-}CCR2\textsuperscript{-/-}ApoE\textsuperscript{-/-} mice have significantly less atherosclerotic lesions than single deficiencies of CX3CR1 and CCR2\textsuperscript{120}. It was found that retention of monocytes is at least in part mediated by their loss of CCR2 and gain of CX3CR1, which results from exposure to oxidized lipids in the vessel wall\textsuperscript{121}.

Additionally, CCL5 has been associated with an unstable atherosclerotic lesion phenotype in carotid endarterectomy patients\textsuperscript{122}. It is thought that CCL5/CCR5
interactions are needed for firm adhesion of rolling monocytes\textsuperscript{123}. However, while some studies indeed indicate that CCR5 expression is pro-atherogenic\textsuperscript{124–126}, others show that CCR5 deficiency does not affect atherosclerosis\textsuperscript{127}. We have shown that an HIV entry inhibitor (TAK-779), a CCR5 antagonist, shows a significant reduction of atherosclerosis\textsuperscript{128}, further suggesting CCR5 as a potential therapeutic target. However, it should be noted that CCR5 is highly expressed by pro-atherogenic T helper (Th)1 cells and is involved in their recruitment to lesions. To what extent reduction of macrophage recruitment by CCR5 contributes to effects of lesion development still needs to be addressed. A clue might be provided in a study by Combadière et al. where CCL2\textsuperscript{-/-}CX3CR1\textsuperscript{-/-}ApoE\textsuperscript{-/-} mice were treated with a CCR5 antagonist. These mice showed a significant additional reduction of circulating (Ly-6Ch) monocytes compared to non-treated mice, indicating that all three chemokine receptors are involved in monocyte recruitment\textsuperscript{129}. However, again it cannot be excluded that the additional decrease in lesion size of CCR5 antagonist-treated CCL2\textsuperscript{-/-}CX3CR1\textsuperscript{-/-}ApoE\textsuperscript{-/-} mice is not due to an additional reduction in Th1 recruitment.

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

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

### 2.3 Macrophages

Macrophages are found at all stages of atherosclerotic lesion development and outnumber any other cell type in the lesion\textsuperscript{136,137}. The majority of lesional macrophages is derived from circulating monocytes\textsuperscript{26}. The important role of macrophages during atherosclerosis is exemplified by ApoE\textsuperscript{-/-} mice deficient in M-CSF, which show reduced macrophage numbers with altered functions and a dramatic 86% reduction in atherosclerosis\textsuperscript{138}. However, it should be noted that these mice also already have reduced monocytes. The same accounts for studies that have used clodronate liposomes. These liposomes deliver clodronate to macrophages, resulting in their apoptotis. This treatment has been shown to reduce neointimal hyperplasia, but again circulating monocytes are also significantly reduced by this treatment\textsuperscript{139}. CD11b-diphtheria toxin receptor (DTR) mice, which display a depletion of macrophages and monocytes, show a similar profound reduction of initial lesion development\textsuperscript{140}. However, no effect on lesion progression is observed, indicating a less pronounced role of monocytes and
macrophages in established lesions\textsuperscript{140}. The role of macrophages was more specifically assessed by systemic administrations of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which only resulted in apoptosis of macrophages but not of other cells. This significantly reduced progression of advanced atherosclerotic lesions\textsuperscript{141}.

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

The accumulation of lipid droplets in macrophage results in a shutdown of endogenous cholesterol biosynthesis and LDLr expression by inhibition of the sterol regulatory element-binding protein (SREBP) pathway\textsuperscript{149}. However, this does not restore cholesterol homeostasis in the macrophage as cholesterol is continuously taken up by SRs. Excessive cholesterol accumulation results in the activation of liver X receptor (LXR) signaling. LXRα and LXRβ induce specific target genes upon binding of oxysterols (oxygenated derivatives of cholesterol). While LXRβ is widely expressed, LXRα is mostly expressed in the liver and the intestine, but gets significantly induced in macrophages upon lipid accumulation. LXRs and retinoid X receptors (RXRs) form heterodimers, which induce three classes of target genes: (1) ATP-binding cassette (ABC) transporter genes, (2) ApoE in macrophages, both promoting cholesterol efflux, and (3) fatty acid synthesis genes, promoting re-esterification of cholesterol. ABC transporters are crucially involved in cholesterol efflux from the macrophages and strongly induced by LXR and RXR signaling. The most studied cholesterol transporters are ABCA1 and ABCG1\textsuperscript{26}. Cholesterol efflux from macrophages can occur passively or actively via high-density lipoprotein (HDL) or apolipoprotein A-I (ApoA-I) cholesterol acceptors. ApoA-I is the major apolipoprotein of HDL and is produced in the liver or intestine. ApoA-I can accept cholesterol esters from ABCA1, thereby forming nascent HDL, which is subsequently converted to mature HDL by esterification of free cholesterol by lecithin cholesterol acyltransferase (LCAT). Mature HDL can in turn accept free cholesterol from ABCG1 and SR-B\textsubscript{I}\textsuperscript{28}. HDL then transports the cholesterol esters back to the liver, where they are either excreted into the bile or reused for lipoprotein assembly. For this beneficial role in maintaining cholesterol homeostasis, HDL is also referred to as the "good cholesterol". Several epidemiological studies have shown an inverse correlation between plasma HDL and cardiovascular diseases\textsuperscript{150}. We
and others have demonstrated the important role of cholesterol efflux in macrophages by reconstituting LDLr−/− mice with ABCA1−/− or ABCA1−/−ABCG1−/− bone marrow. These mice show increased foam cell formation and atherosclerosis151,152. In contrast, results of ABCG1 deficiency are less clear, possibly due to a compensatory upregulation of ABCA1 or a less pronounced role of ABCG1. LDLr−/− mice transplanted with ABCG1−/− bone marrow either showed no effect on atherosclerotic lesions151,153, only moderately increased lesion formation154, or even decreased lesion development155,156 and progression157. Reduced atherosclerosis was attributed to increased macrophage apoptosis upon ABCG1 deficiency156,157, since apoptotic cell clearance induces anti-inflammatory responses158,159. However, because apoptotic cell clearance in advanced human and murine lesions is impaired160-162 it is likely that other processes, such as a previously observed compensatory ABCA1 and ApoE upregulation155, also contributed. Interestingly, it has recently been suggested that desmosterol, a cholesterol-precursor present in oxLDL, can induce an anti-inflammatory macrophage phenotype through activation of LXR163. VLDL has also been associated with cardiovascular disease risk and VLDL levels are often increased in metabolic syndrome. VLDL is mostly processed in adipose tissue and muscle by vascular endothelial cell-anchored lipoprotein lipase (LPL)164. The remaining cholesterol-rich remnants are readily cleared by the liver or result in the formation of LDL. VLDL can also enter lesions and can be taken up by macrophages, which can generate cholesterol ester-rich remnants via LPL. Therefore, while LPL has beneficial effects in the periphery165, its expression in lesions seems pro-atherogenic by production of cholesterol ester-rich lipoproteins, which in turn can be oxidized and taken up by macrophages166.

In addition to their role in local lipid metabolism in the arterial wall, macrophages are involved in modulating immune responses. They express several pattern recognition receptors (PRRs) by which they can sense PAMPs or DAMPs in the lesions. This results in their activation and production of pro-inflammatory cytokines. TLR4 in particular has been implicated to induce pro-inflammatory cytokine production in lesional macrophages167,168. Moreover, they can present antigens derived from the recognized PAMPs/DAMPs on their major histocompatibility complex (MHC) I and MHCII molecules and interact with memory/effector CD8+ T cells and CD4+ T cells, respectively25. Additionally, foam cells were found to express CD1d169, which allows them to interact with NKT cells170.

Macrophages are influenced by their microenvironment: inflammatory mediators and microbial products can modulate macrophage phenotype. Naïve macrophages (M0) are unpolarized macrophages generated by M-CSF, which can then be polarized based on the cues from their environment. However, it has to be acknowledged that macrophage phenotypes are only a snap-shot of a current situation and are a simplification of a continuum of different functions that macrophages can adopt. In fact, macrophages have been shown to be able to re-polarize under certain micro-environmental circumstances171-174. This, in addition to the fact that many subsets express similar markers, makes it difficult to assess the specific role of each macrophage subset in atherosclerosis (Figure 2). Moreover, the similarity of macrophages and DCs, especially upon hypercholesterolemia, has to be noted175.
Nonetheless, current studies suggest that macrophages in early lesions are of an M2 phenotype and switch to an M1 phenotype as lesions progress. Moreover, M1 macrophages were found to localize to rupture-prone regions, while M2 macrophages are located in the adventitia. Induction of an M2 phenotype by Schistosoma mansoni eggs was found to reduce atherosclerotic lesion development, further suggesting an anti-atherogenic role of M2 and a pro-atherogenic role of M1 macrophages.

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

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

2.3.3 Mox, Mha and M4 Macrophages
As mentioned, the microenvironment is crucial in determining the macrophage phenotype. Therefore, it has been investigated whether the atherosclerotic...
environment can result in specific macrophage subsets. Indeed, oxidized phospholipids present in the arterial wall or oxidized red blood cells, associated with hemorrhages in lesions, can influence macrophage phenotype. Both Mox macrophages (induced by oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine, OxPAPC\(^{171}\)) and Mha macrophages (resulting from intraplaque haemorrhage\(^{182}\)) show increased expression of heme oxygenase-1 (HO-1), vascular endothelial growth factor (VEGF) and IL-10. It is likely that these macrophages function to protect other cells from...
oxidative stress by their production of anti-oxidizing enzymes and help to suppress other immune cells by their production of anti-inflammatory mediators. Interestingly Mox macrophages were shown to have a significantly different gene expression than M1 and M2 macrophages and only showed approximately 26% and 12% similar genes expressed, respectively. This suggests that oxLDL does induce a distinct gene signature in macrophages. The exact contribution of these macrophages to the atherosclerotic disease process however still remains elusive. CXCL4, deposited by platelets onto the inflamed endothelium, plays a role in recruiting monocytes to the atherosclerotic lesion\textsuperscript{183}. The deficiency of CXCL4 has been found to result in reduced atherosclerotic lesions\textsuperscript{184}, suggesting a pro-atherogenic effect of this chemokine. Recently, it was shown that CXCL4 can directly affect the macrophage phenotype by inducing a robust expression of CD86, ABCG1, CCL18, CCL22, while inhibiting expression of CD36, HO-1 and CD163 expression\textsuperscript{185,186}. M4 macrophages show reduced cholesterol uptake and increased efflux, culminating in decreased foam cell formation. However, whether this has any implications for atherosclerosis has yet to be established\textsuperscript{186}. Mha and M4 macrophage phenotypes are specifically defined by factors in the microenvironment that induce distinct functions. However, if and to what extend they also show characteristics of M1 or M2 macrophages, and could thus be classified as specialized M1/M2 macrophages remains to be established.

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

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

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

### 2.4 Dendritic Cells

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

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

Certain DCs in peripheral tissues, e.g. Langerhans cells in the skin and intestinal lamina propria DCs, can be derived from monocytes. Additionally, under inflammatory conditions, monocytes can differentiate into DCs. For example monocytes can differentiate into Tip-DCs (TNF-α/iNOS-producing DCs) which show a strong anti-microbial defense\textsuperscript{197}. MoDCs, which are of myeloid origin, can adapt multiple different phenotypes depending on cues from the microenvironment. MoDCs are in general recognized by a high expression of CD11c and MHCII molecules, and can express other markers, such as CD11b, F4/80, CD103, DEC-205. Most of these markers, including CD11c and MHCII, are however also expressed by macrophages and monocytes, especially under inflammatory and hyperlipidemic conditions, making it extremely difficult to identify moDCs. Recently other markers, such as Flt3, c-kit, and CD26 have been suggested to be exclusively expressed by DCs and not...
macrophages. Additionally, the zinc finger transcription factor Zbtb46 was recently identified only in cDCs and not pDCs or monocytes. pDCs in contrast to other DC subsets express low levels of CD11c and MHCII and undergo terminal differentiation in the bone marrow, while other DC subsets do so in the periphery. They are poor APCs in steady state, but respond with strong type I interferon responses to pathogen recognition and then become fully capable of antigen presentation.

2.4.1 DC presence in healthy and diseased aorta

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

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

To establish the contribution of DCs to atherosclerosis, the CD11c-DTR LDLr-/- mouse model was used to deplete DCs and a 55% decrease of lesion development was found. Conversely, increased DC numbers due to an expanded lifespan of DCs (CD11c-specific expression of anti-apoptotic hBcl-2) resulted in increased numbers of Th1 and Th17 cells, and increased Th1-driven IgG2c autoantibodies, while interestingly lesion size was not affected. These studies indicate a pro-atherogenic role for DCs in atherosclerosis. However, it has to be noted that monocytes and macrophages also express CD11c under hypercholesterolemic conditions, making it difficult to determine whether the effects are due to a depletion and expanded lifespan of DCs only. Recently, Choi et al. established that at least two phenotypes of DCs exist in the intima of murine aortas: bona fide classical Flt3-Flt3L signaling-
dependent CD103+Langerin+CD11b+F4/80-CD8-CD205+CX3CR1+33D1+DCs and M-CSF-dependent CD14+CD11b+F4/80+DC-SIGN+TLR4+ monocyte-derived DCs. While both cells expand during atherosclerosis, the Flt3-Flt3L signaling-dependent DCs are most likely tolerogenic and protect from atherosclerosis by inducing Tregs, while M-CSF-dependent monocyte-derived DCs exacerbate atherosclerosis. CCL17-expressing DCs in atherosclerotic lesions were found to limit the induction of Tregs, while at the same time recruiting and activating CD4+ T cells, rendering them pro-atherogenic.

However, whether they are a unique subset or directly related to M-CSF-derived DCs remains to be established. Additionally, pDCs have been found to cluster with cDCs in shoulder regions of the lesions. Their production of IFN-α enhances TRAIL expression by CD4+ T cells (enabling an efficient killing of SMCs) and induces the production of pro-inflammatory cytokines in other APCs. As pDCs specifically express TLR7 and TLR9, they are equipped to recognize nucleotides or DNA from cellular debris in lesions. These studies suggest that different types of DCs that control T cell homeostasis exist in atherosclerotic lesions and that while some are resident DCs, others are derived from recruited monocytes.

The accumulation of moDCs in atherosclerotic lesions has been shown to critically depend on CX3CR1, indicating a possible role for Ly-6C low monocytes in lesional DC accumulation. Indeed, high cholesterol levels were found to increase CD11c expression on Ly-6C low monocytes, which was found to be crucial for monocyte adherence to endothelium (via VCAM-1 and E-Selectin). Hypercholesterolemia also results in an increased recruitment of pro-inflammatory Ly-6C hi monocytes, which can also give rise to DCs when exposed to granulocyte-macrophage colony-stimulating factor (GM-CSF), a growth factor inducing DC development. However, it has to be noted that increased GM-CSF-dependent DC proliferation in the lesions also persists, and even increases over time, when monocyte recruitment is inhibited. While it will need to be clarified to what amount moDCs contribute to lesional DCs, it is clear that GM-CSF is specifically needed to maintain lesional DCs, as GM-CSF deficiency results in a significant reduction of DCs in lesions without any effects on other monocyte-derived cells. A future challenge will be to decipher the relationship of various DC phenotypes, as well as their origin, in atherosclerotic lesions (Figure 3).

Furthermore, studies have demonstrated that under hypercholesterolemic conditions, DCs are impaired in their migration towards draining lymph nodes, as a result of inhibitory signals from platelet-activating factor (PAF) or oxLDL that acts as a PAF mimetic. This appears to be due to impaired CCR7 upregulation, as in the aorta transplantation model for regression (transplantation of atherosclerotic aorta into wild type mice) emigration of DCs was strongly dependent on CCR7, which was also shown to be involved in DC emigration from the skin. Additionally statins were shown to promote lesion regression via activation of CCR7-dependent emigration of cells from lesions. However reconstitution of ApoE expression in ApoE-/- mice, which induces regression in these mice, did not show an involvement of CCR7. Therefore, it still remains to be established if CCR7 is the main chemokine receptor involved, and if other factors besides high cholesterol levels determine DC emigration. Nonetheless,
despite their reduced capacity to emigrate from lesions, DCs still retain their capacity to activate T cells\textsuperscript{214}. Monocyte recruitment, local DC proliferation, and reduced DC emigration result in increased lesional DC content over time and eventually DCs can comprise up to 20% of lesion volume\textsuperscript{224}.

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

**2.4.2 DC function in steady state**

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

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

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

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

**2.4.3 DC maturation and immune responses in atherosclerosis**

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

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

DC-induced T cell activation in draining lymph nodes is crucially dependent on three signals of the DCs: 1) antigen presentation, 2) co-stimulatory molecule
expression, and 3) cytokine production by DCs (Figure 5). One of the most well-established and confirmed antigens in atherosclerosis is oxLDL. Interestingly, oxLDL results in the differentiation of monocytes to mature DCs that secrete IL-12, but does not result in the maturation of DCs\textsuperscript{245}. Other antigens such as β-2 glycoprotein I (β2GPI), HSP60 and HSP65 have been implicated as pro-atherogenic. OxLDL and HSP60 remain the most prominent antigens in humans and experimental models\textsuperscript{246}. Native LDL can also provide antigens for atherosclerosis development and it remains interesting that the immune response in atherosclerosis is directed towards components of native LDL\textsuperscript{247}, suggesting that fully oxidized LDL might not induce an immune response. MHCI molecules acquire peptides from intracellular antigens, which are generated by proteasomal processing, in the endoplasmatic reticulum; while MHCII molecules acquire peptides from extracellular antigens in the endosome. Some extracellular antigens can also escape into the cytosol and be degraded by the proteasome and then be presented on MHCI molecules by cross-presentation\textsuperscript{248}. The vital importance of MHCII-mediated antigen presentation to CD4\textsuperscript{+} T cells in atherosclerosis was shown in mice deficient in the invariant chain CD74, mediating antigen loading on MHCII, which show decreased atherosclerosis. This study assessed both initial and advanced lesions and showed that while deficiency of antigen presentation already reduced early lesions, it much more dramatically halted lesion progression. Advanced lesions showed significant reductions in activated T cells\textsuperscript{249}, further substantiating that antigen presentation and ensuing adaptive T cell responses critically promote atherosclerotic lesion progression. Moreover, a specific subset of MHCII molecules (IAb, expressed in atherosclerosis-susceptible C57BL/6 mice) was found to be essential for inducing Th1 responses\textsuperscript{250}. The identification of involved MHCII molecules will enable a more directed search for atherosclerosis-specific antigenic epitopes by binding prediction models, which has already resulted in the discovery of two peptide fragments\textsuperscript{251}.

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

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

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

Besides their function as APCs, DCs also engulf lipids (via micropinocytosis, endocytosis, scavenger receptors, and efferocytosis) and form foam cells, contributing to atherosclerotic lesion development\textsuperscript{161,209,269}. As they share phenotypic and functional properties with macrophages, which attain a DC-like phenotype upon foam cell formation\textsuperscript{211}, it has been difficult to dissect the role of DCs and macrophages in lipid uptake in atherosclerosis and the contribution of DCs to foam cell formation in atherosclerotic lesions. As discussed, the uptake of oxLDL results in DC maturation, enhanced antigen presentation to T cells and increased T cell proliferation\textsuperscript{245,270}. On the other hand, it was found that uptake of oxidized phospholipids by DCs can impair maturation upon CD40 and TLR2, TLR3 and TLR4 ligation\textsuperscript{271}; possibly providing a control for excessive DC activation\textsuperscript{271}. Therefore, it will be interesting to determine which components of oxLDL are responsible for both pro-inflammatory as well as anti-inflammatory responses and whether these responses correlate with macrophage function. As mentioned, in macrophages desmosterol was shown to induce an anti-inflammatory response\textsuperscript{162}. Additionally, oxysterols, which are found at high concentrations within oxLDL, have been found to activate LXR and similar to macrophages it has been found that LXR can induce an anti-inflammatory phenotype in DCs\textsuperscript{272}. As LXR can modulate DCs, it will also be interesting to determine if cholesterol efflux pathways exist in DCs.

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

2.4.5 DCs as a therapy for atherosclerosis

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

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

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

![Diagram of immune system]

**Figure 6. Different approaches for DC-based vaccines in cancer that can be potentially translated to the field of atherosclerosis.**

1. **Random DC targeting**
   - Peptides with adjuvants
   - Viral vectors
   - DNA vaccines
   - Transduced tumor cells

2. **Ex vivo generated cytokine-driven DCs**
   - Ex vivo instruction to generate appropriate cytotoxic effectors and helper T cells

3. **Targeting specific DC subsets in vivo**
   - Anti-DC antibody fused to pathogen and/or cancer antigens and DC activators

4. **Therapeutic vaccines**
   - i. Optimized DC-based vaccines
   - ii. Blockade of regulatory or suppressive pathways
   - iii. Breakdown of tumor environment

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

To generate immature tolerogenic DCs, the majority of studies have assessed the feasibility of biological factors (e.g. IL-10, 1,25-dihydroxyvitamin D3, TGF-β, M-CSF, or vasoactive intestinal peptide) or pharmacological drugs (e.g. dexamethasone, aspirin or rapamycin)\textsuperscript{199,280}. However, also pathogen-derived molecules, e.g. of *C. albicans*, or tumor-derived molecules, e.g. mucins, have been shown to induce...
immature tolerogenic DCs$^{280}$. Some studies have also used the approach of genetically modifying DCs, e.g. by knocking out suppressor of cytokine signaling 3 (SOCS3) or RelB$^{280}$. These DCs all show low co-stimulatory molecules, increased anti-inflammatory cytokine production, while pro-inflammatory cytokines are reduced, and can induce anergy in T cells and/or Tregs.

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

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

### 2.5 T cells

Both CD4$^+$ and CD8$^+$ T cells have been found in human atherosclerotic lesions, with the majority being CD4$^+$ T cells$^{289}$. T cell infiltration already occurs in early stages of lesion development, in a similar manner as monocyte recruitment$^{290}$. It was found that T cells present in lesions are in an activated state$^{291}$, with more than 60% of CD4$^+$ T cells being memory T cells$^{292}$. Recently, increased memory CD4$^+$ T cells and reduced naïve T cells were associated with coronary artery calcification and carotid artery intimal media thickness in subclinical atherosclerosis$^{293}$, indicating a pro-atherogenic role of these cells. The presence of such a high number of memory T cells suggests an antigen-specific response as these cells have previously encountered their cognate
antigen. Indeed, a fraction of CD4⁺ T cells isolated from human atherosclerotic lesions were found to be oxLDL-specific²⁹⁴.

The overall role of T cells was established in murine models where T cell depletion by anti-CD3 treatment resulted in a reduction of initial lesion development²⁹⁵, a reduction of lesion progression²⁹⁵, as well as increased lesion regression²⁹⁶. Additionally, nude mice (which are deficient in T cells) were found to have an almost complete (90%) reduction in lesion size compared to their heterozygote littermates²⁹⁷. Similarly, ApoE⁻/⁻ mice crossed to Severe combined immunodeficiency (SCID) mice (deficient in B and T cells)²⁹⁸, LDLr⁻/⁻ mice crossed with Rag1⁻/⁻ mice (no mature B and T cells)²⁹⁹,³⁰⁰, and ApoE⁻/⁻ mice crossed with Rag2⁻/⁻ mice show significant inhibition of atherosclerotic lesion development³⁰¹.

2.5.1 Cytotoxic T cells

CD8⁺ T cells (cytotoxic T cells) kill their targets by release of cytotoxins, such as granzymes and perforins, and expression of FasL. They have also been found to produce large amounts of IFN-γ upon activation, such as in atherosclerosis³⁰². Studies have shown a correlation between CD8⁺ T cells and coronary heart disease³⁰³,³⁰⁴ and up to 50% of lymphocytes found in human lesions are CD8⁺ T cells³⁰⁵. In animal models there is contradictory evidence for the role of CD8⁺ T cells. CD8⁺ T cells were found to be the first responders to high fat diet, upregulating their IFN-γ expression weeks before CD4⁺ T cells were activated³⁰⁶. In line with this, antibody-mediated CD8⁺ T cell depletion in ApoE⁻/⁻ mice improved atherosclerosis³⁰⁷, while adoptive transfer of CD8⁺ T cells into Rag-2⁻/⁻ApoE⁻/⁻ mice aggravated atherosclerosis³⁰⁷. In contrast, CD8⁻/⁻ mice backcrossed to ApoE⁻/⁻ mice showed no significant effects on lesion size⁶⁹. Other studies indicate an atheroprotective effect of CD8⁺ T cells, as MHCI deficient C57BL/6 mice show increased atherosclerosis³⁰⁸ and ApoB-100 peptide immunization mediates protective effects in ApoE⁻/⁻ mice via CD8⁺ T cells³⁰⁹. The reason for these observed differences could be due to the heterogeneity of CD8⁺ T cells. CD8⁺ T cells can be further subdivided into type 1 (Tc1) and type 2 (Tc2), with Tc1 being predominant in human lesions³¹⁰. A recent study found that adoptive transfer of the CD25-positive fraction of CD8⁺ cells, possibly including regulatory CD8⁺ T cells³¹¹, reduced atherosclerotic lesion development in ApoE⁻/⁻ mice³¹². This indicates that indeed different subsets of CD8⁺ T cells likely have distinct functions in atherosclerosis. It is therefore of significant interest to further dissect the role of different CD8⁺ T cell subsets in atherosclerosis.

2.5.2 Helper T cells

The crucial role of CD4⁺ T cells (T helper cells) in atherosclerosis has been established in several mouse models. CD4⁺ T cell depletion was shown to reduce lesions by 70% in C57BL/6 mice²⁹⁷. Adoptive transfer of CD4⁺ T cells into SCID mice dramatically increased lesions by 1.6-fold²⁹⁸. Moreover, MHCI transgenic mice (expressing other MHCI molecules than the IAb of athero-susceptible C57Bl/6 mice) were found to have a dramatically reduced atherosclerotic lesion development²⁵⁰, further confirming the crucial role of CD4⁺ T cells.
Interestingly, recent evidence suggests that the lipid rich environment in atherosclerotic lesions can directly modulate T cell function. Lysophosphatidylcholine, one of the main phospholipid components of oxLDL, can enhance the expression of CD40, CXCR4, and IFN-γ in human CD4<sup>+</sup> T cells, in the absence of APCs<sup>313</sup>. Moreover, increased membrane cholesterol was found to activate T cells, which were more prone to differentiate towards Th1 cells<sup>314</sup>. It will be interesting to establish how hypercholesterolemia affects T cell metabolism, and thereby T cell activation and proliferation. T cell deficiencies were also associated with a significant reduction of total cholesterol levels<sup>300</sup>, predominantly due to decreased VLDL, indicating that T cells might not only be affected by cholesterol levels, but can also directly affect these themselves.

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

### 2.5.2.1 Th1 cells

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

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

### 2.5.2.2 Th2 cells

Th2 cell numbers in atherosclerotic lesions is low, but gradually increases with disease progression<sup>316</sup>. When comparing atherosclerosis development in LDLr<sup>-/-</sup> mice on a C57BL/6 (Th1 dominated) versus BALB/c (Th2 dominated) background, it was found that atherosclerosis development was significantly increased in Th1 biased mice, indicating that Th2 cells may be atheroprotective or at least do not contribute to the
same extent as Th1 cells to atherosclerosis development\textsuperscript{324}. Indeed, in humans it was found that Th2 cells were associated with a reduced risk of MI\textsuperscript{325}. However, in mice the role of Th2 cells has not been perfectly elucidated. Our laboratory showed that Th2 cells might actually promote atherosclerosis progression, as interference of the OX40-OX40L pathway was associated with a reduced amount of Th2 cells and reduced atherosclerotic lesion progression\textsuperscript{256}. Most studies have assessed the role of Th2 cells by studying the cytokines that induce Th2 cells (IL-4, IL-33) and the cytokines that Th2 cells produce themselves (IL-4, IL-5, IL-10 and IL-13). IL-4 induces GATA-binding protein 3 (GATA-3) which increases IL-4 expression of the T cells and inhibits IFN-γ\textsuperscript{226}.

IL-4 administration or deficiency was found to have no effect on early atherosclerosis in ApoE\textsuperscript{-/-} mice\textsuperscript{327}. While IL-4 deficiency in LDLr\textsuperscript{-/-} mice was also found to have no effect on early lesions in the aortic arch\textsuperscript{327}, the same group showed later on that transplantation of bone marrow from IL-4\textsuperscript{-/-} mice into LDLr\textsuperscript{-/-} mice resulted in reduced lesions in the aortic arch and the thoracic aorta, while aortic root lesions were not affected\textsuperscript{328}. This could be due to differences induced by the bone marrow transplantation, as aortic root lesions develop faster and thoracic aorta lesions develop slower after γ-irradiation\textsuperscript{129}. This already indicates that the role of Th2 cells seems to critically depend on the site of lesion development, the stage of the lesions and the animal model used.

Two other cytokines that Th2 cells produce are IL-5 and IL-13. IL-5 has been shown to be anti-atherogenic by promoting the development of B-1 cells that produce protective IgM antibodies, resulting in reduced atherosclerosis\textsuperscript{51}. Recently anti-IL-5 autoantibodies have been shown to be associated with human atherosclerosis, as antibody titers were much higher in patients with coronary and peripheral artery disease than in controls, and additionally correlated with well-known risk factors, such as plasma LDL-cholesterol\textsuperscript{330}. It was found that IL-33 can specifically induce IL-5 production in CD4\textsuperscript{+} T cells\textsuperscript{331,332} and that administration of IL-33 reduces atherosclerosis development in ApoE\textsuperscript{-/-} mice\textsuperscript{332}. In line with this, anti-OX40L treatment, which results in IL-5 producing T cells (via IL-33) and increased IgM levels results in reduced atherosclerotic lesion progression\textsuperscript{256}. IL-13 has also been shown to reduce atherosclerotic lesion development by skewing macrophages towards an M2 phenotype\textsuperscript{333}.

The different effects of IL-4, IL-5 and IL-13 on atherosclerosis indicate the difficulty of dissecting the role of Th2 cells in atherosclerosis. This is further complicated by the fact that the studied cytokines are not solely produced by Th2 cells and also affect other cell types. Moreover, innate lymphoid cells have also been found to express Gata-3\textsuperscript{334}. Overall, there is a necessity for specific markers and mouse models to establish the role of Th2 cells in atherosclerosis in more detail. The general consensus at the moment is however that Th2 cells are anti-atherogenic in early lesion development and pro-atherogenic in later stages of atherosclerosis.
2.5.2.3 Th17 cells
Th17 cells are found in both human and murine lesions. In mice, TGF-β and IL-6 results in the activation of signal transducer and activator of transcription (STAT)3 and subsequently retinoic acid-related orphan receptor γT (RORγT) and RORα, resulting in Th17 induction. IL-21 is required for Th17 proliferation, while IL-23 is necessary for their maintenance. In humans, IL-1β, IL-6 and IL-23 result in the induction of Th17 cells. Moreover, also the combination of IL-21 and TGF-β was found to induce Th17 cells in humans. Th17 cells produce IL-17A/F, IL-21 and IL-22. The effect of Th17 cells in atherosclerosis remains controversial. T cells isolated from human atherosclerotic coronary arteries were found to produce IFN-γ as well as IL-17 upon stimulation. Moreover, Th17 cells were found to be increased in unstable angina patients and increased IL-17 mRNA levels in unstable lesions were found. While both these findings indicate a pro-atherogenic role for Th17 cells, circulating IL-17A levels showed no association with carotid intima-media thickness in humans. As with Th2 cells, studies in mice have investigated the role of cytokines that are produced by Th17 cells. Blocking IL-17A in ApoE-/- mice was found to significantly reduce atherosclerosis. In line with this, both IL-17A-/-ApoE-/- mice and IL-17RA-/-ApoE-/- mice were found to have reduced atherosclerosis. However, other groups found that IL-17A deficiency in ApoE-/- mice had either no effect or resulted in significantly more atherosclerosis. Similar contradictory data exist for LDLr-/- mice. We have shown that transplantation of IL-17R-/- bone marrow into LDLr-/- mice resulted in significantly reduced atherosclerosis, indicating a pro-atherogenic role for Th17 cells. However, SOCS3-/-LDLr-/- mice, which have increased STAT3-mediated expression of IL-17A in T cells, were found to have a significant 50% reduction in atherosclerosis. Additionally, the same study showed that an IL-17A blocking antibody increased atherosclerosis, indicating a protective role for Th17 cells. Future studies will have to resolve this issue; again T cell specific knockout models should prove crucial to rule out the effect of other immune cells, such as innate lymphoid cells, NKT cells, NK cells and γδ T cells.

2.5.2.4 Regulatory T cells
Regulatory T cells (Tregs) are, as the name suggests, regulators of immune responses and their main function is thought to be the inhibition of self-reactive T cells in the periphery. Tregs that derive from the thymus are referred to as natural Tregs (FoxP3+CD25hi) and they produce IL-10 and TGF-β. The constitutive high expression of CD25 (the α-subunit of the IL-2 receptor) enables them to respond to physiologically low IL-2 levels. IL-2 is crucial for Treg generation and maintenance, and induces FoxP3, which controls key molecules expressed in Tregs, such as CD25, CTLA4, GITR and ICOS. Genetic defects in FoxP3 are the cause of a severe autoimmune disease in humans, the X-linked autoimmune syndrome (IPEX). Scurfy mice and FoxP3-null mice, both lacking Tregs, as well as neonatal thymectomy were all found to result in spontaneous autoimmune manifestations. Helios has recently been suggested to be a specific marker of natural Tregs. However, at least in humans, it was found that natural Tregs do not all express Helios and in mice Helios has been also found to be
expressed in Th2 cells\(^\text{352}\). Additionally, neuropilin 1 has been suggested as a marker for natural Tregs in mice\(^\text{353}\). However, in humans neuropilin 1 was found to be less specific as it could also be detected on other T cells in secondary lymphoid organs and its expression could be induced in vitro upon activation of T cells\(^\text{354}\). Tregs can also be induced from naïve T cells, by ligation of the T cell receptor (TCR), CD28 ligation and/or TGF-β and IL-2, IL-10 or retinoic acid\(^\text{326,355}\) and these are thus referred to as inducible Tregs (iTregs). These are further subdivided into Tr1 (mainly IL-10-producing and induced by IL-10) and Th3 cells (mainly TGF-β-producing and induced by TGF-β). iTregs also express CD25, but do not need FoxP3 expression to be functional\(^\text{326}\). So far, no exclusive markers for these cells exist making it difficult to dissect their specific roles in immune responses and atherosclerosis. Currently, the distinction between natural Tregs and iTregs can only be derived from the methylation profile of the FoxP3 promoter\(^\text{356}\).

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

Tregs can suppress numerous immune cells, such as (naïve, effector, and memory) T cells, B cells, monocytes, macrophages, DCs, NK cells, and NKT cells. Treg production of IL-10 and TGF-β plays a crucial role in inhibiting immune responses, most likely through the inhibition of APC function and direct inhibition of T cell proliferation\(^\text{346}\). Their production of TGF-β can e.g. induce apoptosis in activated T cells\(^\text{363}\). Treg suppression requires cellular interactions and results in reduced proliferation of target T cells\(^\text{346}\). Addition of IL-2 can overcome Treg suppression, suggesting that limiting IL-2 is crucial for the T cell suppression\(^\text{364}\). As IL-2 is crucial for Treg maintenance and function, the current understanding is that Tregs sequester IL-2 produced by target T cells\(^\text{355}\). Another molecule that has been shown to be involved in the suppression of target T cells is CTLA-4. CTLA-4 can bind CD80 and CD86 on either APCs or target T cells and induce a suppressive signal in these cells or prevent binding of CD28 and activation via this molecule\(^\text{365}\). Tregs can also exert direct cytotoxicity by their
expression of granzymes, which can kill activated CD4+ and CD8+ T cells, monocytes, and B cells. Tregs were also found to suppress CCL2 production, resulting in reduced recruitment of Ly-6C+ monocytes. Tregs are capable of suppressing immune responses via multiple mechanisms and which mechanism is predominant likely depends on the microenvironment and the presence of antigens and APCs.

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

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

atherosclerotic lesion development in ApoE−/− mice. Reconstitution of LDLr−/− mice with bone marrow from depletion of regulatory T cell (DEREG) mice (expressing DTR under the control of FoxP3) and depletion of Tregs by diphtheria toxin injection were found to significantly increase atherosclerotic lesions373,374. Interestingly also increased VLDL and plasma cholesterol levels were found in these mice indicating a link between Tregs and cholesterol homeostasis374. Our laboratory has shown that vaccination against FoxP3, resulting in the depletion of Tregs, significantly increases atherosclerotic lesion development375. The opposite approach to increase Tregs by adoptive transfer also dramatically decreased atherosclerosis258,371,376. Moreover, treatment of LDLr−/− mice with an IL-2/anti-IL-2 antibody complex, which induces Treg expansion, dramatically reduced lesion development377. It has recently been shown that the presence of CD103+ DCs217 and MyD88+ DCs368 is needed within aortas to maintain functional Tregs and that loss of these DCs results in increased atherosclerosis. All of these studies clearly demonstrate a protective role of Tregs. Additionally, a surfeit of studies that used approaches to induce/expand Tregs have all been shown to be able to reduce atherosclerosis, such as oral tolerance induction378,379, anti-CD3 antibody treatment380, and tolerogenic DC therapy288. Interestingly, statins were also shown to increase Tregs373. Overall, Treg expansion has been shown to be a feasible goal of any immune therapy to induce tolerance.

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

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

In chapter 3, we determined whether enhanced β-catenin signaling in DCs, shown to induce a tolerogenic phenotype, can reduce atherosclerosis. We generated CD11c-βcatEX3/LDLr+/− bone marrow chimeras to assess the effect of overall stabilization of β-catenin in DCs and also adoptively transferred DCs from CD11c-βcatEX3 mice into LDLr+/− mice. CD11c-βcatEX3 mice have a CD11c-promoter-driven Cre recombinase and the exon 3 of the β-catenin gene floxed, which results in excision of the sequence encoding the ubiquitination site needed for degradation of β-catenin. We show that these DCs result in the induction of Tregs and reduce overall inflammation in mice, indicating that strategies to enhance β-catenin signaling in DCs are feasible to reduce atherosclerotic lesion development.

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

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

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

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

Overall we provide strategies to decrease inflammatory monocyte responses, enhance anti-inflammatory cytokine production by macrophages, increase the presence of tolerogenic DCs and increase the amount of Tregs. Moreover, we show that adoptive transfer of tolerogenic DCs and MSCs may also prove beneficial in treating atherosclerosis. Interestingly, while our strategies aimed to modulate immune responses in atherosclerosis, we observe that MSC therapy and proteasomal inhibition
dramatically affect VLDL metabolism. This as well as other conclusions that can be drawn from our studies for future immune therapies will be discussed in Chapter 8.

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

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