# CONVENTIONAL DENDRITIC CELLS IN ATHEROSCLEROSIS: FROM PATHOGENIC PLAYERS TO TARGETS FOR THERAPY?

Proefschrift voorgelegd tot het behalen van de
graad van doctor in de Farmaceutische Wetenschappen
aan de Universiteit Antwerpen te verdedigen door

MICHE ROMBOUTS

**COLOFON**

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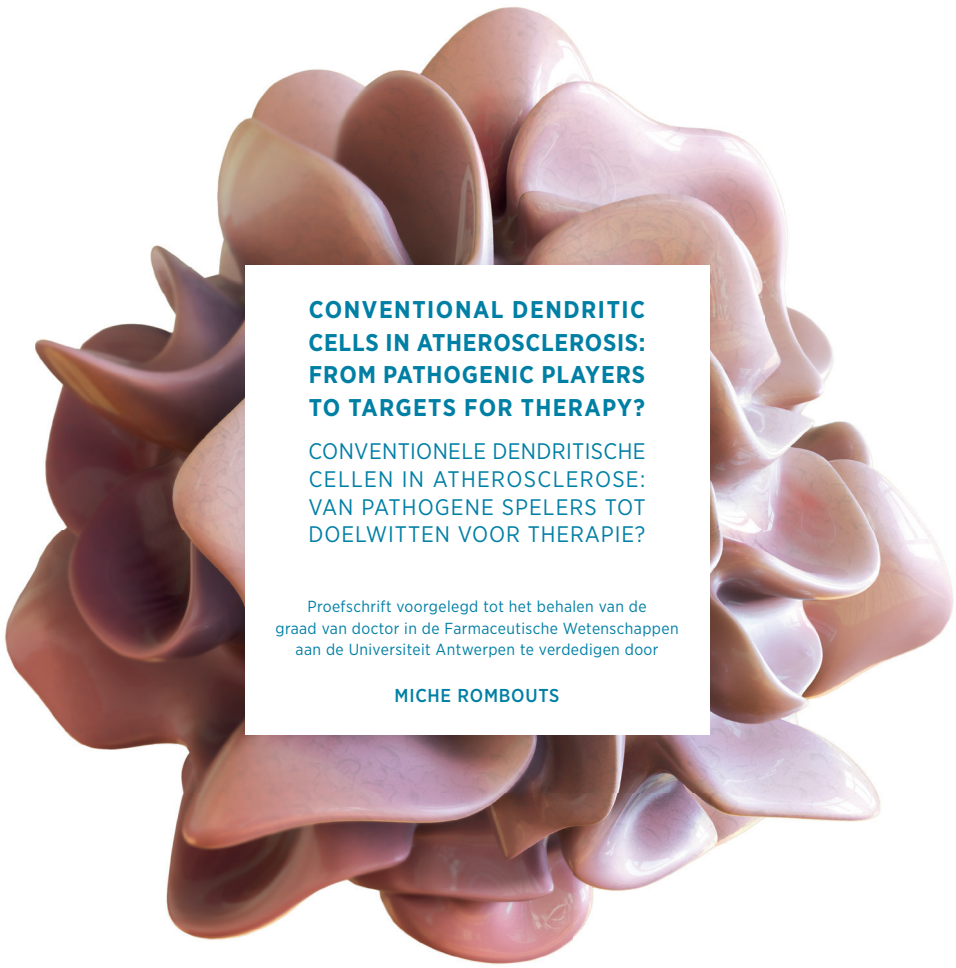

# CONVENTIONAL DENDRITIC CELLS IN ATHEROSCLEROSIS: FROM PATHOGENIC PLAYERS TO TARGETS FOR THERAPY?

## CONVENTIONELE DENDRITISCHE CELLEN IN ATHEROSCLEROSE: VAN PATHOGENE SPELERS TOT DOELWITTEN VOOR THERAPIE?

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Farmaceutische Wetenschappen aan de Universiteit Antwerpen te verdedigen door

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**“THE SCARIEST MOMENT IS ALWAYS JUST BEFORE YOU START. AFTER THAT, THINGS CAN ONLY GET BETTER.”**

~ STEPHEN KING

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α-GalCer | α-galactosylceramide |
| APCs | antigen-presenting cells |
| ApoE | apolipoprotein E |
| ASA | acetylsalicylic acid |
| BDCA | blood dendritic cell antigen |
| BMDCs | bone marrow-derived dendritic cells |
| BMT | bone marrow transplantation |
| CAD | coronary artery disease |
| CCL | CC chemokine ligand |
| CCR | CC chemokine receptor |
| CD | cluster of differentiation |
| cDCs | conventional dendritic cells |
| CDP | common dendritic cell progenitor |
| CLP | common lymphoid progenitor |
| CMP | common myeloid progenitor |
| cMoP | common monocyte precursor |
| CTL | cytotoxic T lymphocytes |
| CVD | cardiovascular disease |
| $CX_3CR1$ | CX3C motif chemokine receptor 1 |
| DAMP | damage-associated molecular pattern |
| DCs | dendritic cells |
| DT(R) | diphtheria toxin (receptor) |
| EC | endothelial cell |
| Flt3(L) | FMS-like tyrosine kinase 3 (ligand) |
| FoxP3 | forkhead box P3 |
| FSC | forward scatter |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| HB-EGF | heparin-binding epidermal growth factor-like growth factor (or DTR) |
| hs-CRP | high sensitivity C-reactive protein |
| HDL | high-density lipoprotein |
| H-E | haematoxylin-eosin |
| HSP | heat shock proteins |
| iDCs | immature dendritic cells |
| IFN-γ | interferon-γ |
| IL | interleukin |
| IDO | indoleamine 2,3-dioxygenase |
| iNOS | inducible nitric oxide synthase |
| LCs | Langerhans cells |
| LDL(r) | low-density lipoprotein (receptor) |
| LMPP | lymphoid-primed multipotent progenitor |
| LN | lymph nodes |
| LPS | lipopolysaccharide |
| MCP-1 | monocyte chemoattractant protein-1 |
| M-CSF | macrophage colony-stimulating factor |
| MDA | malondialdehyde |
| mDCs | mature dendritic cells |
| MDP | common macrophage-DC progenitor |
| Mφ | macrophage |
| MHC | major histocompatibility complex |
| MI | myocardial infarction |
| Mo | monocyte |
| moDCs | monocyte-derived dendritic cells |
| MyD88 | myeloid differentiation factor 88 |
| NF-κB | nuclear factor-κB |
| NKT | natural killer T |
| oxLDL | oxidized low-density lipoproteins |
| PAMP | pathogen-associated molecular pattern |
| PBMCs | peripheral blood mononuclear cells |
| PCs | principal components |
| PCA | principal component analysis |
| pDCs | plasmacytoid dendritic cells |
| PDCA-1 | plasmacytoid dendritic cell antigen-1 |
| PD-L | programmed death-ligand |
| PPIA | peptidylprolyl isomerase A |
| PRR | pattern recognition receptor |

| | |
|---|---|
| RA | rheumatoid arthritis |
| SiglecH | sialic acid binding Ig-like lectin H |
| siRNA | small interfering ribonucleic acid |
| SIRPα | signal regulatory protein α |
| SMC | smooth muscle cell |
| SR | scavenger receptor |
| SSC | side scatter |
| T-bet | T-box transcription factor expressed in T cells |
| TCR | T cell receptor |
| TGF-β | transforming growth factor-β |
| Th | T helper cell |
| TipDCs | TNF-α/iNOS-producing dendritic cells |
| TLR | toll-like receptor |
| TNF-α | tumor necrosis factor-α |
| tolDCs | tolerogenic dendritic cells |
| TRAIL | tumor necrosis factor-related apoptosis inducing ligand |
| Tregs | regulatory T cells |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VLDL | very low-density lipoprotein |
| WD | Western-type diet |
| XCR1 | chemokine XC receptor 1 |
| Zbtb46 | zinc finger and BTB domain containing transcription factor 46 |

## SCIENTIFIC CURRICULUM VITAE


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**Rombouts M***, Nuyts AH*, Derdelinckx J, Peeters K, Van Camp K, De Meyer GR, Goossens H, Berneman ZN, Van Brussel I, Schrijvers DM**, Cools N**. Silencing RNA as a tool to overcome the Th1-polarizing capacity of mature dendritic cells. *(manuscript submitted)*

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Vermeulen Z, Hervent A, Vandekerckhove L, **Rombouts M**, Beyens M, Dugaucquier L, Schrijvers DM, De Meyer GR, Maudsley S, De Keulenaer GW, Segers V. The neuregulin-1/ErbB4 pathway counterbalances inflammation and fibrogenesis in heart, skin and lung. *(manuscript in preparation)*

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## ABSTRACTS FOR ORAL PRESENTATION

**Rombouts M**, Cools N, Schrijvers DM. Conventional dendritic cells in atherosclerosis: from pathogenic players to targets for therapy. Research Day of the Department of Pharmaceutical Sciences, Antwerp, Belgium, 2016.

**Rombouts M**, Van Brussel I, Gorrebeeck S, Ruyssers NE, Heylen M, De Winder BY, Cools N, Schrijvers DM. Effects of immunosuppression on allogeneic T cell activation by mature conventional dendritic cells. Autumn Meeting Belgian Society of Fundamental and Clinical Physiology and Pharmacology, Brussels, Belgium, 2013.

## ABSTRACTS FOR POSTER PRESENTATION

**Rombouts M**, Nuyts AH, Derdelinckx J, Peeters K, Van Camp K, De Meyer GR, Goossens H, Berneman ZN, Van Brussel I, Schrijvers DM and Cools N. Silencing RNA as a tool to overcome the Th1-polarizing capacity of mature dendritic cells. 30th Annual Conference of the European Macrophage and Dendritic Cell Society (EMDS), Amsterdam, The Netherlands, 2016.

**Rombouts M**, de Bakker F, Van Brussel I, Grootaert MO, Wouters A, Staelens S, De Meyer GR, De Winter BY, Cools N, Schrijvers DM. Depletion of conventional dendritic cells in atherosclerosis using the Zbtb46-DTR mouse model. 22nd Annual Scandinavian Atherosclerosis Conference, Humlebæk, Denmark, 2016.

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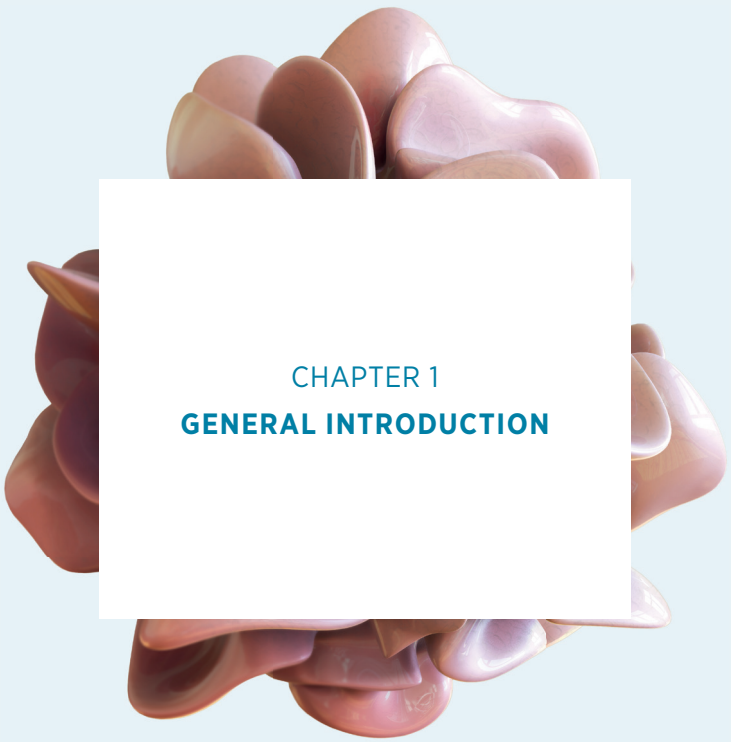

CHAPTER 1

# GENERAL INTRODUCTION

## CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a general term that describes a range of conditions that affect the heart or circulation. It includes numerous problems, of which many are related to the process of atherosclerosis, also known as the gradual build-up of plaques within vessel walls (Figure 1.1).[1-3] This build-up narrows the arteries (stenosis), making it harder for blood to flow through. Rupture of the plaque surface may cause formation of a thrombus, the main cause of ischemic events in both coronary and brain vessels.[4,5] The most important behavioural risk factors that facilitate atherosclerosis are unhealthy diet, physical inactivity, smoking and harmful use of alcohol, resulting in increased blood pressure, elevated blood glucose and lipids (mainly triglycerides and low density lipoproteins), overweight and obesity. Additionally, genetic predisposition, prior infections, underlying auto-immune diseases and aging were shown to substantially increase the risk for CVD.[1-3]

The probability at birth of eventually dying of major CVD is 47%.[1] Even more, it was estimated that 17.5 million people died from CVD in 2013, representing 31% of all global deaths.[1,6] Also among Europeans, CVD is responsible for 45% of all deaths.[7] In Belgium, heart disease is still the leading cause of death among women. In addition, the disease is associated with a very high cost for the Belgian health care system, including the high price of treatments before and after a myocardial infarction (MI) or stroke (EUR 3.5 billion in 2008).[8] In industrialized countries, improved survival after MI has led to a decrease in mortality from CVD. However, as more individuals become at risk of developing CVD because of an aging population, the prevalence and consequently the economic burden of CVD are likely to increase.[9] Hence, there is a high unmet need for continued research for novel and enhanced therapeutic strategies for atherosclerosis, the main underlying cause of CVD.

## ATHEROSCLEROSIS

Since the demonstration of the role of cholesterol in the development of atherosclerosis, the classical concept of atherosclerosis as a disorder of lipid metabolism and accumulation has gained wide acceptance.[10] However, from 1958 onwards, histological signs of inflammation in early atherosclerotic lesions have been observed, both in animals and humans, suggesting a pathophysiology that is much more complex than mere lipid storage.[11-13] Until recently, the cellular composition, including detection of different types of immune cells within atherosclerotic plaques was limited to immunohistochemical methods.[14] The development of flow cytometry, in which a panel of markers enables unequivocal positive

or negative detection of various cell types, helped to further define the cellular infiltrate in atherosclerotic lesions and paved the way for studies determining the origin, lineage, phenotype, and function of distinct inflammatory cells involved in plaque development.[14,15]

Atherosclerosis develops from early ages (Figure 1.1). Even coronary arteries of 6 month old children already contain small amounts of macrophages and macrophages filled with lipid droplets.[16] The earliest atheromas, which emerge in some adolescents and young adults, can either remain asymptomatic throughout an entire lifetime or can develop into larger lesions which will ultimately result in vascular remodelling, flow-limiting stenosis, and possibly acute complications caused by thrombus formation.

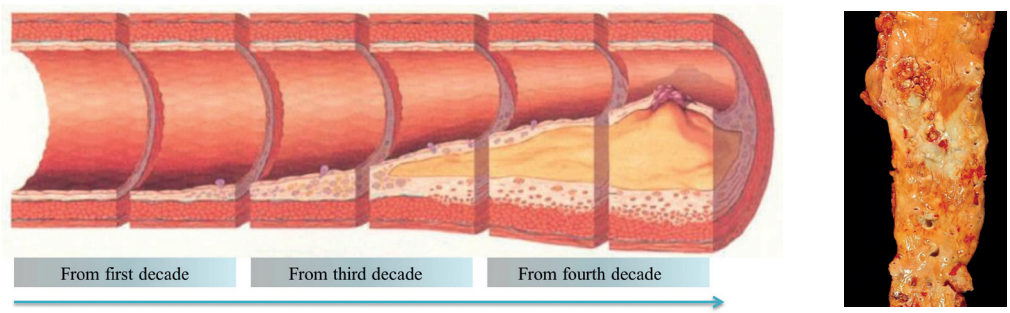


**FIGURE 1.1. Left**: Atherosclerosis timeline, showing the progression of atherosclerosis from initial lesions to complicated lesions. Adapted from[17]. **Right**: Autopsy specimen of aorta opened lengthwise to reveal luminal surface studded with lesions of atherosclerosis. From the Public Health Image Library.[18]

## Atherosclerotic plaque initiation and development

The emergence of atherosclerotic lesions at very specific sites of the vasculature is inherent to the arterial geometry and closely linked to local hemodynamic factors.[19,20] Before development of any lesions, these sites are characterized by changes in endothelial turnover, altered gene expression and presence of dendritic cells (DCs).[19-21] Endothelial dysfunction, characterized by a reduction in the bioavailability of nitric oxide, is a key event in atherogenesis and occurs at branching points and curvatures of the arterial tree where the blood flow pattern is disturbed, and wall shear stress is low or oscillatory.[22] In addition, harmful stimuli such as hyperlipidaemia, hypertension, smoking or pro-inflammatory mediators, are associated with enhanced endothelial dysfunction.[19,20,23-25] Changes in endothelial cell (EC) morphology and permeability promote the entry and retention of cholesterol-containing low-density lipoproteins (LDL) (Figure 1.2A).[23,24,26] Subsequent modifications of trapped LDL by oxidation or aggregation induce ECs and smooth muscle cells (SMCs)

to increase their expression of adhesion molecules (e.g. vascular cell adhesion molecule-1; VCAM-1), chemo-attractants (e.g. monocyte chemoattractant protein-1; MCP-1), and growth factors (e.g. macrophage colony-stimulating factor; M-CSF) (Figure 1.2B).[20,25,27,28] Whereas VCAM-1 mitigates the binding of leukocytes to the intimal surface, separate signals provided by chemokine receptors/chemokines (such as CC chemokine receptor (CCR) 2/ CC chemokine ligand (CCL) 2, $CX_3C$-chemokine receptor 1 ($CX_3CR1$)/$CX_3CL1$ and CCR5/CCL5) promote transmigration of bound leukocytes into the vessel wall.[29,30]

At this stage, the atherosclerotic lesions are referred to as fatty streaks.[20] These fatty streaks can either remain asymptomatic or they can develop into progressive atherosclerotic lesions. Nowadays it has become widely accepted that chronic inflammation of the arterial intima is the principal driving force behind the development of atherosclerosis.[31] This inflammatory response is initially considered to be protective, aiming to clear the arterial wall of potentially harmful antigens (e.g. damaged and modified self-structures). In a time span of years, a chronic state of inflammation turns this initially protective reaction into a damaging process inside the arterial wall.[31,32]

In the intima, exposure of monocytes to M-CSF results in their differentiation into macrophages.[33] In addition, M-CSF elicits the expression of scavenger receptors (SRs) on macrophages that mediate the uptake of modified LDL particles.[34] Cholesterol, derived from these ingested particles, will accumulate as cytolytic droplets, resulting in the formation of foam cells. In addition, monocyte-derived (mo)DCs were also shown to contribute to foam cell formation (Figure 1.2C).[35] Similar to monocytes, T cells also penetrate the intima where they recognize modified LDL and other autoantigens related to the atherosclerotic process presented by macrophages and DCs, and participate in the development of lesions (Figure 1.2C).[36] T helper type 1 (Th1) cells are among the earliest cells to accumulate in atherosclerotic lesions where they secrete proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) which in turn stimulate macrophages as well as ECs and SMCs.[33,37,38]

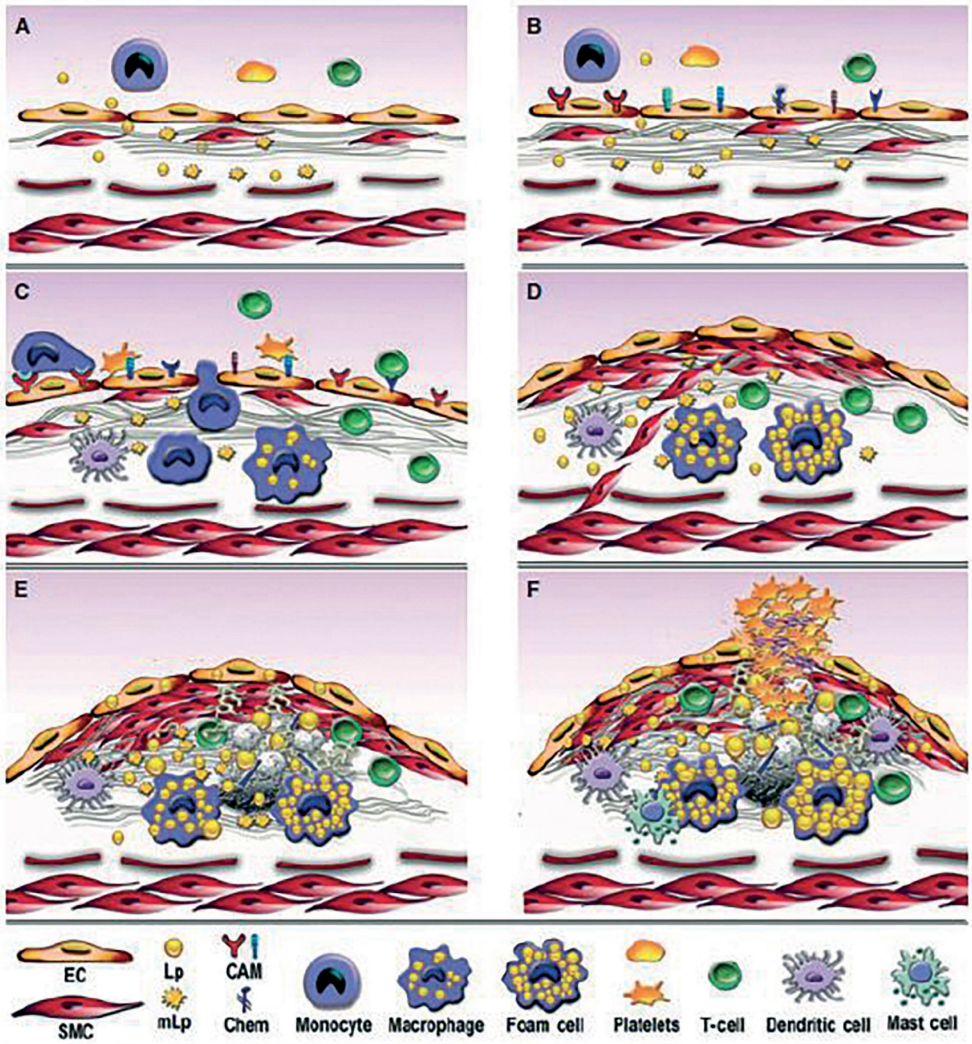


**FIGURE 1.2. Inflammation is implicated in all stages of atherosclerotic lesion development.** (A) Entry of lipoproteins (Lp) and their housing in the intima where they become modified (m)Lp. (B) ECs express cell adhesion molecules (CAM), cytokines and chemokines (Chem). (C) Recruitment of blood immune cells to the intima. Monocytes differentiate into macrophages which take up mLp and form foam cells. DCs contribute to T cell recruitment and activation in the plaque. (D) Migration and proliferation of SMCs leads to the formation of a fibrous cap that is accompanied by increased synthesis of extracellular matrix components. (E) Dead foam cells contribute to the formation of a necrotic core. (F) EC apoptosis and erosion, thinning of the fibrous cap, macrophage- and DC-secreted pro-inflammatory mediators generate rupture of the plaque. Contact between tissue factors, platelets and blood coagulation components trigger thrombus formation that may partially or totally impede the blood flow. Adapted from[39].

### Advanced atherosclerotic plaques

The transition from fatty streaks to advanced and more complicated lesions is marked by the formation of a thick fibrous cap, which surrounds the necrotic core. ECs, activated T cells, DCs and macrophages, present in the fatty streak, secrete inflammatory cytokines, fibrogenic mediators and growth factors promoting migration and proliferation of SMCs. These SMCs produce a dense extracellular matrix consisting of collagen and elastin fibres (Figure 1.2D).[33,40] Continuous accumulation of extracellular lipids, infiltration (and proliferation) of leukocytes, and production of extracellular matrix lead to expansion of the lesion and restructuring.[25,40] To compensate for the progressive luminal narrowing by growing lesions, the artery wall gradually dilates (remodelling). Thereafter, stenosis formation may occur through continued plaque growth which can cause ischemic symptoms (e.g. stable angina pectoris).[3,28,40]

Apoptosis of foam cells and SMCs contributes to the formation of a necrotic core in the lesion (Figure 1.2E).[20] Many factors, such as damaged and modified self-structures, that are capable of inducing apoptosis are present in plaques. When removal of apoptotic remnants (i.e. efferocytosis) by neighbouring phagocytes is impaired, they are left to undergo secondary necrosis, which contributes to the growth of the necrotic core and promotes further inflammation.[41,42]

It is now evident that plaque activation and stability rather than stenosis are more decisive for ischemia and infarction.[3,28] While a thick fibrous cap is essential for the stability of an atherosclerotic plaque, vulnerable lesions usually have a thin fibrous cap with low numbers of SMCs and reduced collagen, a relatively high concentration of lipid-filled macrophages within the shoulder region, and a large necrotic core.[43] Plaque inflammation appears to be a key factor in thinning of the fibrous cap. Activated T cells not only stimulate lesional macrophages to produce collagen-degrading proteolytic enzymes (e.g. matrix metalloproteinases), they also secrete IFN-γ that can halt collagen synthesis by the SMCs, rendering the plaque more rupture prone (Figure 1.2F).[25,28,43] This degradation process may be accompanied by the production of inflammatory cell-derived tissue factor which is a key contributor to plaque thrombogenicity. Inflammation has also been implicated in intraplaque neovascularisation and haemorrhage, common features of advanced lesions, which also influence plaque stability.[20,43] Upon rupture, the thrombogenic core of the plaque, enriched in tissue factor, becomes exposed to blood components, initiating the coagulation cascade and thrombosis formation (Figure 1.2F).[3,43] Thrombi can also be formed on lesions without rupture (plaque erosion), though the underlying mechanism

remains largely unknown.[20,43] The outcome of the thrombotic process is unpredictable. A major and life-threatening luminal thrombus can cause complete blockage of an artery and result in a fatal MI or stroke. More often, the evolution of advanced plaques may involve repetitive cycles of micro-haemorrhage and thrombosis. These micro-thrombi can travel via the blood flow and obstruct smaller arteries downstream in the heart (coronary heart disease), brain (ischemic stroke), or lower extremities (peripheral vascular disease).[3,20]

### Mouse models of atherosclerosis

Over the years, animal models have provided us with valuable insight into the mechanisms responsible for atherogenesis. Historically there has been a predominance of studies in monkeys, pigs and rabbits, because of their sensitivity to diet-induced hypercholesterolemia. The majority of contemporary atherosclerosis research, however, is dominated by the use of mouse models. Wild-type mice are relatively resistant to the development of atherosclerosis because of the lack of cholesteryl ester transfer protein. As a result, wild-type mice carry cholesterol mainly via high-density lipoproteins (HDL) and contain only very low concentrations of atherogenic LDL and very low-density lipoprotein (VLDL), which explains the need for genetically-modified mice.

The two most commonly used transgenic mouse strains mimicking atherosclerosis are apolipoprotein E-deficient ($ApoE^{-/-}$) and LDL receptor-deficient ($LDLr^{-/-}$) mice.[44] ApoE and the LDLr are both crucially involved in the clearance of chylomicrons and lipoproteins from the circulation. ApoE is found on chylomicrons and VLDL, which facilitates their uptake by the LDLr. Hence, $ApoE^{-/-}$ mice have VLDL dominated hyperlipidaemia, and readily develop lesions on normal chow diet, but more pronounced on a Western-type diet (WD).[44] Interestingly, ApoE also has other functions affecting macrophage biology and immune function.[45] The LDLr is expressed on liver cells and binds ApoE as well as ApoB (found on the outer layer of LDL particles) to clear lipoproteins from the circulation. $LDLr^{-/-}$ mice display minimal hypercholesterolemia on normal chow and require a WD for the development of significant lesions. In humans, familial hypercholesterolemia is caused by the absence of a functional LDLr.[44] The advantage of the $LDLr^{-/-}$ model is that it does not have the multitude of bystander effects as described for ApoE.[44,45]

By genetically manipulating the expression of specific gene products in $ApoE^{-/-}$ and $LDLr^{-/-}$ mice, the impact of several genes and signaling pathways on atherosclerosis has been explored. Alternatively, transplantations of bone marrow from other lines of mice carrying additional genetic alterations, can be used to increase our understanding of disease. The choice of

mouse model much depends on the experimental design of the atherosclerosis study, in particular when using bone marrow transplantation. While the LDLr phenotype of donor cells does not influence the size of atherosclerotic lesions when used to repopulate LDLr$^{-/-}$ mice, the transfer of bone marrow from a mouse expressing ApoE into an ApoE$^{-/-}$ recipient reduces atherosclerosis and plasma lipid levels.[44,46,47]

Although mouse models have become a convenient tool to evaluate the complex interactions in atherosclerosis, one needs to keep in mind that mice and humans differ in several parameters that may influence atherogenesis. In addition, the morphological characteristics and distribution of lesions is not identical. In humans, lesions occur more frequently in the coronary arteries, carotids, and peripheral vessels. Lesions in mice develop predominantly in the aortic root, aortic arch, and brachiocephalic artery. Nonetheless, many of the critical features of the atherosclerotic process are shared, and studies using mouse models have suggested biological processes and interactions that underlie atherosclerosis.[44] Interestingly, comparative genetics identified similar atherosclerosis-modifying genes that are important for atherosclerosis progression in both mice and men. Furthermore, mouse models are useful to study the mechanisms that induce vulnerable plaques such as necrotic core formation and fibrous tissue degradation.[48] Thus, mouse models have proven to be of value for translational research, especially when the primary goal of the research is to establish a mechanistic proof of concept or characterize the cause and effect pathways that link drug administration to therapeutic effect.

## THE IMMUNE SYSTEM IN ATHEROSCLEROSIS

Both innate and adaptive immune responses (Figure 1.3) greatly contribute to the development of atherosclerotic lesions as well as changes in lesion phenotype that lead to clinical cardiovascular events. Indeed, almost all immune cells have been found to either play a pro- or anti-atherogenic role. In general, cells of the innate immune system provide a first-line defence against many common microorganisms. However, they cannot always eliminate infectious organisms. Therefore, the cells of the adaptive immune system have evolved to provide a more versatile means of defence. In addition, adaptive immunity provides immunological memory after an initial response to a specific pathogen, leading to an enhanced response to subsequent reinfection with the same pathogen.[49] Importantly, activation of specialized antigen-presenting cells (APCs) is a necessary first step for the induction of adaptive immunity. Professional APCs such as monocytes, macrophages, B cells, and DCs, are able to activate a resting Th cell when the matching antigen is presented, although the role of monocytes per se as APCs is rarely considered.[50] Relative to other APCs, DCs are the most potent and adept at stimulating naive T cells. DCs also control the quality of the T cell response, driving naive lymphocytes into distinct classes of effectors.[51] Because of this unique ability we chose to focus on DCs. However, it is impossible to describe the function of DCs without spending some attention to their main effector cells, T cells. In addition, natural killer (NK) T cells, a unique subset of T cells that are located at the interface between innate and adaptive immunity (Figure 1.3), have been recently raising attention in atherosclerosis as lipid-responsive cells. Indeed, NKT cells recognize glycolipid antigens and are able to produce both pro- and anti-inflammatory cytokines upon activation. Because of these unique characteristics, NKT cells have recently been ascribed a role in the regulation of immunity and inflammation, including atherosclerosis.[52]

The role of DCs, T cells, and NKT cells will be further discussed in detail. However, other immune cells including B cells, mast cells, neutrophils, eosinophils, NK cells and γδ T cells have each been shown to be involved in the disease process in their own way. It is beyond the scope of this thesis to discuss all these cells but an extensive description is reviewed in literature.[49,53,54]

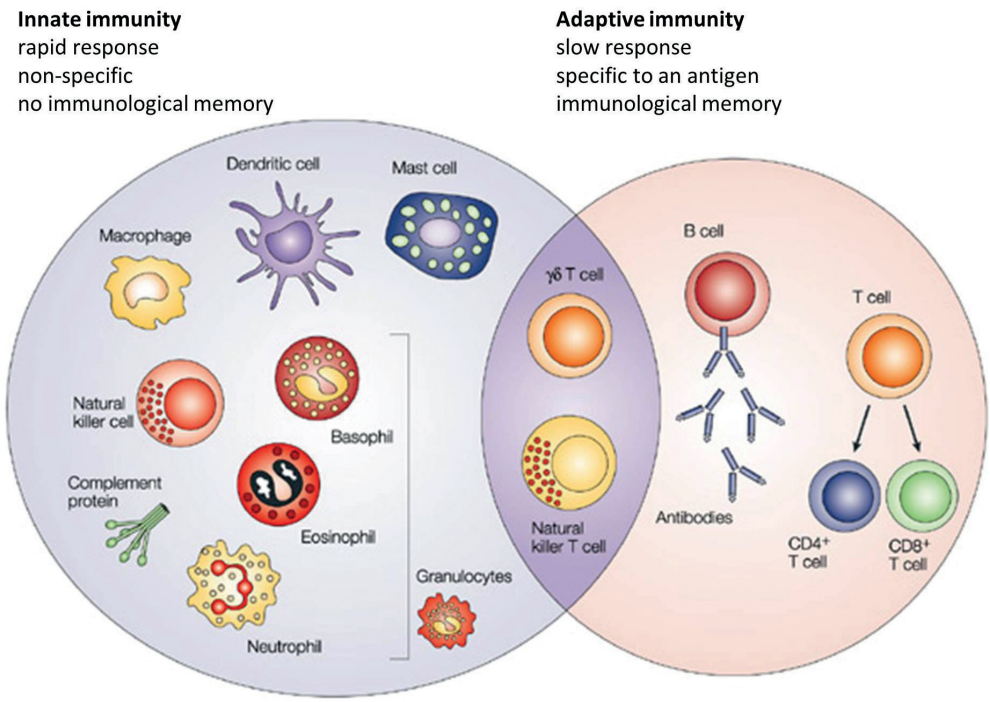


**FIGURE 1.3. Innate and adaptive cellular response.** The innate immune response functions as the first line of defense against infection and is characterized by rapid but poorly specific responses. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes, mast cells, macrophages, dendritic cells and natural killer cells. Adaptive immune responses are more delayed but manifest increased antigen specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T cells. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that are positioned at the interface between both systems. Adapted from[55].

## Dendritic cells

### *DC biology*

The discovery of DCs by Steinman and Cohn in 1973 led to decades of research into understanding DC biology.[56] For this discovery, Prof. dr. Steinman received the Nobel Prize in Physiology or Medicine in 2011. DCs comprise a heterogeneous population of professional APCs that have the unique ability to induce primary T cell responses. In doing so, they provide an essential link between innate and adaptive immune responses and permit establishment of immunological memory. DCs originate from a common $CD34^+$ progenitor in the bone marrow, from where they are released into the blood after differentiation into pre-DCs. Thereupon, they can be found in all lymphoid, and most non-lymphoid tissues

that are in contact with the external environment, where they relentlessly sample the antigenic content of their (micro)environment. In the steady state, immature (i)DCs capture harmless self-antigens in the absence of inflammatory signals. The antigenic sample is then degraded into short peptides that are loaded onto the DCs surface molecules CD1d or major histocompatibility complex (MHC) class I or MHC class II. iDCs might enter the regional lymph nodes or the spleen to present the self-antigen to naïve or resting T cells. Consequently, they can induce immunological tolerance by eliminating self-reactive naïve T cells by means of apoptosis (deletion), anergy (silencing), or induction of regulatory T cells (Tregs).[57,58] In contrast, any encounter with invading pathogens or other danger signals during infection or tissue damage induces a complex maturation process, including the initiation of migration to the lymphoid tissues. Besides downregulation of the endocytotic capacity, the maturation of DCs is further accompanied by an upregulation of the expression of costimulatory molecules (CD40, CD80/B7.1, CD86/B7.2), required for effective interaction with T cells, chemokine receptors (e.g. CCR7) and adhesion molecules (e.g. CD11a, CD50). Consequently, mature (m)DCs will efficiently trigger an immune response by (naïve) T and B cells that recognize the presented antigen.[58,59]

There are four main types of DCs, namely conventional DCs (**cDCs**), plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs) and Langerhans cells (LCs, DCs of the skin). However, recent data suggests that LCs may derive from yolk sac progenitors or from fetal liver–derived monocytes that take residence in the skin prior to birth, questioning if LCs truly belong to the DC family of cells.[60] In **mice**, cDCs subtypes can be divided into lymphoid tissue-resident cDCs and migratory (nonlymphoid tissue) cDCs (Table 1.1). Lymphoid tissue-resident cDCs are recognized as $CD8\alpha^+CD4^-CD11b^-CD11c^+$ cDCs, $CD8\alpha^-CD4^+CD11b^+CD11c^+$ cDCs, and $CD8\alpha^-CD4^-CD11c^+$ cDCs, which can either be CD11b positive or negative. $CD8\alpha^+$ DCs have the ability to efficiently process and load exogenously acquired antigens on MHC I molecules, referred to as cross-presentation, and a superior ability to prime $CD8^+$ T cells.[60] $CD4^+CD11b^+CD11c^+$ cDCs and $CD4^-CD11b^-CD11c^+$ cDCs efficiently induce $CD4^+$ T cell responses. cDCs in nonlymphoid tissues consist of two major subsets: $CD103^+CD11b^-$ DCs and $CD11b^+$ DCs. Lymphoid tissue $CD8\alpha^+$ DCs and nonlymphoid tissue $CD103^+CD11b^-$ cDCs have the same origin and share a similar phenotype and function. $CD103^+CD11b^-$ cDCs are capable of cross-presentation, but can also induce tolerance via the induction of Treg or Th2 responses. $CD11b^+$ DCs are found in most peripheral tissues and are thought to have a predominant role in MHC class II presentation and $CD4^+$ T cell priming. They mainly promote Th1 responses.[60]

TABLE 1.1. MARKERS USED FOR THE CHARACTERIZATION OF DC SUBTYPES.

| DC subtype | Subdivision according to localization | DC subsets | |
|---|---|---|---|
| | | Mouse | Human |
| cDCs | lymphoid tissue-resident | $CD8\alpha^+CD4^-CD11b^-$ | $CD141^+$ |
| | | $CD8\alpha^-CD4^+CD11b^+$ | $CD1c^+$ |
| | | $CD8\alpha^-CD4^-CD11b^{+or-}$ | $CD1c^+$ |
| | nonlymphoid tissues | $CD103^+CD11b^-$ | $CD141^+$ |
| | | $CD103^-CD11b^+$ | $CD1c^+$ |
| pDCs | | $PDCA\text{-}1^+SiglecH^+$ | $CD303^+CD304^+CD123^+$ |
| moDCs | | $CD11b^+CD64^+$ | $CD11b^+CD205^-CD64^+$ |
| | DC phenotype | Markers | |
| Activated DCs | costimulatory molecules | CD40 CD70 CD80 CD86 | |
| | activation molecules | CD83 | |
| | migratory molecules | CX3CR1 CCR5 CCR7 CCR9 | |
| | inhibitory molecules | PD-L1 PD-L2 IDO | |

cDCs, conventional DCs; pDCs, plasmacytoid DCs; IDO, Indoleamine 2,3-dioxygenase.

In **humans**, two cDCs subsets expressing the non-overlapping markers CD1c (BDCA-1) or CD141 (BDCA-3) are present in blood and lymphoid tissues (Table 1.1). Although a direct comparison between mouse and human cDCs subsets is hampered by the lack of CD8α expression on human DCs, it is believed that the two subsets share homology with mouse cDCs expressing either CD11b ($CD1c^+$ DCs) or CD8α/CD103 ($CD141^+$ DCs).[60,61] $CD141^+$ cDCs also uniquely express Clec9A and XCR1, and have superior cross-presenting potential compared with $CD1c^+$ DCs.[60]

**pDCs** represent a small subset of DCs that, unlike other DC subsets, undergo terminal differentiation in the bone marrow. pDCs express low levels of MHC II and are low (mouse) or negative (human) for CD11c. They are poor APCs but potent inducers of the IFN type I response upon recognition of viruses and bacteria through Toll-like receptors (TLR)7 and 9.[60] pDCs are further distinguished by the expression of murine SiglecH and Bst2 (PDCA-1), or human CD123, CD303 (BDCA-2) and CD304 (BDCA-4) (Table 1.1).[60,61]

DCs can also directly differentiate from monocytes, also known as **moDCs**.[59,60,62] The capacity of monocytes to behave as DC precursors was firstly evidenced by *in vitro* systems relying on the culture of monocytes with granulocyte–macrophage colony-stimulating factor (GM-CSF).[63] Later, experimental models have proven the differentiation of DCs from monocytes

*in vivo*, during inflammatory reactions[64], infections[65], and in the steady state[66]. Circulating Ly-6C$^{high}$ (inflammatory) monocytes in mice are considered to be the direct precursors of moDCs in tissues.[29] However, Ly-6C is quickly downregulated upon tissue entry, making it difficult to distinguish moDCs from tissue-resident CD11b$^+$ cDCs.[60] It was suggested that CD64 expression may distinguish the CD11b$^+$-type DCs from moDCs.[67] Furthermore, monocytes can differentiate into Tip-DCs (TNF-α/iNOS-producing DCs) which show a strong antimicrobial defense.[60,62] The capacity of cDCs to trigger specific immunity or tolerance has not been described for these inflammatory DCs, which are mainly involved in innate defense and in T cell activation.[62] Although it has been known for many years that monocytes have the potential to differentiate into DCs *in vitro*, the challenge remains to identify the *in vivo* counterpart of human moDCs. It was reported that human inflammatory exudates contain distinct inflammatory DC-like cells. Transcriptional profiling suggests that they most likely represent the *in vivo* equivalents of moDCs.[68] Furthermore, CD14$^+$DC-SIGN$^+$ DCs have been described in humans as monocyte-like CD11c$^+$ cells lacking typical cDC markers, that may arise from classical monocytes.[61]

The similarities in phenotype between cDCs and monocytes/macrophages has made it difficult to distinguish their individual contributions to immune responses *in vivo*.[69] For example, CD11c has frequently been used as a surrogate marker to identify DC lineage, but this marker can also be expressed on macrophages, activated monocytes and pDCs.[69,70] Recently, a novel and evolutionarily conserved zinc finger transcription factor was identified, Zbtb46 (also known as Btbd4 or zDC), which is exclusively expressed by pre-cDCs, and lymphoid organ- and tissue-resident cDCs, but not by other immune populations.[69,70] Zbtb46 is not required for the development of cDCs, but acts as negative regulator preventing activation of cDCs in the steady state, for instance by repressing several MHC II genes. Upon stimulation of cDCs with TLR agonists, Zbtb46 protein is downregulated, allowing MHC II molecules to be expressed at higher levels.[71] This discovery has led to the development of a new mouse model in which the receptor for diphtheria toxin (DTR) was inserted into the 3' untranslated region of the Zbtb46 locus to serve as an indicator of Zbtb46 expression and as a means to specifically deplete cDCs.[69]

### *DCs in atherosclerosis*

In the vasculature, DCs are found to localize mostly in atheroprone regions that are subjected to major hemodynamic stress and their numbers increase substantially during lesion progression.[15,21,72,73] Millonig *et al.* proposed that these intimal DCs, together with macrophages

and T cells, constitute a vascular-associated lymphoid tissue, serving as a local defense of the vascular system.[21] DCs form close interactions with T cells in advanced atherosclerotic lesions, mostly in the shoulder and rupture-prone regions, suggesting *in situ* T cell activation and indicating their crucial role in ongoing immune responses.[74,75] In line with this, DCs in advanced lesions were found to have a mature phenotype. Moreover, remarkably higher amounts of DCs, alongside other inflammatory cells, can be found in vulnerable than in stable plaques.[76] Although the intima itself may be a site of DC-mediated antigen presentation and T cell activation, priming of naïve T cells will mostly occur in peripheral lymphoid tissues.[77]

Research into establishing the contribution of DCs to atherosclerosis is hampered by the lack of a universal marker that is expressed by all DCs. Nevertheless, a first model for depletion of DCs was established by Jung *et al.*, who generated mice that carry a simian DTR under control of the murine CD11c promoter.[78] When bred onto the LDLr$^{-/-}$ background and fed a Western-type diet, DT treatment resulted in a marked reduction in intimal CD11c$^{+}$ cells and a 55% decrease in accumulation of lipids during the earliest stages of plaque formation.[35] Prolonging the lifespan of DCs (and CD11c-expressing macrophages) in mice that carry CD11c-specific expression of the anti-apoptotic hBcl2, did not accelerate plaque progression,[79] albeit that cholesterol levels were reduced. Moreover, depletion of DCs resulted in enhanced cholesterolemia, implicating that DCs can control cholesterol homeostasis.[79] These findings could also be attributed to the critical role of DCs in the control of steady-state myelogenesis.[80] It needs to be noted that monocytes and macrophages also express CD11c under hypercholesterolemic conditions, making it difficult to discriminate the contribution of DCs alone.

By crossing mice deficient in FMS-like tyrosine kinase 3 (Flt3$^{-/-}$) to LDLr$^{-/-}$ mice, a selective deficiency of CD103$^{+}$ aortic cDCs is achieved, which is associated with aggravated atherosclerosis. Hence, CD103$^{+}$ cDCs are likely tolerogenic and protect from atherosclerosis by regulating local Treg homeostasis.[81] CD11b$^{+}$ DCs are most abundant in mouse aorta and have been shown to rapidly increase during atherogenesis. Recently, Busch *et al.* showed that CD11b$^{+}$ cDCs can also derive from pre-DCs depending on Flt3-Flt3L signalling.[82] A study by Weber *et al.* demonstrated the presence of CCL17-expressing DCs (Figure 1.4), which express high levels of maturation markers and were found to prevent the differentiation of naïve T cells into Tregs and promote apoptosis of Tregs, while at the same time recruiting and activating CD4$^{+}$ T cells.[83]

pDCs represent another subset of DCs that are present in the healthy vessel wall and within atherosclerotic lesions of ApoE$^{-/-}$ and LDLr$^{-/-}$ mice, albeit in smaller numbers than cDCs (Figure 1.4). In human tissue, pDC numbers and transcripts of pDC markers were increased in advanced compared with early plaques.[84] However, there is no consensus yet on the contribution of pDCs to atherosclerosis. Studies in mice using pDC depletion antibodies yielded discrepant results, with pDCs either promoting or reducing atherosclerotic lesion development.[85-87] A major drawback in these studies is the fact that the antibody used not exclusively recognizes pDCs.[88,89] In a study by Sage *et al.*, selective MHC II deficiency in pDCs from LDLr$^{-/-}$ mice pointed to a proatherogenic role for pDCs.[90] By contrast, aortic pDCs with tolerogenic capacities were also described. These pDCs express CCR9 and indoleamine 2,3-dioxygenase, and protect against atherosclerosis by the induction of Tregs. In line with this, depletion of pDCs during atherosclerosis resulted in decreased Treg numbers, albeit only in the aorta.[89]

As mentioned, lesional DCs may arise from blood-derived monocytes that infiltrate the intima (Figure 1.4). Deficiency in $CX_3CR1$ in ApoE$^{-/-}$ mice correlated with decreased accumulation of DCs in the aortic wall which also reduced the atherosclerotic burden.[91] However, $CX_3CR1$ is also used by monocytes to adhere to the vascular wall. There are two blood monocyte subsets in mice, Ly-6C$^{high}$$CX_3CR1^{low}$CCR2$^{+}$ "inflammatory" monocytes (which roughly correspond to "classical" human CD14$^{+}$CD16$^{-}$ monocytes) and Ly-6C$^{low}$$CX_3CR1^{high}$CCR2$^{-}$ "resident" monocytes (which share properties with human CD14$^{low}$CD16$^{+}$ monocytes).[92] It is not yet clear which subset preferentially differentiates into lesional moDCs as hypercholesterolemia triggers recruitment of both Ly-6C$^{low}$ and Ly-6C$^{high}$ monocytes.[29,92] A combined inhibition of CCL2 (the main ligand for CCR2), CCR5, and $CX_3CR1$ almost completely abrogates lesion formation in ApoE$^{-/-}$ mice.[29]

In addition to the recruitment of pre-DCs and monocytes, GM-CSF-dependent local proliferation of recruited or resident CD11c$^{+}$ cells may also contribute to increased numbers of DCs in nascent and early lesions, as demonstrated by 5-bromo-2′-deoxyuridine (BrdU) labelling (Figure 1.4).[93] This proliferation also persists when monocyte recruitment is inhibited.[93] Moreover, GM-CSF is an essential regulator of DC formation in lesions, as GM-CSF deficiency results in a significant reduction of DCs in lesions without any effect on other monocyte-derived cells.[94]

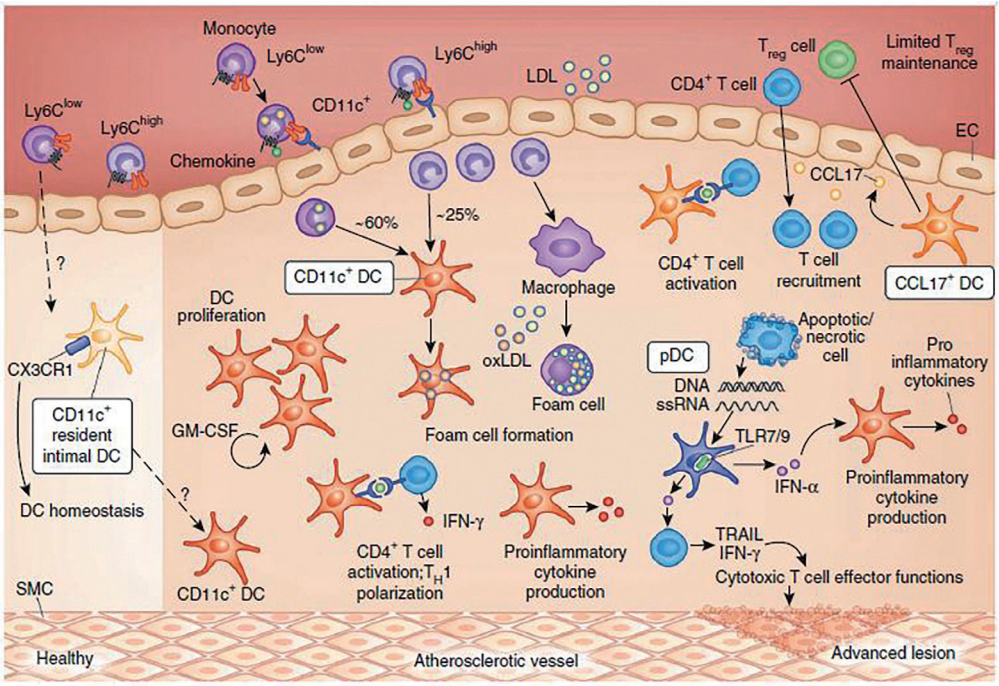


**FIGURE 1.4. DCs in an atherosclerotic lesion.** Resident intimal DCs are present in atherosclerotic-prone regions of vessels and their numbers increase upon lesion formation and progression in a GM-CSF-dependent manner. Circulating monocytes can give rise to $CD11c^+$ moDCs in lesions. A distinction between lesional macrophages and DCs, which both express $CD11c^+$ upon lipid-loading, remains difficult because of the lack of specific markers. Lesional DCs also take up lipids and become foam cell-like cells. Activated DCs produce proinflammatory cytokines, activate T cells and induce Th1 cell polarization. $CCL17^+$ DCs limit Treg cell expansion and recruit $CD4^+$ T cells. pDCs stimulate naïve $CD4^+$ T cells to express IFN-γ and TRAIL and trigger APCs to produce proinflammatory mediators. TRAIL, tumor necrosis factor–related apoptosis-inducing ligand. Adapted from[95].

Furthermore, arterial DC numbers are also affected by egress. DC emigration from aortic lesions to draining lymph nodes is impaired in hypercholesterolemic mice.[96] This appears to be due to impaired CCR7 upregulation, as oxLDL also seems to block CCR7 expression on DCs *in vitro* and to downregulate expression of the latter in atherosclerotic lesions.[97] Findings of attenuated CCR7 expression might point to retention of DCs in plaques and hence increased inflammation. In the aorta transplantation model for regression (transplantation of diseased aorta into healthy mice), emigration of DCs was strongly dependent on CCR7.[98] Moreover, inhibition of CCR7 signalling using antibodies against CCR7 ligands CCL19 and CCL21 inhibited DC-foam cell egress and prevented lesion regression.[98] By contrast, in one study, lesion regression was equal in $ApoE^{-/-}$ or $CCR7^{-/-}ApoE^{-/-}$ mice treated

with adenoviral vectors encoding ApoE, indicating that CCR7 did not affect myeloid cell egress from plaques.[99] However, CCR7 is also important for homing of T cells [100], which could have influenced the phenotype of the CCR7-deficient lesions.

Increased recruitment of pre-DCs and monocytes from the circulation, local DC proliferation or defective emigration of DCs from plaque to lymphoid organs may all result in increased DC numbers in atherosclerotic lesions over time.[58] Moreover, it is clear that different DC subsets may exert distinct functions in the pathogenesis of atherosclerosis. However, due to the use of non-selective depletion models, the relative contribution of each of these subsets in the context of atherosclerosis remains elusive.

### *DC function in atherosclerosis*

#### Immune dysregulation in atherosclerosis

Over the last decades, it has become apparent that patients with systemic autoimmune diseases, which are characterized by chronic inflammation and immune dysregulation, have accelerated atherosclerosis.[101-103] Moreover, it was shown that autoimmune diseases and atherosclerosis have a number of pathogenic similarities.[104] In general, autoreactive responses that occur in autoimmune disorders cause inflammation and lead to irreversible tissue damage. Yet, it is still poorly understood how autoreactive T cells in the normal human repertoire remain in a tolerant state, even though they continuously encounter self-antigens, and why these cells are persistently activated in patients with a chronic autoimmune disease. Previous research by our lab demonstrated that blood DC compartments were disturbed in patients with coronary artery disease (CAD), a clinical manifestation of atherosclerosis.[105,106] It is hypothesized that a regulatory mechanism exerted by DCs contributes to the onset of autoimmune atherosclerosis. Indeed, DCs play a central role in maintaining a homeostatic balance between the initiation of immunity (against potentially dangerous foreign antigens) and immune tolerance (against harmless self-proteins). Disruption of this balance may trigger clinically overt atherosclerosis.

#### DC function in steady state

In the steady state, DCs play an important role in the maintenance of arterial homeostasis (Figure 1.5A). Immature and semi-mature DCs prevent inflammation in the arterial wall by taking up lipids, apoptotic bodies and cellular debris arising from normal cell turnover, which are cleared from the intima by egress and efferocytosis.[107] Extracellular lipid uptake induces the formation of DC-foam cells. As mentioned above, migratory egress of DCs is

most likely dependent on CCR7. Efferocytosis prevents secondary necrosis of apoptotic cells which, in turn, prevents the release of potentially damaging intracellular molecules into the extracellular milieu. Additionally, engagement of efferocyte receptors by its ligands often triggers an anti-inflammatory response, and antigen processing by DC efferocytes can play a part in T cell-mediated immune recognition processes.[108] Ultimately, DC migration and presentation of self-antigens to draining lymph node T cells in the steady state is an essential aspect of peripheral immune tolerance limiting the risk of developing autoimmunity and chronic inflammation.[57,107] However, increased subendothelial retention of inflammatory lipid mediators (e.g. oxLDL), pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) drives increased vascular DC expansion and maturation (Figure 1.5B). Furthermore, modification of auto-antigens and molecular mimicry may lead to breakdown of self-tolerance. For example, endogenous stress-induced proteins called heat shock proteins (HSP), which are expressed by cells within atherosclerotic plaques, resemble those expressed by *Chlamydia pneumoniae* and *Helicobacter pylori*.[109] In pro-atherosclerotic conditions, DCs may fail to distinguish between self and foreign antigens, and present self-antigens to T cells which subsequently promote inflammatory responses in the plaque.[110] As mentioned, defective clearance of apoptotic cells can result in the onset of inflammation and autoimmunity, including atherosclerosis.[42,111]

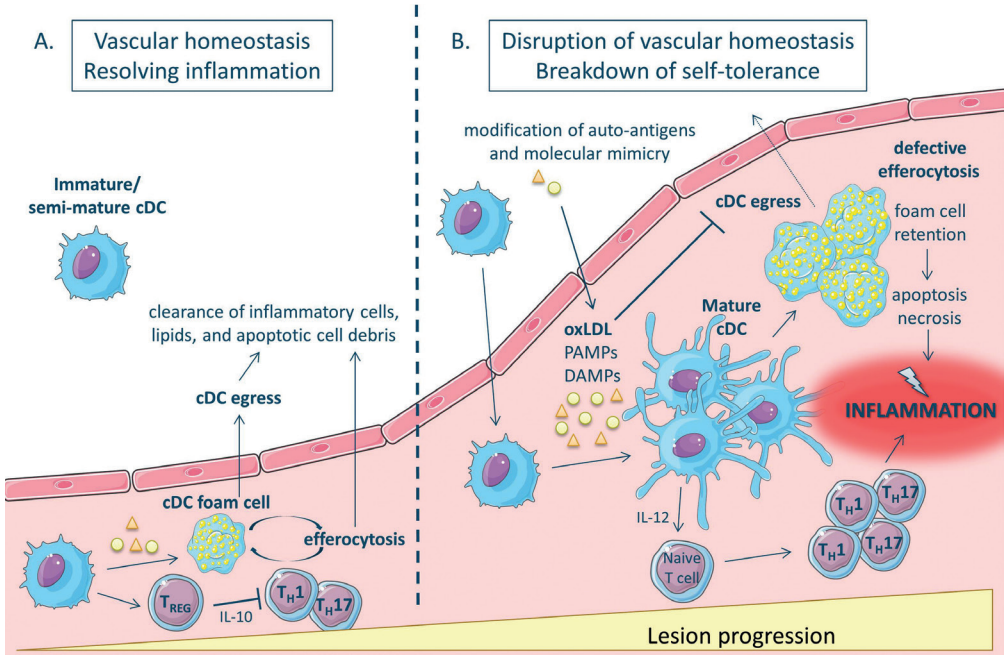


**FIGURE 1.5. Disruption of vascular cDCs homeostasis during atherogenesis.** (A) Immature and semi-mature cDCs take up lipids and other intimal antigens, thereby preventing them from eliciting proinflammatory signaling in other arterial wall cells. Extracellular lipid uptake induces the formation of cDCs foam cells which are cleared from the intima by egress and efferocytosis. cDCs induce tolerance against auto-antigens by silencing T-cell responses. (B) Increased subendothelial retention of inflammatory lipid mediators (oxLDL), PAMPs, and DAMPs drives increased vascular cDC expansion and maturation. Furthermore, modification of auto-antigens and molecular mimicry may lead to breakdown of self-tolerance resulting in the presentation of auto-antigens to T cells which promote inflammatory responses in the plaque. Blockage of cDC emigration by oxLDL and defective efferocytosis lead to increased foam cell retention, apoptosis, and necrosis, further increasing inflammation in the arterial wall.

## DC maturation and immune responses in atherosclerosis

DCs express pattern recognition receptors (PRRs) such as TLRs, which they use to sense PAMPs of bacteria and viruses, or DAMPs of damaged cells. Different DC subsets have distinct repertoires of TLRs. For example, TLR2 and TLR4 are expressed by CD11b$^+$ cDCs, but not by CD103$^+$ cDCs, which express TLR3.[107] This implicates a role for CD11b$^+$ cDCs in the pathogenesis of atherosclerosis as TLR2 and TLR4 expression were shown to exert an overall proatherogenic effect in response to hyper-cholesterolemia.[107,112] Mouse moDCs, derived from monocytes in the presence of GM-CSF, show increased expression of TLR4 and TLR7. However, only injection of LPS, the natural ligand of TLR4, increased the

numbers of moDCs in the lymph nodes.[113] *In vitro* differentiated human moDCs express the same spectrum of TLRs as cDCs with, in addition, TLR1 and TRL3.[114] Upon maturation of moDCs, TLR3 expression is decreased while TLR4 expression is increased.[115]

Downstream signalling molecules of TLRs include, among others, the myeloid differentiation factor 88 (MyD88) and Toll/IL-1 receptor domain-related adaptor protein inducing IFN-β.[112] Engagement of TLRs ultimately activates the nuclear factor-κB (NF-κB) pathway which controls the expression of an array of inflammatory cytokine genes.[116] In atherosclerosis, TLR4 expression was observed after oxLDL stimulation which is detected through complexes formed from TLR2, TLR4 and CD36.[117]

Maturation of DCs further includes down regulation of the endocytic capacity and the up regulation of antigen-presenting molecules as well as morphological changes (formation of dendrites) and an increased motility. Maturing DCs will present the processed antigen to either naïve, memory or effector T cells which undergo clonal expansion and proliferation. To achieve this, DCs upregulate the expression of several products necessary to supply T cells with the three signals that will determine their activation status and fate: 1) antigen presentation, 2) co-stimulatory molecule expression, and 3) cytokine production. Although oxLDL is one of the most accepted disease-associated antigens in atherosclerosis, evidence on the role of oxLDL on the maturation and function of DCs is conflicting.[97,118,119] Alderman *et al.* demonstrated that oxLDL are capable of promoting DC phenotypic maturation, while Perrin-Cocon *et al.* showed that oxLDL results in the differentiation of monocytes into mature DCs, but does not trigger maturation of iDCs.[118,119] Furthermore, native LDL can also be recognized by DCs.[119,120] Other candidate DAMPs and (auto-)antigens include HSP60 and HSP65, β2-glycoprotein I, DNA and RNA fragments derived from dying cells, high-mobility group box 1 protein, and an array of inflammatory cytokines.[112,121]

However, the exact nature of the antigen(s) contributing to DC activation in atherosclerosis remains elusive.[121] Processed antigens are presented by DCs via MHC class I (for intracellular antigens) or MHC class II molecules (for extracellular antigens) to $CD8^+$ and $CD4^+$ T cells, respectively. Alternatively, extracellular antigens can also be presented by MHC I molecules via cross-presentation. Deficiency of the invariant chain CD74, which mediates antigen loading on MHC II, in $LDLr^{-/-}$ mice reduced atherosclerosis and the number of activated T cells in advanced plaques.[122] This demonstrates the vital importance of adaptive immune responses via MHC class II-mediated antigen presentation to $CD4^+$ T cells in atherosclerosis. Furthermore, atherosclerosis-susceptible C57Bl/6 mice express the MHC II molecule $I\text{-}A^b$,

which has been reported to predominantly induce the proliferation of Th1 cells.[123] DCs are also capable of presenting glycolipids in the context of CD1d to NKT cells.

As mentioned, not only the specific recognition of antigens presented by DCs, but also T cell co-stimulation and co-inhibition direct T cell function and determine T cell fate.[124] Data from animal models show that deficiencies or blockade of important co-stimulatory molecules in mice such as CD80, CD86, CD40L and OX40L, among others, reduce T cell infiltration/activation and lesion development.[124,125] In addition to co-stimulatory molecules, deficiency of co-inhibitory molecules such as programmed death-ligand 1 (PD-L1) and/or PD-L2 in hypercholesterolemic mice reduces Treg function and leads to increased atherosclerosis.[125]

Signal three of DC-induced T cell activation concerns the production of T cell polarizing cytokines by DCs that shape the T cell response towards Th1, Th2, Th17 or Treg development. One of the best explored cytokines is interleukin (IL)-12, which drives the development of naïve T cells into proatherogenic and IFN-γ-producing Th1 cells.[107,124] Indeed, IL-12 deficiency as well as vaccination against IL-12 reduces atherosclerosis, while exogenous administration of IL-12 accelerates atherosclerosis.[126-128] Additionally, DCs produce chemokines in an atherosclerotic environment. As mentioned, vascular $CCL17^+$ DCs can produce CCL17 which attracts T cells to the lesions.[83] Furthermore, DC-derived CCL19 and CCL21 have been implicated in the recruitment of CCR7-expressing DCs and naïve T cells.[107]

pDCs have also been implicated in atherosclerosis but behave differently than cDCs. pDCs have a different TLR expression profile (TLR7, TLR8, and TLR9) which recognize RNA and DNA derived from (viral) pathogens. In the atherosclerotic plaque, they may also recognize nucleotides from dying cells. Activated pDCs mediate IFN-α production which has been shown to enhance atherosclerosis in mice by amplifying the inflammatory capacity of several different cell types present during atherosclerosis.[107] Besides its strong antiviral effects, IFN-α also induces upregulation of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) on $CD4^+$ T cells, which might lead to killing or apoptosis of plaque-resident cells, rendering the plaque more vulnerable.[129]

### *DCs as biomarkers of atherosclerotic disease*

Because of the sensitivity of DCs in peripheral blood to chronic low-grade inflammation, monitoring the frequency and phenotype of DCs in blood may provide a predictive tool for assessing disease and chronic inflammatory risk.[130] Indeed, we and others previously found

that circulating DCs (cDCs and pDCs) were markedly decreased in patients with CAD, the clinical manifestation of atherosclerosis. This decrease was irrespective of CAD grade or the number of affected arteries.[106,131] In a subsequent more extended study, Yilmaz *et al.* found that the decline in blood DCs correlated with disease severity and that circulating DCs may serve as independent predictors of the presence of CAD when several risk factors (age, male gender, diabetes, and hypertension) were included.[132] Later, several studies confirmed or rejected these findings, but in general, the majority of studies show declined blood DC numbers in CAD patients.[133] The mechanisms responsible for their decline remain elusive but there are indications pointing to enhanced recruitment to inflammatory sites, or impaired differentiation from bone marrow progenitors.[58] Additionally, it would be even more interesting to verify whether the immune cell populations and activation status in the periphery correlate with plaque inflammation and may be indicative of patients "at risk". Combining the inflammatory phenotype with patient characteristics may result in a better classification of "high-risk" patients who would benefit from additional, more targeted immunomodulatory strategies to prevent or treat atherosclerosis.

### *DCs as a therapeutic target for atherosclerosis*

DCs are central in directing innate and adaptive immunity. Being the upstream component in the chain of immune cell activation, they are an important target when considering potential strategies to modulate atherosclerosis.[134,135] Up till now, the treatment of atherosclerosis is based on reducing risk factors, such as lowering lipids and decreasing hypertension. Some of the pharmacological agents, such as statins and aspirin, also show anti-inflammatory properties and might affect DC maturation and function, although results remain rather conflicting.[134]

Because DCs are the most efficient APCs and coordinators of the immune response, they seem extremely suitable as cell-based vaccine, an approach similar to what is employed nowadays for cancer immunotherapy. This is achieved by transferring autologous or syngeneic DCs, loaded with an appropriate antigen *ex vivo*, to naïve recipients (Figure 1.6).[134-136] Several groups have already tested this technique in pre-clinical studies using different antigen formulations, but the effect on atherosclerosis has been inconsistent. One critical issue remains the identification of antigens relevant to atherogenesis that can be used to load the DCs. Intravenous immunisation of $LDLr^{-/-}$ mice with oxLDL-pulsed DCs stimulated with lipopolysaccharide (LPS) prior to the induction of atherosclerosis attenuated lesion development.[137] In contrast, multiple subcutaneous injections of DCs that were simulta-

neously pulsed with LPS and malondialdehyde-modified (MDA-)LDL into ApoE$^{-/-}$ mice increased the lesion size in the aortic root.[138] Using ApoB100 as the antigen, Hermansson *et al.* treated DCs with LPS but added IL-10 to induce a tolerogenic phenotype. Intravenous immunisation of "humanized" mice, expressing the full-length human ApoB100 in the liver, with ApoB100-loaded tolerogenic DCs proved effective in attenuating atherosclerosis development.[139] Furthermore, Pierides *et al.* observed that immunisation of ApoE$^{-/-}$ mice with DCs loaded with different sets of ApoB100-derived peptides induced a differential response. While DCs pulsed with p210 induced an increase in the number of Tregs accompanied by an increase in the circulating levels of IL-10, DCs loaded with p45 had an inhibitory effect on Tregs and reduced IL-10 levels in immunised mice, suggesting that this peptide may enhance DC maturation.[140]

There are many questions and obstacles that must be solved before (tolerogenic) DCs can be used as a treatment for atherosclerosis, including route of administration, timing of the treatment and choice of antigen.[141] Many foreign antigens including, bacteria, viruses, and even periodontal pathogens, have been identified in atherosclerotic plaques. However, it is unclear if these pathogens are causative agents, or if they simply skew the immune response towards inflammation through molecular mimicry.[135] Among the endogenous antigens, LDL and other ApoB100-containing lipoproteins have the strongest causative link with atherosclerosis. Besides naïve and modified LDL, and ApoB100 or peptides derived from ApoB100, other atherosclerosis-associated antigens, such as double-stranded DNA, or HSP60/65, could be used to modulate immune responses in atherosclerosis.[135] The particular effects of these antigens on the modulation of immune responses and the potential induction of Tregs is an element that merits further investigation.

To circumvent the problem of selecting the appropriate antigen, DCs could be loaded *ex vivo* in medium containing a total extract or suspension of atherosclerotic plaque material, for example, from patients undergoing carotid endarterectomy. Such an *ex vivo* DC immunotherapy can schematically be presented as shown in Figure 1.6. Starting from the patient's own white blood cells, maturation-resistant DCs can be grown *in vitro*, pulsed with total plaque material or tissue extract, and returned to the patient. This approach would mimic events as they occur in plaques *in situ* in the patient, thereby increasing efficacy and reducing the risk of unwanted side effects.[134,136]

Being an inflammatory disease, induction of tolerance by DCs has been put forward as a promising approach to ensure immune protection against atherosclerosis (Figure 1.6). DCs can be converted into 'tolerogenic (tol)DCs' by the addition of various immune-modulating biological agents (e.g. IL-10, transforming growth factor-beta (TGF-β), or 1,25-dihydroxy-vitamin D3) or pharmacological agents (e.g. dexamethasone, or aspirin).[59,134] As mentioned above, Hermansson, *et al.* used IL-10 to induce tolDCs.[139]

Alternatively, tolerance can also be induced indirectly, for instance by adoptively transferring oxLDL-induced apoptotic DCs.[142]

TolDCs typically show some similarities with iDCs, including reduced expression of co-stimulatory molecules and reduced production of pro-inflammatory cytokines, while maintaining the capacity to migrate to secondary lymphoid tissue in order to induce tolerance. However, the main feature of tolDCs is that they are maturation resistant and cannot be activated in an inflammatory setting. In this they differ from conventional iDCs. Down modulation of the expression of co-stimulatory molecules on DCs, or the production of pro-inflammatory cytokines by DCs *ex vivo* prior to the vaccination might be an attractive approach to ensure the induction of immunological tolerance against atherosclerosis.[134,141] However, it needs to be noted that co-stimulatory molecule blockade can impair both effector T cell and Treg differentiation and function. Indeed, CD80 and CD86 also appear to be crucial for the homeostasis and development of Tregs.[143] Although CD80 and CD86 bind the same receptors on T cells, it is now known that CD80 and CD86 have opposing functions through CD28 and CTLA-4 on Tregs.[144] For example, blocking CD86 protected nonobese diabetic mice from diabetes while blocking CD80 accelerated the disease, even though they display a similar reduction in the frequency of Tregs.[143] This emphasizes the complexity of these interactions and the difficulty to predict the outcome of blocking co-stimulatory molecules. Therefore, modulation of these molecules poses challenges for the design of therapeutic strategies in atherosclerosis.

Furthermore, a critical phase in the development of autoimmune disease comprises a period of autoreactivity against specified protein autoantigen(s). Work in animal models indicates that this phase may be susceptible to regulation. Therefore, therapy timing and the immunological status of a patient are additional critical issues that need to be considered carefully when designing proper clinical applications.[133,145]

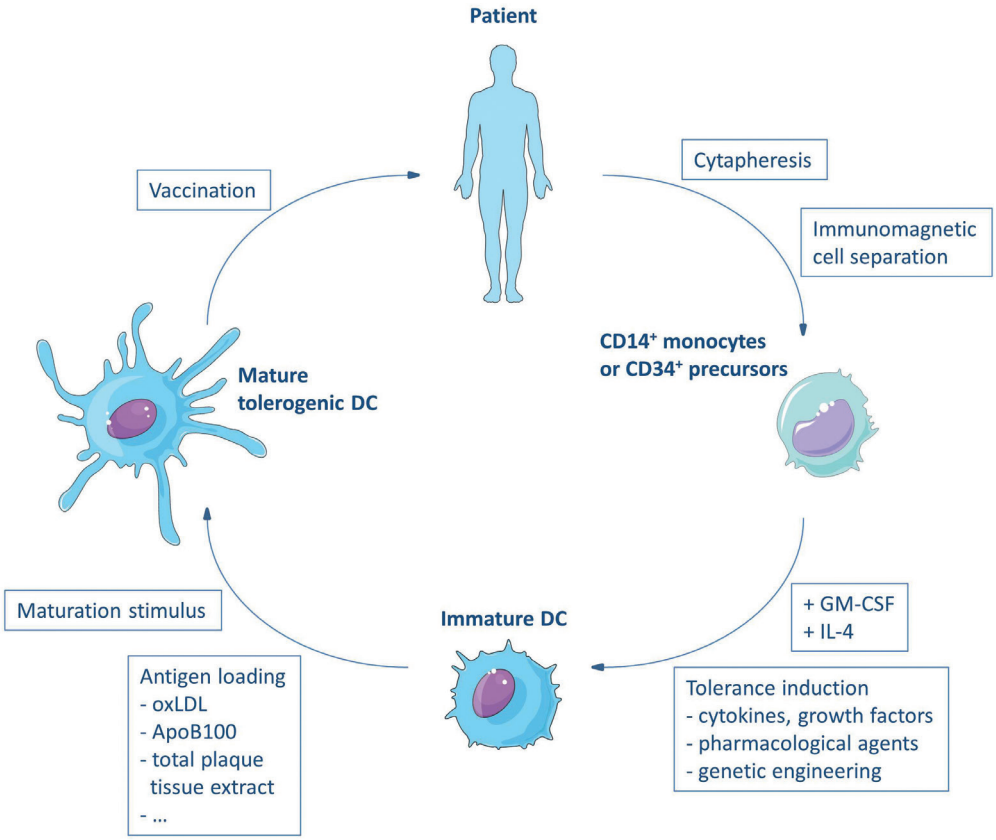


**FIGURE 1.6. (Tolerogenic) DC-based immunotherapy to treat atherosclerosis.** Immunisation of patients with autologous, monocyte-derived or $CD34^+$ precursor-derived DCs that are loaded with appropriate antigens *ex vivo* are suggested as potential new strategy in the battle against atherosclerosis. Furthermore, DCs can be converted to 'tolerogenic DCs' by addition of various immunomodulating agents, including cytokines and growth factors (e.g. IL-10, and TGF-β) and pharmacological agents (e.g. 1,25-dihydroxyvitamin D3, and aspirin), or they can be generated by using small interfering RNA (siRNA). Tolerogenic DC-based immunotherapy has recently been successfully tested in mice as a possible novel approach to induce immunological tolerance for prevention or treatment of atherosclerosis.

### T cells

In the 1980s, Jonasson *et al.* demonstrated the presence of activated $CD4^+$ and $CD8^+$ T cells in human atherosclerotic lesions.[13] Later, it was found that the majority of human plaque T cells are memory cells.[146] Research by the same group also revealed that a fraction of $CD4^+$ T cells cloned from human atherosclerotic plaques recognized oxLDL presented by autologous APCs via HLA-DR, a human equivalent of mouse MHC class II that is highly expressed on DCs.[147] More recently, chronic immune activation, as reflected by higher proportions of circulating $CD4^+$ memory T cells and lower proportions of naïve T cells, was found to be associated with subclinical atherosclerosis.[148] T cell depletion by anti-CD3 treatment[149], targeted gene deletion and subsequent adoptive transfer experiments[150], also revealed a net atherogenic effect for T cells in hyperlipidemic mice.

Functionally distinct $CD4^+$ T helper (Th) cell subsets, defined largely by the cytokines they produce, differentiate from naïve $CD4^+$ T cells, and have specialized roles in atherogenesis (Figure 1.7). The three best-characterized Th cell subsets are Th1 cells, which secrete IFN-γ, Th2 cells, which secrete IL-4, IL-5, and IL-13, and Th17 cells, which secrete IL-17, IL-22, and IL-23.[151] It is widely accepted that **Th1 cells** and IFN-γ are fundamental in promoting atherogenesis and inflammation. In mice, Th1 cells predominate over other Th cell subsets during early lesion formation, deficiency of Th1 cells attenuates atherosclerosis, and exogenously administered Th1 cell-derived IFN-γ promotes atherosclerosis.[54,151,152] The Th1 cell response perpetuates the adaptive immune response during atherosclerotic plaque formation through the activation of ECs and APCs, and the stimulation of SMC proliferation.[54] It is well established that IL-12 is the polarizing cytokine that drives Th1 differentiation from naïve T cells.[126,151]

The development of **Th2 cells** presumably involves the cytokines IL-4, IL-6, and IL-10.[134] Data on the influence of Th2 cells on the pathogenesis of atherosclerosis are limited and inconsistent.[151,152] Several studies show that IL-5, IL-10, and IL-13 can suppress vascular inflammation and atherosclerosis[54,152], while other reports suggest that Th2 cells or IL-4 may be associated with advanced atherosclerosis in hypercholesterolemic mice.[126,151,153] The fact that other types of immune cells are also able to secrete Th2 cytokines makes a unified interpretation of the role of Th2 cells in atherosclerosis difficult.[124] Mice that lack the Th1-driving transcription factor T-bet display a Th2 cell bias and decreased lesion development.[54,124,151] Also, $ApoE^{-/-}$ mice on a BALB/c background, which prevailingly display Th2 responses, showed reduced lesion sizes. These findings demonstrate that a Th1/Th2 switch alleviates the proatherogenic effects of Th1, although it does not constitute proof of an antiatherogenic effect of Th2 cells.[124]

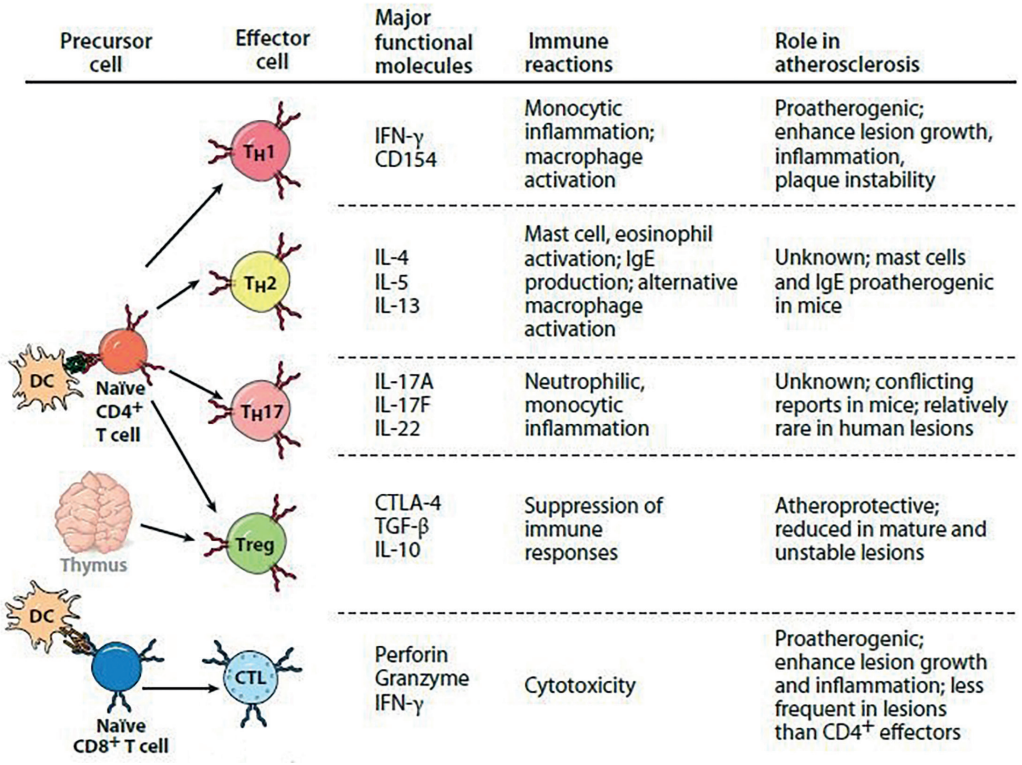


**FIGURE 1.7. T effector cells and their role in atherosclerosis.** $CD4^+$ Th cells differentiate from naïve $CD4^+$ T cells in response to antigen presentation by DCs and include, among others, Th1, Th2, and Th17 subsets defined by the cytokines they produce. Cytotoxic T lymphocytes (CTLs) are derived from naïve $CD8^+$ T cell precursors. The evidence from mouse models has clearly established that Th1 cells exert proatherogenic effects, but the impact of Th17 or Th2 cells is not clear. CTLs contribute to lesion growth and inflammation. $CD4^+$ Tregs, which may be derived from naïve T cells in peripheral lymphoid tissues or develop in thymus, protect against lesion growth and inflammation. Adapted from[151].

**Th17 cells** are induced by a combination of TGF-β and the inflammatory cytokines IL-6, IL-21, IL-1β, and IL-23. They are found in both human and murine lesions, but reports on the role of Th17 cells or IL-17 in atherogenesis are inconsistent.[154] Most of the available data indicate a pathogenic role for IL-17 in human CVD.[155-157] While some groups reported that inhibition of IL-17 signalling reduced atherosclerosis in $ApoE^{-/-}$[158,159] and $LDLr^{-/-}$[160] mice, others reported that IL-17 deficiency accelerates atherosclerotic plaque formation supporting for an atheroprotective role for IL-17.[161,162] In contrast, it was demonstrated that IL-17 is produced concomitantly with IFN-γ by coronary artery–infiltrating T cells and that these cytokines act synergistically to promote lesion progression and instability.[156]

**Tregs** play an important role in atherogenesis inhibiting self-reactive T cells through suppression of T cell proliferation and secretion of inhibitory cytokines, such as IL-10 and TGF-β.[163] Naturally-occurring Tregs ($FoxP3^+CD25^{hi}$) originate during T cell development in the thymus, whereas induced Tregs (which also express CD25, but do not need FoxP3 expression to be functional) can be generated in the periphery from naïve $CD4^+$ T cells.[124,163] It is hypothesized that an imbalance between Th1/Th17 responses and Tregs is one of the reasons for the local inflammation and proinflammatory response in atherosclerosis.[53,163] Moreover, clinical studies showed reduced frequencies of Tregs in cardiovascular patients.[163] As mentioned, Treg survival and homeostatic proliferation critically depend on continued interactions between costimulatory molecules of the B7 family (CD80, CD86) and CD28/CTLA-4.[143] Depletion of Tregs by combined deficiency of CD80 and CD86, deficiency of CD28, or with a CD25 neutralizing antibody also aggravates lesion development in $LDLr^{-/-}$ mice.[164] Similarly, blocking DC maturation through CD11c-specific knockdown of MyD88 alters the Treg pool and accelerates atherosclerosis.[165] Other strategies for Treg ablation, including selective deletion of $FoxP3^+$ cells using a DTR transgenic approach[166] or vaccination against FoxP3[167], also lead to more severe atherosclerosis in hypercholesterolemic mice. On the contrary, adoptive transfer of Tregs reduces lesion development in $ApoE^{-/-}$ mice.[164] Overall, approaches that induce/expand Tregs could provide a valuable tool in the battle against atherosclerosis.[163]

$CD8^+$ cytotoxic T lymphocytes (**CTLs**) directly kill antigen-bearing target cells via a perforin/granzyme–dependent mechanism, and they also secrete IFN-γ that activates macrophages.[151] $CD8^+$ CTLs are generally less numerous than $CD4^+$ T cells in human lesions. CAD patients display a persistent higher amount of $CD8^+$ CTLs in blood compared with healthy controls.[168] Although few lesional $CD8^+$ CTLs are present in murine plaques, antibody-mediated depletion of $CD8^+$ CTLs attenuated atherosclerosis in $ApoE^{-/-}$ mice, while transfer of $CD8^+$ CTLs into lymphocyte-deficient $ApoE^{-/-}$ mice aggravated atherosclerosis.[169] Additionally, similar to $CD4^+$ T cells, regulatory subtypes of $CD8^+$ T cells may also function to down-regulate immune responses in atherosclerotic diseases, as adoptive transfer of $CD8^+CD25^+$ T cells into $ApoE^{-/-}$ mice reduced atherosclerosis.[170]

### NKT cells

NKT cells represent a subset of T cells, co-expressing NK cell markers (NK1.1 in mice and CD56 and/or CD161 in humans) and a functional T cell receptor (TCR) complex, that respond in an innate-like manner to danger signals and pro-inflammatory cytokines.

They recognize glycolipid antigens presented by CD1d molecules (on APCs) via their semi-invariant TCR (predominantly Vα14Jα18, with a limited Vβ chain repertoire for mice, and Vα24Jα18/Vβ11 for humans).[171] Upon recognition of a glycolipid antigen, they rapidly produce large amounts of both Th1 and Th2 cytokines. Although NKT cells represent a minor population among T cells (0.3-2% in the human plaque)[172], they became of great interest in the past few years considering that retention and subsequent oxidative modification of lipids and lipoproteins in the vessel wall are one of the earliest events in the pathogenesis of atherosclerosis.

CD1d is expressed by APCs in mouse and human atherosclerotic lesions, and DCs and NKT cells were shown to co-accumulate within rupture-prone regions in human atherosclerotic plaques.[172,173] Furthermore, circulating NKT cell levels were reduced in blood of patients with previous cardiovascular events.[173] Most experimental data from animal models attribute a proatherogenic role to NKT cells. NKT cell-deficient ApoE$^{-/-}$ and LDLr$^{-/-}$ mice (CD1d$^{-/-}$ ApoE$^{-/-}$ or LDLr$^{-/-}$) exhibited reduced atherosclerotic lesion development compared with controls.[174,175] Exogenous administration of α-galactosylceramide (α-GalCer), a synthetic glycolipid that activates NKT cells via CD1d, resulted in accelerated atherosclerosis in ApoE$^{-/-}$ mice, whereas treatment of CD1d$^{-/-}$ ApoE$^{-/-}$ mice had no impact on lesion progression.[174,176] However, whether high reactivity to α-GalCer mirrors high reactivity to lipids accumulating within plaques is unknown since endogenous antigens for CD1d remain largely unidentified.[173] Adoptive transfer of NKT cell-enriched splenocytes from Vα14Jα18 TCR transgenic mice caused significant progression of aortic root atherosclerosis in recipient immune-deficient, atherosclerosis-susceptible RAG1$^{-/-}$LDLr$^{-/-}$ mice.[177] Similarly, increased numbers of NKT cells in Vα14 transgenic mice on a LDLr$^{-/-}$ background and placed on an obesogenic diet, aggravates the metabolic, inflammatory and atherosclerotic features of these obese mice.[178] Importantly, the influence of NKT cells on atherosclerosis seems transient and limited to early fatty streak lesions.[175,176] In contrast, there is a study showing that administration of α-GalCer into LDLr$^{-/-}$ mice reduced plaque formation.[179] In line with this study, it was reported that repeated injection of α-GalCer into mice led to changes in their cytokine profile, and induced regulatory properties in splenic DCs in an IL-10-dependent manner.[180]

As with DCs, there is a growing interest in using NKT cells as biomarkers for prognostic purposes (as proposed for type 1 diabetes and asthma), as well as in developing new treatments that exploit the unique functions of these cells.[181] α-GalCer, used in clinical trials to promote NKT cell proliferation and cytokine release, is safe in humans but is largely

ineffective when delivered intravenously. On the contrary, patients who are injected with α-GalCer-pulsed DCs, showed a transient increase in NKT cell frequency and an improved antitumor response as a result of the production of large amounts of IFN-γ. Indeed, IFN-γ released by NKT cells was shown to be important in several models, leading to the stimulation of NK cells, the licensing of DCs to release IL-12 and the promotion of the development of antitumor CTLs.[181,182] In a recent study, a CD1d-dependent lipid antagonist to NKT cells reduced atherosclerosis development and delayed progression of established lesions in $ApoE^{-/-}$ mice without affecting NKT cell or other lymphocyte numbers.[183] This suggests that targeting lesion inflammation via CD1d-dependent activation of NKT cells has a therapeutic potential in treating atherosclerosis and underlines the importance of the CD1d-NKT cell axis in this disease.

## CONCLUSION AND RESEARCH QUESTIONS

To this day, atherosclerosis, a complex multifactorial disease that involves dysregulated immune responses at every stage, is associated with an enormous socio-economic burden. The treatment of atherosclerosis is currently based on lipid lowering. However, despite optimal cholesterol reduction, residual cardiovascular risk remains a major concern, which highlights room for additional improvement.[184,185] Over the past few years it has become clear that atherosclerosis is more than a mere lipid-related disorder. In fact, the complex interplay between immunity, inflammation and dyslipidaemia was shown to determine the course of the disease. Because of the increasing evidence for a role of immune processes as the underlying disease mechanism in atherosclerosis, research concerning the feasibility and applicability of immunomodulatory treatments for atherosclerosis is of major importance.

A growing body of evidence designates DCs as the chief orchestrators of immune responses. However, the origin and functional role of DC (subsets) in the pathogenesis of atherosclerosis is not clear. Although experimental models for the selective depletion of moDCs are still lacking, the recent development of the Zbtb46-DTR mouse model allows for the first time to unravel the contribution of cDCs to atherogenesis. In the current dissertation, we aim to identify new targets for therapy by further unraveling the contribution of cDCs in the pathogenesis of atherosclerosis and to investigate if cDCs can be modulated for the treatment of atherosclerosis. Therefore, the main research questions of this thesis were:

1. How do local and systemic immune cell profiles in atherosclerosis develop during plaque progression, and do certain immunological cell profiles correlate with the progression of atherosclerotic disease?
2. What is the contribution of *bona fide* cDCs in the development of atherosclerosis?
3. Can cDCs be modulated in order to move toward novel therapeutic approaches for plaque stabilization?

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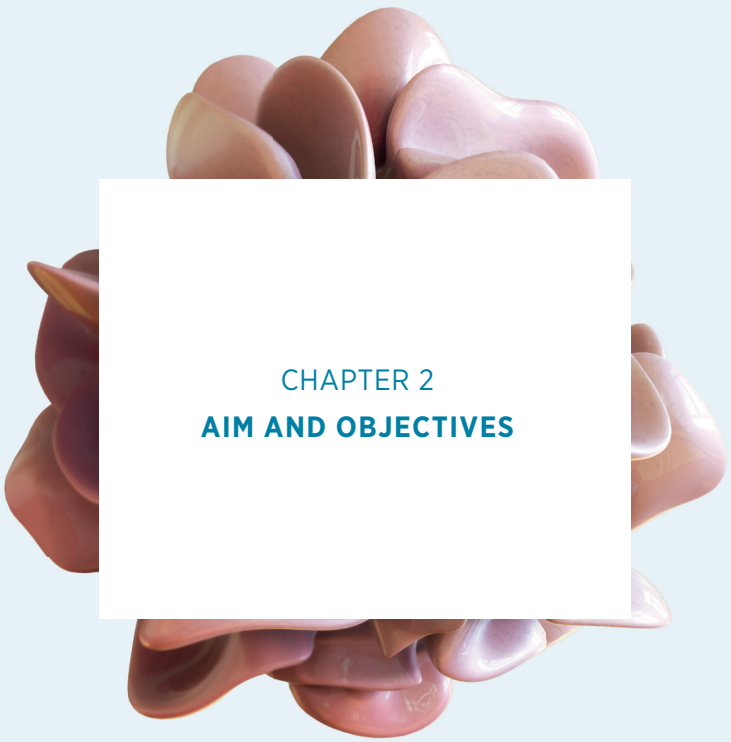

CHAPTER 2

# AIM AND OBJECTIVES

As outlined in **chapter 1**, it has been repeatedly demonstrated that ongoing dysregulated immune responses play a fundamental role in all stages of atherosclerosis, making immune-modulatory therapy a potentially attractive way of managing this disease. Within the entire spectrum of immune cells, DCs possess the unique ability of shaping immune responses. This makes them a highly appealing cell population to investigate and an attractive target for therapy. Indeed, DC-based therapies are on the rise, emerging as new therapeutic strategies for various auto-immune diseases and cancer. The promising outcomes from preclinical experiments in animal models have resulted in a number of currently ongoing clinical trials to treat autoimmune diseases such as type 1 diabetes and rheumatoid arthritis.[1] Although these are primarily safety trials, clinical trials regarding DC-based cancer therapies have already yielded encouraging clinical outcomes.[2,3] Considering the enormous socio-economic burden associated with cardiovascular events, more research is clearly needed to decipher the complex role of the immune system and the potential for immunization as an immune-modulatory therapy for atherosclerosis.[4]

Therefore, the aims of this thesis were (i) to further unravel the contribution of cDCs in the pathogenesis of atherosclerosis and (ii) to examine whether cDCs can be modulated for the treatment of atherosclerosis (Figure 2.1).

In order to investigate the contribution of cDCs and effector cells to atherogenesis we first conducted a longitudinal study (**chapter 3**). For this, we analysed innate and adaptive immune cell distributions in blood, plaques and lymphoid tissue reservoirs in ApoE$^{-/-}$ mice at different time points during lesion development, and in blood and plaques from patients undergoing endarterectomy. Subsequently, correlations between immunological parameters in blood and plaque were studied to determine whether or not blood parameters could be predictive for plaque growth or inflammation during atherosclerosis. Linking peripheral immune cell profiles to the stage of the disease could provide a useful tool to identify potential immunotherapeutic targets and determine the optimal timing for immunotherapy.

Next, we aimed to elucidate the role of cDCs, derived from pre-cDCs, in the pathogenesis of atherosclerosis by selective depletion of this cell type. **Chapter 4** reports the *in vivo* targeting of cDCs in atherosclerosis by using the recently developed Zbtb46-DTR mouse model.[5,6] For this, lethally irradiated, atherosclerosis-prone, LDLr$^{-/-}$ mice were transplanted with bone marrow from Zbtb46-DTR donor mice, which allows for specific depletion of cDCs by administration of DT.

In a second part of this thesis, we explored the potential of cDCs as targets for immunomodulation by using a combination of *in vitro* and *in vivo* analyses, allowing us to assess novel DC-targeted therapeutic strategies for atherosclerosis.

It was reported that the immunogenic phenotype of DCs can be modulated pharmacologically by acetylsalicylic acid (ASA). However, the dose used to induce a tolerogenic phenotype *in vitro* may have toxic effects upon administration *in vivo*. In **chapter 5** we determined if chronic low-dose ASA treatment, used for many years in the treatment and prevention of cardiovascular disease,[7,8] has immune modulatory capacities and, consequently, the potential to reduce atherosclerosis. For this, the effect of ASA, administered for 15 weeks to the drinking water of ApoE$^{-/-}$ mice upon 10 weeks of a Western-type diet was evaluated. Atherosclerotic plaque composition and immune cell profiles in blood, spleen and mediastinal lymph nodes were studied in this chapter.

Another way to modify the immunogenic phenotype of DCs is to prevent the transcription of genes involved in the generation of an immune response by means of selective interference with siRNA. A common technique used to transfect DCs *in vitro* with antigen or with immune-modulatory molecules is electroporation.[9-11] In **chapter 6**, we aimed to investigate the feasibility and efficacy of electroporation of siRNA in order to generate DCs with modified T cell-polarizing function. After extensive validation of the culture of mouse bone marrow-derived (BM)DCs, immature BMDCs were genetically modified *in vitro* by means of siRNA to transiently inhibit the expression of IL-12p35, the unique subunit of the biologically active IL-12p70, a protein that is mandatory for immunogenic DC functions.[12] We hypothesize that silencing of IL-12p70 production interferes with the Th1 cell-stimulatory capacity of DCs and has the future potential to generate regulatory DCs that ultimately can be used to restore the immunological imbalance in atherosclerosis.

The overall results of this thesis are discussed in **chapter 7**. A summary is given in **chapter 8**.

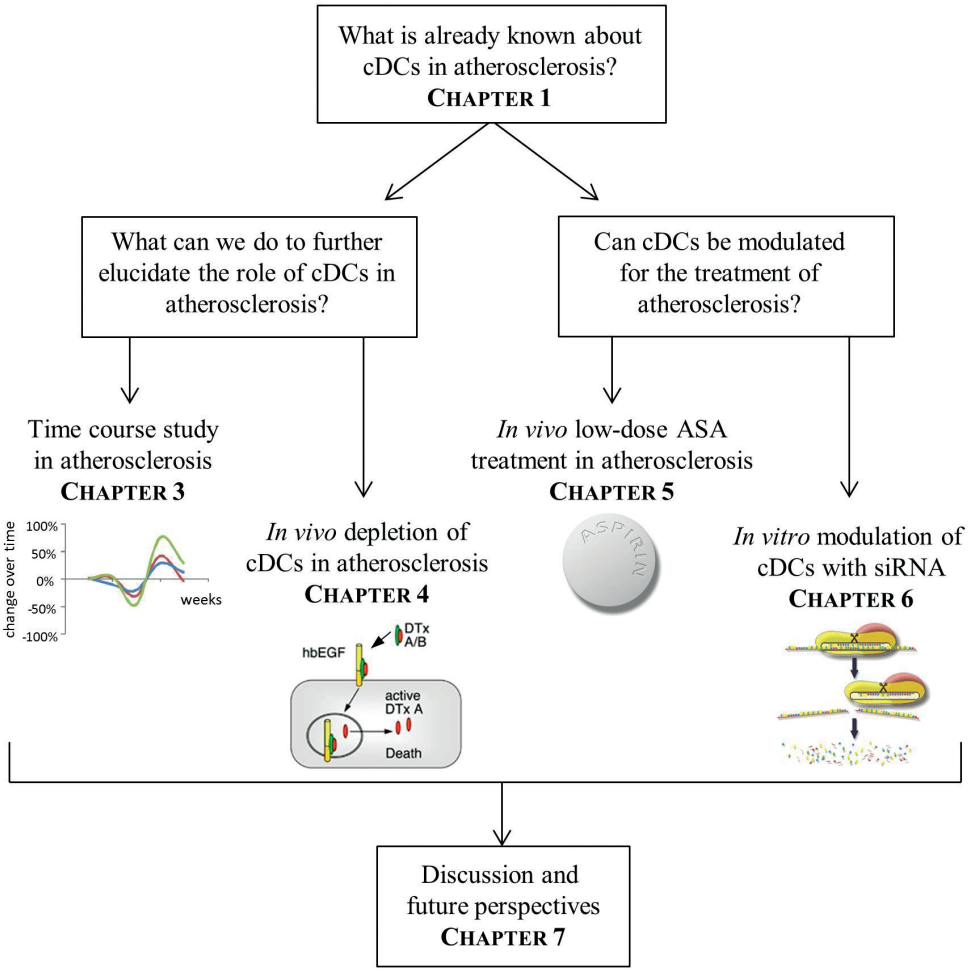


FIGURE 2.1. Schematic outline of the thesis.

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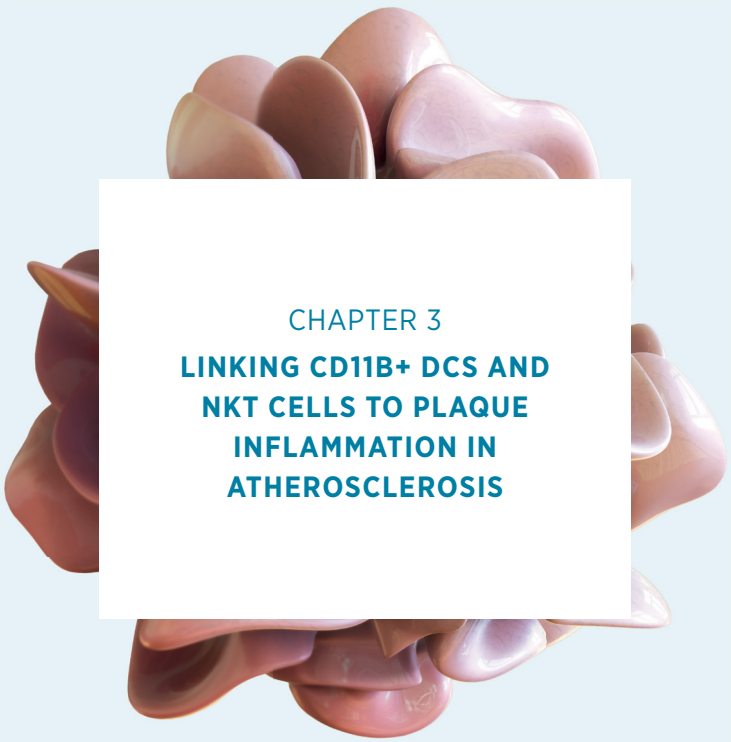

CHAPTER 3

# LINKING CD11B+ DCS AND NKT CELLS TO PLAQUE INFLAMMATION IN ATHEROSCLEROSIS

ADAPTED FROM

**Rombouts M**, Ammi R, Van Brussel I, Roth L, De Winter BY, Vercauteren SR, Hendriks JMH, Lauwers P, Van Schil PE, De Meyer GRY, Fransen E, Cools N, and Schrijvers DM. Linking $CD11b^{+}$ dendritic cells and natural killer T cells to plaque inflammation in atherosclerosis. *Mediators of Inflammation*. Volume 2016 (2016), Article ID 6467375, 12 pages.

## INTRODUCTION

In the past two decades, the innate and adaptive immune system have been identified as major players in the development, progression and rupture of atherosclerotic plaques, both in mice and humans.[1-4] Still, there is a great need for the identification of suitable biomarkers that could provide a useful complementary criterion to ensure better risk stratification and optimize clinical management (e.g. follow-up and therapeutic interventions), as well as the development of new therapies leading to the stabilization or regression of atherosclerotic plaques.[5] Therefore, detailed knowledge of the local and systemic immune mechanisms in atherosclerotic plaques is pivotal in order to identify patients with vulnerable, highly inflammatory plaques, and to determine optimal timing for immunotherapy. Until now, studies in which the human inflammatory cell subsets in atherosclerosis were characterized mostly focused on systemic immune activation.[6-9] However, whether the circulating immune cell distributions represent local plaque inflammation remains unclear.

Although multiple immune cells are involved in atherosclerosis, most studies focus on a single cell type due to technical limitations. Detailed immune cell phenotyping requires the use of multi-laser flow cytometers.[10] We previously described a protocol and a gating technique to identify and isolate immune cells from human atherosclerotic plaques using multi-parametric flow cytometry.[11] The antigen-specific activation of T cells may not be solely driven from the periphery, but also from within the plaque.[12] In this chapter, local and systemic immune cell distributions in murine and human atherosclerosis were characterized simultaneously using flow cytometry and real-time qPCR. The distribution of cDC (subsets), monocytes, macrophages, NK(T) cells, and T cells, was analysed in blood, plaques and lymphoid tissue reservoirs in ApoE$^{-/-}$ mice, and in blood and plaques from patients undergoing endarterectomy. Furthermore, to investigate whether changes in immune cell dynamics could be associated with or are predictive for plaque growth or inflammation during atherosclerosis, possible correlations between immunological parameters in blood and plaque were investigated. Additionally, we assessed the expression of different chemokine receptors during disease development to determine whether the homing capacity of immune cells correlates with changes in immune cell dynamics or plaque development.

## MATERIAL AND METHODS

### Mice

Male and female ApoE$^{-/-}$ mice were fed a Western-type diet (WD, 4021.90, AB Diets) starting at an age of 6 weeks (wk). Mice were sacrificed with sodium pentobarbital (250 mg/kg, i.p.) before onset of atherosclerosis (0 wk of WD) or after 6, 12 and 24 wk of WD. These time points represent healthy artery, fatty streak, fibroatheroma and advanced atherosclerotic plaques in mice. Analysis of total plasma cholesterol was performed by using a commercially available kit (Randox) following the manufacturer's instructions. Age-matched non-atherosclerotic C57BL/6J control mice on chow feeding were used to adjust for changes related to ageing rather than atherosclerosis. The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and had free access to water and food.

### Patients

To characterize the immune cells in human atherosclerosis, 72 patients that were eligible for endarterectomy at the carotid (n = 35; 49%) and femoro-popliteal level (n = 37; 51%) were recruited from the clinical departments of Thoracic and Vascular surgery of the Antwerp University Hospital and ZNA Middelheim. From 57 (79%) of the included patients, peripheral blood samples were collected as well, to study the systemic immune cell distribution. High sensitivity C-reactive protein (hs-CRP) levels were measured in serum from 35 patients by the clinical lab of the Antwerp University Hospital. Patient characteristics are shown in Table 3.1.

### Ethics statement

The mouse protocols were approved by the Antwerp University Ethics Committee on Animal Experiments (permit number: 2013-68). The animals received human care and were treated according to the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, 1985). Protocols involving patients were approved by the local Ethics Committee (n° 12/25/212), and all research was based on written informed consent with proper arrangements for the protection of the confidentiality of personal data of the individuals concerned.

TABLE 3.1. PATIENT CHARACTERISTICS.

| Variable | Carotid artery plaque (n=35) | Femoral artery plaque (n=37) | Significance† |
|---|---|---|---|
| Age (years) | 72 ± 2 | 71 ± 2 | NS |
| Male gender (%) | 60 | 68 | NS |
| Degree of artery stenosis (%) | 82 ± 2 | 87 ± 1 | NS |
| **Risk factors (%)** | | | |
| Family history | 20 | 46 | $P < 0.05$* |
| Hypertension | 71 | 78 | NS |
| Hypercholesterolemia | 80 | 75 | NS |
| Diabetes mellitus | 42 | 27 | NS |
| Smoking | 46 | 70 | $P < 0.01$** |
| Obesity | 29 | 22 | NS |
| Prior vascular intervention | 46 | 68 | NS |
| **Medication (%)** | | | |
| Acetylsalicylic acid | 98 | 92 | NS |
| NSAIDs | 3 | 3 | NS |
| Beta-blockers | 46 | 65 | NS |
| Calcium channel blockers | 34 | 22 | NS |
| ACE-inhibitors | 43 | 35 | NS |

† Significant differences between plaque location; NS: no significance.

### Cell isolation and flow cytometry from murine blood and tissues

After sacrifice of the mice, blood was obtained by cardiac puncture. Single cell suspensions of the aorta-draining mediastinal lymph nodes (LN)[13] and the spleen were prepared by passage through a 40 μm cell strainer. Erythrocytes were lysed using a red blood cell-lysing buffer (Hybri-Max, Sigma-Aldrich). Remaining leukocytes were counted using a hemocytometer and labelled with anti-mouse monoclonal antibodies (Table 3.2) at 4°C in FACS buffer (PBS supplemented with 0.1% BSA (Sigma- Aldrich) and 0.05% $NaN_3$ (Merck)) in the presence of CD16/32 Fc-receptor blocker (BioLegend). Cells were analysed on a BD Accuri C6 cytometer (BD Biosciences). Debris and dead cells were excluded based on forward scatter (FSC), side scatter (SSC) and positive staining for propidium iodide (Life Technologies). The gating strategy is depicted in Figure 3.1. Data analysis was performed with FCS Express 4 (De Novo Software).

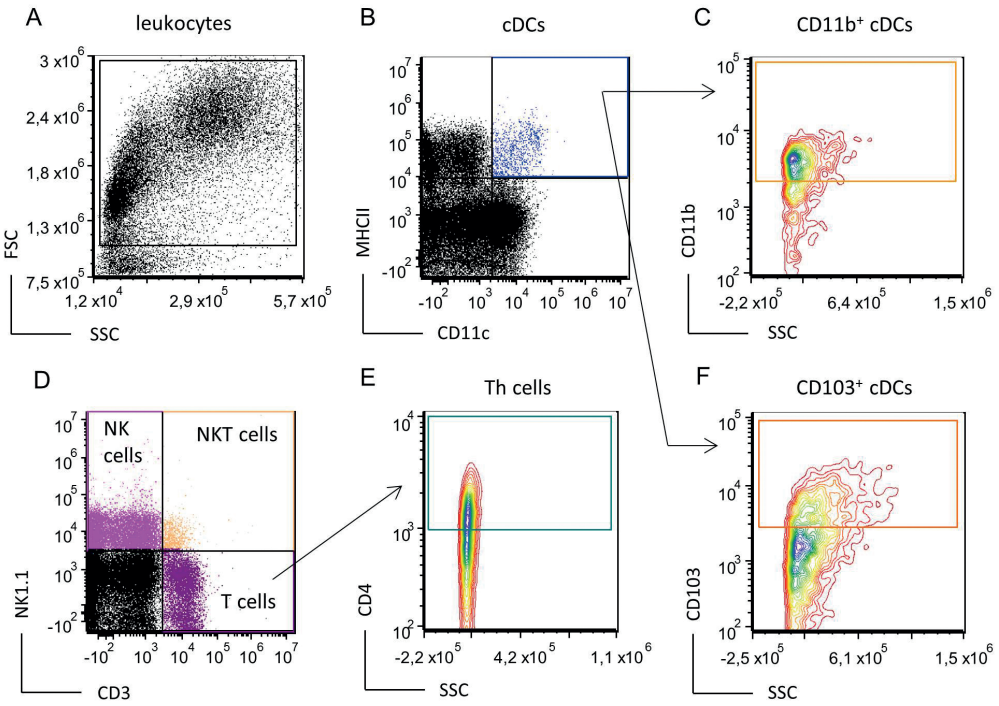


**FIGURE 3.1. Gating strategy for the analysis of immune cells in murine atherosclerosis.** Gates are set on isotypes to correct for non-specific binding. (A) Plots are gated on FSC and SSC to define the total percentage of leukocytes from cell debris. (B) The total cDC population was identified based on the expression of CD11c and MHCII. (C,F) Based on their expression of CD11b (C) and CD103 (F) two cDC subsets were identified. A distinction was made between circulating Ly-6C$^{low}$ and Ly-6C$^{high}$ monocytes. Macrophages (Mφ) were identified as CD68$^{+}$ cells within the total leukocyte population (plots not shown). (D) Lymphocyte subsets were identified as T cells (CD3$^{+}$NK1.1$^{-}$), NK cells (CD3$^{-}$NK1.1$^{+}$) and NKT cells (CD3$^{+}$NK1.1$^{+}$). (E) Th cells were defined as CD4$^{+}$ cells within the total T cell population.

## Cell isolation and flow cytometry from human atherosclerotic plaques and peripheral blood

Atherosclerotic plaques were collected in RPMI 1640 medium (Life Technologies) and kept at room temperature until processing. Cell isolation was performed as previously described.[11] Briefly, within 2 h after surgery the plaque specimens were dissected into small pieces, followed by an enzymatic digestion with 2.5 mg/ml collagenase IV (Life Technologies) and 0.2 mg/ml DNase I (Roche) for 2 h at 37°C. After digestion, the residue was filtered over a 40 µm cell strainer. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll (GE Healthcare) density gradient centrifugation.

After isolation, cells from plaque and blood were blocked with mouse gamma globulins and stained with an optimized 9-color panel of mouse anti-human monoclonal antibodies (Table 3.2). To eliminate the abundance of cell debris and extracellular lipids in the digested plaque suspensions, we used a gating strategy as described previously.[11] All measurements were performed on the FACSAria II (BD Biosciences). Data acquisition and analysis were done using FACSDiva 6.1.2 (BD Biosciences). The gating strategy is depicted in Figure 3.2.

**TABLE 3.2. MONOCLONAL ANTIBODIES USED FOR THE FLOW CYTOMETRIC ANALYSIS IN THIS STUDY.**

| Marker | Conjugate | Clone | Specificity | Source |
|---|---|---|---|---|
| **Anti-mouse antibodies** | | | | |
| CD11c | APC | N418 | cDCs | BioLegend |
| I-$A^b$ | FITC | KH74 | cDCs, B cells | BioLegend |
| CD11b | PerCP | M1/70 | cDC2, Mφ | BioLegend |
| CD103 | PerCP-Cy5.5 | 2E7 | cDC1 | BioLegend |
| CD3ε | APC | 145-2C11 | T cells | BioLegend |
| CD4 | PerCP | GK1.5 | Th cells | BioLegend |
| CD19 | PE | 6D5 | B cells | BioLegend |
| NK1.1 | FITC | PK136 | NK(T) cells | BioLegend |
| Ly-6C | APC | HK1.4 | Mo | BioLegend |
| CD68 | APC | FA-11 | Mφ | BioLegend |
| **Anti-human antibodies** | | | | |
| CD45 | APC-H7 | 2D1 | Leukocytes | BD |
| Lineage 2 | FITC | SK7 (CD3), SJ25C1 (CD19), L27 (CD20), MΦP9 (CD14), NCAM16.2 (CD56) | T cells, B cells, Mφ, Mo, NK cells neutrophils, eosinophils | BD |
| HLA-DR | V500 | G46-6 | Total DCs | BD |
| CD11c | PE-CF594 | B-ly6 | cDCs | BD |
| CD11b | BV421 | ICRF44 | BDCA-$1^+$ cDCs | BD |
| CD16 | PerCP-Cy5.5 | 3G8 | moDCs | BD |
| CD3 | PE-Cy7 | SP34-2 | T cells, NKT cells | BD |
| CD7 | PE | M-T701 | NK(T) cells | BD |
| CD14 | APC | M5E2 | Mφ, Mo | BD |
| CD68 | APC | Y1/82A | Mφ, Mo | BioLegend |

Abbreviations: BDCA-1, blood dendritic cell antigen-1; HLA-DR, human leukocyte antigen; I-$A^b$, murine major histocompatibility complex class II alloantigen; Mo, monocyte; moDCs, monocyte-derived dendritic cells; Mφ, macrophage.

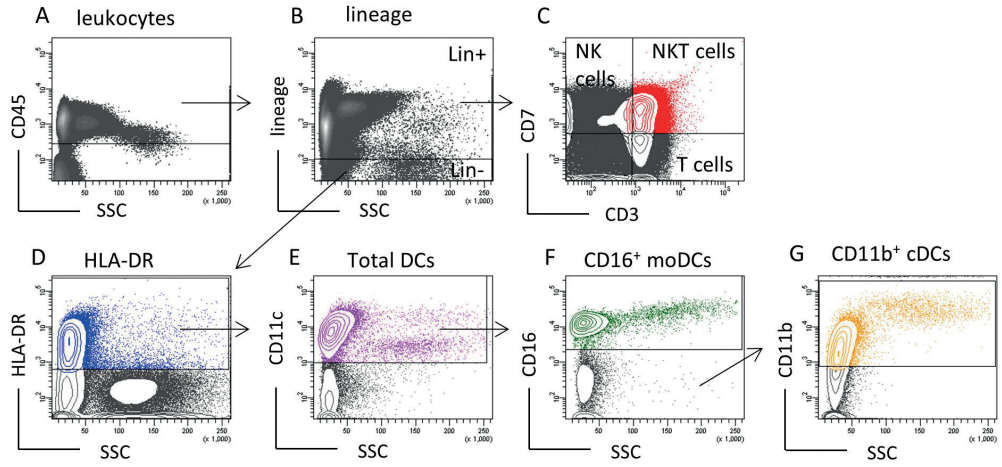


**FIGURE 3.2. Gating strategy for the analysis of leukocyte(s) (subsets) in human plaque and blood samples.** (A) After staining the leukocyte population using a CD45 pan leukocyte antibody, (B) lineage markers (= a CD3, CD14, CD19, CD20, CD56 cocktail) were used to separate the DC (lineage-) from the other immune cells (lineage+). (C) Within the lineage+ cells we identified T cells, NK cells and NKT cells, that were defined as $CD3^+$, $CD3^-$ $CD7^+$ and $CD3^+$ $CD7^+$ respectively. cDCs were then identified as positive for (D) HLA-DR and (E) CD11c. (F) CD16 was used for the staining of monocyte-derived (mo)DCs. Subsequently, (G) $CD11b^+$ cDCs were gated from the CD16-negative population. Within the lineage+ population we identified monocytes in blood as HLA-$DR^+$ $CD11c^+$ $CD11b^+$ $CD14^+$ and macrophages in plaques as HLA-$DR^+$ $CD11c^+$ $CD11b^+$ $CD68^+$ (plots not shown).

## Gene expression analysis

The aortic adventitia of mice was partially digested and removed from the rest of the vessel following incubation in an enzyme digestion solution composed of 781.25 U collagenase II and 14.0625 U elastase (Worthington) in 2.5 ml PBS for 10 minutes at 37°C.[14] Total RNA was extracted from the aorta, stripped from the adventitial layer, using a TRIzol-based RNA isolation protocol (Ambion). RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA was reverse transcribed with the SuperScript II Reverse Transcriptase kit (Life Technologies). Quantitative gene expression analysis was performed on a 7300 Real-Time PCR System (Applied Biosystems) using SYBR green technology (SensiMix, GC Biotech). The parameters for PCR amplification were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 30 s. Melting curves were checked for amplification of a single, specific product. Used primer pairs are summarized in Table 3.3. All data were analysed using qBase+ 3.0 (Biogazelle).

TABLE 3.3. PRIMER SEQUENCES FOR PCR.

| | Sequence sense | Sequence antisense |
|---|---|---|
| **CCR5** | CAAGACAATCCTGATCGTGCAA | TCCTACTCCCAAGCTGCATAGAA |
| **CCR7** | AGAAGAACAGCGGCGAGGA | AGCATAGGCACTAGGAACCCAAA |
| **GAPDH** | CCAGTATGACTCCACTCACG | GACTCCACGACATACTCAGC |
| **PPIA** | GAAGCCATGGAGCGTTTTGG | CAGATGGGGTAGGGACGCTC |
| **SIRPα** | ATACGCAGACCTGAATGTGCCCAA | TGGCCACTCCATGTAGGACAAGAA |
| **T-bet** | GCCAGGGAACCGCTTATATG | GACGATCATCTGGGTCACATTGT |
| **Vα14Jα18** | TGGGAGATACTCAGCAACTCTGG | CAGGTATGACAATCAGCTGAGTCC |
| **XCR1** | CATGGGTTCTTGGCCTCAGT | ACAGTGCTGGATGTCTTCCG |
| **Zbtb46** | TCACATACTGGAGAGCGGC | CCTCATCCTCATCCTCAACC |

The expression of genes was determined relative to the average expression of the two household genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA). Abbreviations: CCR5/7, CC chemokine receptor type 5/7; SIRPα, signal regulatory protein α; T-bet, T-box transcription factor expressed in T cells; Vα14Jα18, invariant TCR-α chain rearrangement specific for NKT cells; XCR1, chemokine XC receptor 1; Zbtb46, zinc finger and BTB domain containing transcription factor 46.

## Histological analysis

After sacrifice of ApoE$^{-/-}$ mice, the proximal ascending aorta and brachiocephalic artery were collected, embedded in Neg-50 (Thermo Scientific) and snap frozen in liquid nitrogen. Atherosclerotic plaque size, stenosis and necrotic core (acellular area with a threshold of 3000 μm$^2$) were analysed on haematoxylin-eosin (H-E) stained 5 μm cryosections (Table 3.4). All images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope and were quantified with Image J software (National Institutes of Health).

**TABLE 3.4. CHOLESTEROL AND PLAQUE PARAMETERS OF APOE$^{-/-}$ MICE DURING ATHEROGENESIS.**

| | 0 wk | 6 wk | 12 wk | 24 wk |
|---|---|---|---|---|
| Cholesterol (mg/dl) | 232 ± 11 | 625 ± 97*** | 658 ± 40*** | 698 ± 45*** |
| Stenosis $A_{prox}$ (%) | 0 ± 0 | 1.2 ± 0.4 | 13.8 ± 2.5*** | 23.2 ± 2.3*** |
| Stenosis $A_{br}$ (%) | 0.8 ± 0.8 | 1.8 ± 1.1 | 51.3 ± 7.7*** | 61.5 ± 2.8*** |
| Necrotic core $A_{prox}$ (%) | 0 ± 0 | 0 ± 0 | 0 ± 0 | 3.5 ± 1.1** |
| Necrotic core $A_{br}$ (%) | 0 ± 0 | 0 ± 0 | 1.4 ± 0.7 | 5.5 ± 1.3*** |

Data from proximal ascending aorta ($A_{prox}$) and brachiocephalic artery ($A_{br}$), mean ± SEM, 0 wk n = 9-11, 6 wk n = 11-12, 12 wk n = 10-11 and 24 wk n = 11-12; **p< 0.01, ***p< 0.001.

### Statistical analysis

All data are presented as mean ± SEM. Multiple comparisons of means were performed for the analysis of all mouse data using one-way ANOVA followed by Dunnett's Multiple Comparison test or two-way ANOVA followed by Bonferroni's Multiple Comparison test, where appropriate. Differences between human plaques derived from the carotid and femoral artery were tested with the Student's t-test. Variables that failed normality were logarithmically transformed, or analysed with the nonparametric Mann–Whitney U test. Correlations between local and circulating cells in atherosclerosis were described using the Spearman rank-order correlation coefficient. To extract relationships between changes in aortic gene expression and circulating immune cell dynamics during atherosclerosis, a principal component analysis (PCA) was applied to the quantitative flow cytometry and PCR data of ApoE$^{-/-}$ mice. Statistical analysis was performed using Prism 5.0 (GraphPad) or R version 3.1.2.[15] $p<0.05$ was considered statistically significant.

## RESULTS

### Analysis of immune cells in blood, plaque and lymphoid tissue of ApoE$^{-/-}$ mice

During atherosclerotic plaque development in ApoE$^{-/-}$ mice the percentage of total DCs increased in the spleen but no distinct changes were observed in blood or LN (Figure 3.3A). Interestingly, at all locations and time points the CD11b$^{+}$ cDCs represented the most predominant subset. Furthermore, a significant drop of this subset was seen at all locations after 12 wk of WD (Figure 3.3B, red bars). Regarding the other cDC subset, CD103$^{+}$ cDCs, we found no significant difference over time (Figure 3.3C). Additionally, the percentage of resident Ly-6C$^{low}$ monocytes and their inflammatory counterparts, Ly-6C$^{high}$ monocytes, in blood is decreased after 12 wk of WD. The percentage of both subtypes increased between 12 and 24 wk of WD, which was most pronounced in the Ly-6C$^{high}$ subset (Figure 3.4A).

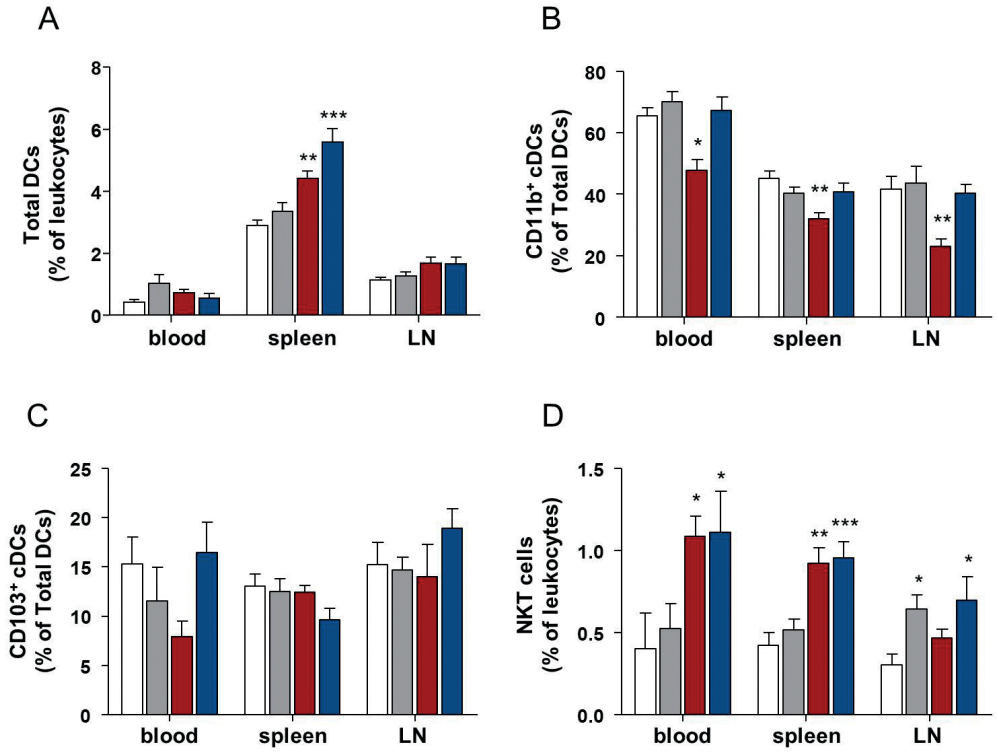


**FIGURE 3.3. Flow cytometry results of DC (subsets) and NKT cells in ApoE-/- mice during atherogenesis.** Bar graphs representing mice sacrificed after 0 wk (white bars, n = 7-12), 6 wk (grey bars, n = 9-12), 12 wk (red bars, n = 9-10) and 24 wk (blue bars, n = 10-12) of WD. (A) Percentages of the total DC population. (B) Percentages of the $CD11b^+$ cDCs subset within the total DC population. (C) Percentages of $CD103^+$ cDCs within the total DC population. (D) Percentages of NKT cells within the total leukocyte population; *p<0.05, **p<0.01, ***p<0.001.

Regarding cells of the adaptive immunity, the percentage of T cells gradually declined in all studied compartments. The reduction in T cells was most pronounced after 24 wk of WD in blood, spleen and mediastinal LN compared with mice sacrificed before the onset of atherosclerosis (Figures 3.4A-C). With regard to T cell subsets, the percentage of total $CD4^+$ Th cells significantly decreased after 12 wk of WD in blood and spleen as compared to mice at 0 wk of WD (Figures 3.4A,B). Interestingly, percentages of NKT cells are increased after 12 wk (in blood and spleen) and 24 wk of WD (in blood, spleen and LN) (Figure 3.3D). All graphs indicate a clear turning point in immune cell dynamics at 12 wk of WD (Figures 3.4A-C, arrows). At this time point, a substantial increase was observed in the size and areas of the atherosclerotic plaques in the brachiocephalic and proximal aorta (Table 3.4). Between 12 and 24 wk of WD systemic immune activation is induced as evidenced by an increase in the majority of immune cells above baseline in all locations investigated.

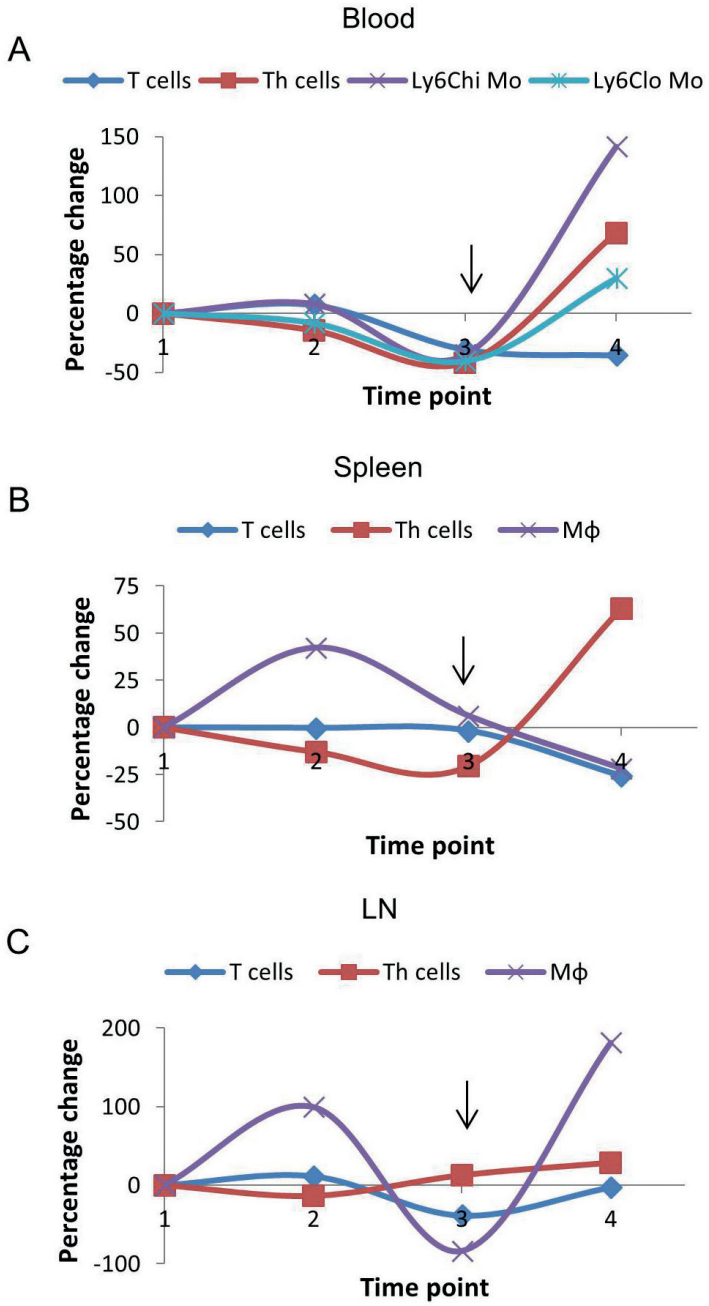


**FIGURE 3.4. Flow cytometry results of leukocyte(s) (subsets) in ApoE$^{-/-}$ mice during atherogenesis.** Graphs showing fluctuations (as percentage change over time) in T cells, Th cells and monocytes (Mo) and macrophages (Mϕ) in blood (A), spleen (B) and mediastinal LN (C) at different time points (1 = 0 wk, 2 = 6 wk, 3 = 12 wk, 4 = 24 wk of WD). The arrow at time point 3 (= 12 wk of WD) indicates a clear turning point in immune cell dynamics.

Expression levels of DC (subset) genes within plaque-containing aortic tissues were measured with qPCR. The expression of Zbtb46, a transcription factor, used to distinguish the total cDCs population from other immune cells, was increased after 6 wk of WD, returned to baseline after 12 wk of WD and was reduced below baseline levels after 24 wk of WD (Figure 3.5A). SIRPα and XCR1 gene expression was used to discriminate between $CD11b^+$ cDCs and $CD103^+$ cDCs, respectively.[16] The expression level of SIRPα increased 11-fold in mice after 12 wk of WD compared to mice sacrificed before the start of the WD (Figure 3.5B). A significant increase (2.6-fold) was also observed in the expression of XCR1 after 6 wk of WD (Figure 3.5C). The same is true for the amount of NKT cells, as detected by Vα14Jα18 mRNA (2-fold increase, Figure 3.5D).

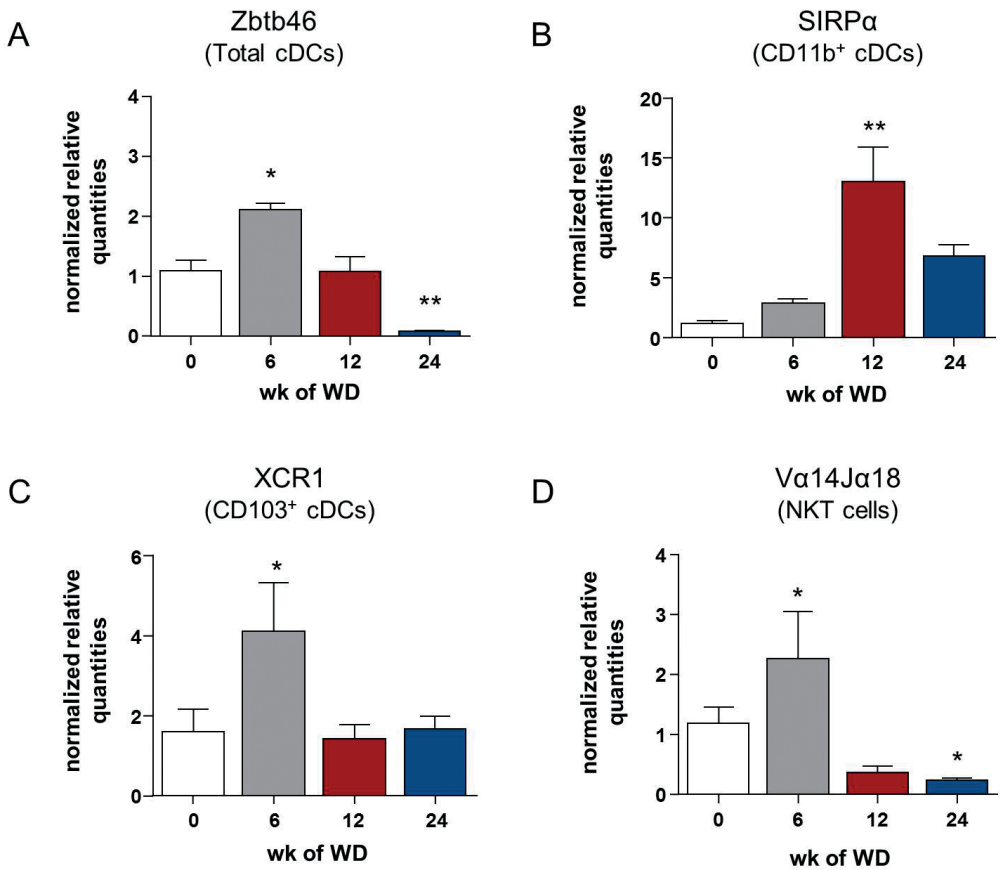


**FIGURE 3.5. Gene expression results of DC (subsets) and NKT cells in $ApoE^{-/-}$ mice during atherogenesis.** Normalized expression levels of Zbtb46 (A), SIRPα (B), XCR1 (C) and Vα14Jα18 (D) mRNA in aortic tissue samples; 0 wk n=6, 6 wk n=4-5, 12 wk n=11, 24 wk n=13-15; *p<0.05, **p<0.01.

### The number of circulating CD11b$^{+}$ cDCs and NKT cells is highly indicative for plaque inflammation in mice during atherosclerosis

In mouse plaques, strong features of inflammation could be detected, including the expression of T-bet, the main director of Th1 lineage commitment.[17] The relative mRNA expression of T-bet was significantly increased (3-fold) after 6 wk of WD feeding compared to mice that had not yet received a WD (Figure 3.6A). Furthermore, the expression of different chemokine receptors, involved in homing of leukocytes to inflammatory sites or lymph nodes, was investigated. Plaque mRNA expression for CC chemokine receptor type 5 (CCR5) and CCR7 was significantly higher (3-fold) after 6 wk of feeding on the WD and returned to baseline when lesions were progressing (Figures 3.6B,C).

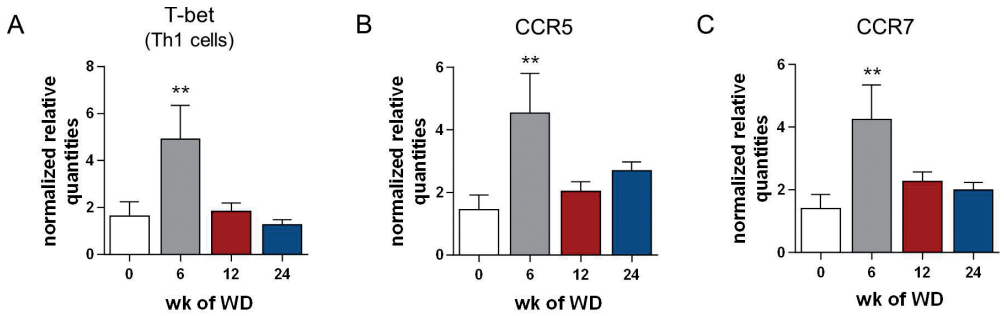


**FIGURE 3.6. Features of inflammation in ApoE$^{-/-}$ aortic plaques during atherogenesis.** Relative mRNA levels of T-bet (A), CCR5 (B) and CCR7 (C) in plaque containing aortic tissue measured by real-time qPCR.; 0 wk n=6, 6 wk n=4-5, 12 wk n=11, 24 wk n=14-15; **p<0.01.

In aortic plaques, the expression of T-bet is positively correlated with CCR5 and CCR7 expression at 6 wk and 12 wk of WD (Table 3.5). Furthermore, there is a strong correlation between the degree of stenosis in the proximal aorta and the mRNA level of CCR7, Vα14Jα18, Zbtb46 and SIRPα in mice sacrificed at 6 wk of WD. No strong correlations were found between the expression levels of CCR5/7 and the numbers of circulating T cells, CD4$^{+}$ Th cells, B cells or NK cells after 6 wk on the WD. In mice from the 12 wk of WD group, Vα14Jα18 expression is correlated to T-bet, CCR5, CCR7, Zbtb46, SIRPα and XCR1 (Table 3.5).

Because blood provides a conduit between all organs and tissues, correlations between local and circulating cells in atherosclerosis were also described using the Spearman's rank-order correlation coefficient (Table 3.5). Plaque development and inflammation were most

pronounced in mice that had a low number of circulating NKT cells at 6 wk of WD. At this time point, a strong inverse correlation was determined between NKT cell numbers in blood and the expression of T-bet, CCR5, CCR7, Zbtb46 and XCR1 in plaque-containing aortic tissue. In line with these findings, the degree of stenosis in the proximal aorta was inversely correlated to circulating NKT cell numbers. Similar observations were seen for the number of circulating $CD11b^+$ cDCs and the expression of the same genes in the aorta at 12 wk of WD.

**TABLE 3.5. CORRELATION ANALYSIS BETWEEN INFLAMMATION MARKERS IN MOUSE PLAQUES AND BLOOD.**

| **6 wk of WD** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | T-bet | CCR5 | CCR7 | Vα14Jα18 | Zbtb46 | SIRPα | XCR1 | Stenosis |
| T-bet$^{(p)}$ | - | 0.900 | 0.700 | 0.200 | -0.200 | -0.400 | 0.900 | 0.354 |
| Stenosis$^{(p)}$ | 0.354 | 0.354 | 0.707 | 0.707 | 0.755 | 0.755 | 0.354 | - |
| Vα14Jα18$^{(p)}$ | 0.200 | 0.500 | 0.300 | - | 0.400 | 0.200 | 0.500 | 0.707 |
| NKT cells$^{(b)}$ | -1.000 | -0.800 | -1.000 | -0.400 | -1.000 | -0.500 | -0.800 | -0.657 |
| CD11b+ cDCs$^{(b)}$ | -0.400 | 0.000 | -0.100 | 0.500 | 0.000 | 0.000 | 0.000 | -0.251 |
| **12 wk of WD** | | | | | | | | |
| | T-bet | CCR5 | CCR7 | Vα14Jα18 | Zbtb46 | SIRPα | XCR1 | Stenosis |
| T-bet$^{(p)}$ | - | 0.855 | 0.855 | 0.891 | 0.758 | -0.818 | 0.952 | -0.261 |
| Stenosis$^{(p)}$ | -0.261 | -0.515 | -0.393 | -0.370 | -0.381 | 0.345 | -0.200 | - |
| Vα14Jα18$^{(p)}$ | 0.891 | 0.721 | 0.879 | - | 0.636 | -0.830 | 0.939 | -0.370 |
| NKT cells$^{(b)}$ | -0.456 | -0.535 | -0.426 | -0.322 | -0.116 | 0.274 | -0.377 | -0.189 |
| CD11b+ cDCs$^{(b)}$ | -0.717 | -0.733 | -0.717 | -0.583 | -0.650 | 0.633 | -0.733 | 0.400 |

The table shows Spearman's rank correlation coefficients (ρ) of associations between gene expression results within plaques or between plaques and circulating NKT cells or CD11b+ cDCs. The magnitude of the correlation coefficient determines the strength of the correlation: $|\rho| > 0.7$ strong correlation; $0.5 < |\rho| < 0.7$ moderate correlation; $|\rho| < 0.5$ weak correlation. 0 wk n = 6, 6 wk n = 5, 12 wk n = 11, 24 wk n = 14-15. Abbreviations: p, plaque; b, blood.

### Principal component analysis for aortic gene expression and circulating immune cells at different stages of atherosclerosis

PCA is a data reduction technique where all information contained in the original variables is re-arranged into a novel set of independent variables called principal components (PCs). These PCs are linear combinations of the original variables, with the first PCs (PC1 and PC2) containing the majority of the information. In this case 42% of the information is contained in PC1, while 25% is contained in PC2. When PC1 is plotted against PC2, a total of 67% of the total information in our dataset is displayed in a single plot (Figure 3.7). Although considerably fewer data points are available for mice that are sacrificed after 6 w of WD (black symbols) compared to those sacrificed after 12 w of WD (red symbols), still we can already observe a separation of clusters that are enriched for similar gene expression and circulating immune cells corresponding to these time points. For example, mice at 6 w of WD (black symbols) have a high score for PC1 indicating that the values for circulating NKT cells and SIRPα mRNA in plaques are low, while numbers of circulating $CD11b^+$ cDCs and levels of CCR5 and CCR7 mRNA in plaques are high (based on the loadings for PC1).

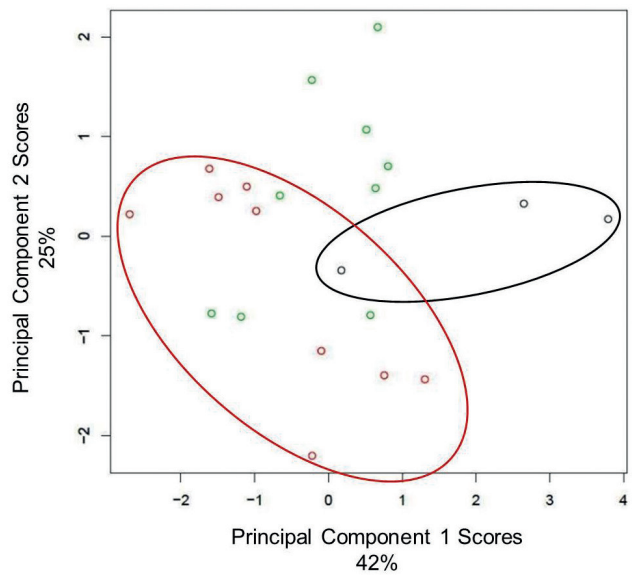


**FIGURE 3.7. PCA score plot for the first two PCs of an analysis of aortic gene expression and circulating immune cells at different stages of atherosclerosis.** The score plot separates clusters that are enriched for similar gene expression and circulating immune cells corresponding to different time points of atherogenesis. Plot shows clusters corresponding to profiles at 6 (black symbols), 12 (red symbols) and 24 (green symbols) weeks of WD. Samples grouped by similar expression profiles are depicted by colored ovals.

### CD11b$^{+}$ cDCs and NKT cell dynamics in ApoE$^{-/-}$ mice are distinct from healthy controls

To correct for changes related to age rather than atherosclerosis, we compared the number of CD11b$^{+}$ cDCs and NKT cells in ApoE$^{-/-}$ mice fed an atherosclerotic diet and age-matched healthy wild-type mice fed a chow diet at two time points which represent early and advanced atherosclerotic lesions. Percentages of circulating CD11b$^{+}$ cDCs are higher in ApoE$^{-/-}$ mice as compared to healthy wild-type controls in blood, spleen and mediastinal LN during early lesion (6 wk of diet) formation (Figures 3.8A-C). In the case of advanced plaques (24 wk of diet), the CD11b$^{+}$ cDCs percentage is also significantly higher in the blood of ApoE$^{-/-}$ mice (Figure 3.8A).

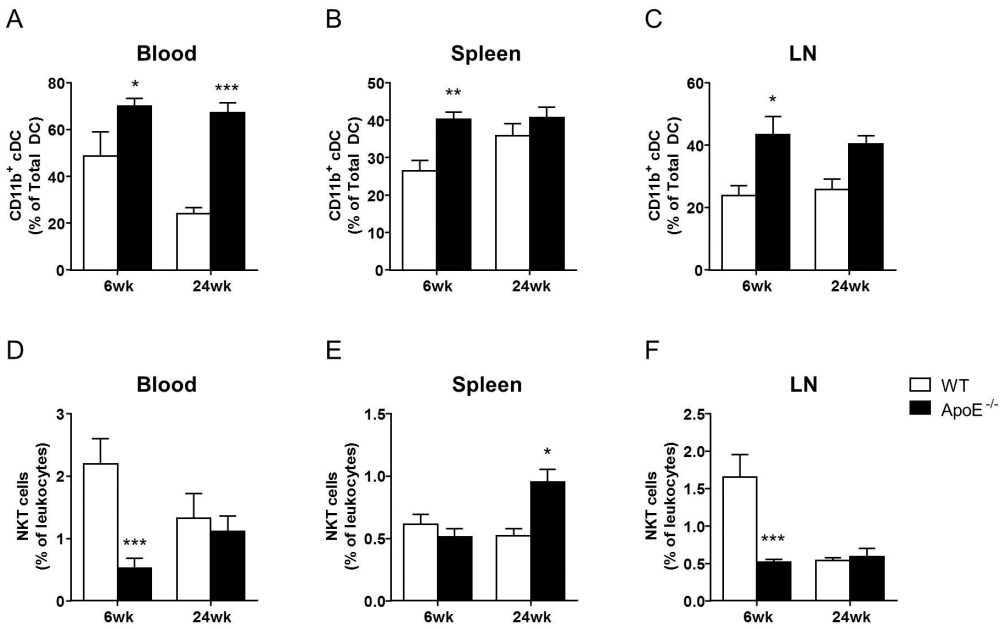


**FIGURE 3.8. Comparison of CD11b$^{+}$ cDCs and NKT cell fluctuations between ApoE$^{-/-}$ mice and age-matched healthy controls.** Percentages of circulating CD11b$^{+}$ cDCs in blood (A), spleen (B) and mediastinal LN (C) of ApoE$^{-/-}$ mice fed a WD and healthy wild-type controls fed a chow diet sacrificed at 6 and 24 wk of diet. Circulating NKT cell numbers in blood (D), spleen (E) and mediastinal LN (F) of ApoE$^{-/-}$ mice fed a WD and healthy wild-type controls (on chow diet) sacrificed at 6 and 24 wk of diet. WT (white bars), n = 4-6; ApoE$^{-/-}$ (black bars), n = 9-12; *p<0.05, **p<0.01, ***p<0.001.

The opposite is seen for the NKT cells: at the initiation of the disease (6 wk of diet) NKT cell numbers in blood (Figure 3.8D) and mediastinal LN (Figure 3.8F) of ApoE$^{-/-}$ mice are low as compared to healthy controls. There is no difference in the NKT cell percentage in the

spleen between ApoE$^{-/-}$ and control mice at the onset of atherosclerosis but they increase with enhanced atherosclerosis (Figure 3.8E). Hence, high numbers of CD11b$^{+}$ cDCs and low numbers of NKT cells at 6 wk of diet are attributable to the induction of atherogenesis in ApoE$^{-/-}$ mice.

### Analysis of immune cells in human atherosclerotic plaques and blood

To analyze different leukocyte subsets in plaque and blood samples from advanced atherosclerosis patients, we used a gating strategy as depicted in Figure 3.2. As observed in mice, and similar to our previous data[11] we observed a predominance of CD11b$^{+}$ cDCs within the CD45$^{+}$ population in the plaques, compared to the CD16$^{+}$ monocyte-derived (mo)DC subset. In contrast to the plaque, the CD16$^{+}$ moDCs was the predominant subset in the blood compared to the CD11b$^{+}$ cDCs (Table 3.6). Clec9A was used as a marker for the human equivalent of CD103$^{+}$ cDCs in mice. However, due to their low numbers[11], we refrained from studying this cDC subset in subsequent analyses in this study. Within the CD45$^{+}$ population, atherosclerotic plaques predominantly contained NK cells. Furthermore, relatively high mean percentages of NKT cells and T cells were also observed, both in blood and plaque, as compared to the DC (subsets) and monocytes/macrophages (Table 3.6).

TABLE 3.6. FLOW CYTOMETRIC ANALYSIS OF IMMUNE CELLS IN HUMAN ATHEROSCLEROTIC PLAQUES AND BLOOD.

| | Blood (% within CD45$^{+}$ population) | Plaque (% within CD45$^{+}$ population) |
|---|---|---|
| **Total DCs** | | |
| CD11c$^{+}$ DCs | 6.5 ± 0.9 | 2.0 ± 0.2 |
| **DC subsets** | | |
| CD11b$^{+}$ cDCs | 2.7 ± 0.4 | 1.4 ± 0.2 |
| CD16$^{+}$ moDCs | 3.7 ± 0.5 | 0.3 ± 0.1 |
| **Other leukocytes** | | |
| CD14$^{+}$ Mo | 0.4 ± 0.1 | NA |
| CD68$^{+}$ Mφ | NA | 1.1 ± 0.8 |
| CD3$^{+}$ T cell | 10.3 ± 1.8 | 5.9 ± 1.0 |
| CD7$^{+}$ NK cell | 9.5 ± 1.7 | 14.1 ± 1.3 |
| CD3$^{+}$ CD7$^{+}$ NKT cell | 8.9 ± 1.8 | 6.7 ± 1.5 |

Abbreviations: Mo, monocyte; Mφ, macrophage; NA, not applicable.

To extend the evaluation of atherosclerotic plaque composition we compared the immune cell distribution in plaques from distinct anatomical locations (carotid versus femoral artery). To correct for size differences between plaques from femoral and carotid artery, the number of cells per gram tissue was calculated. Comparing between plaque locations, the number of cells per gram was significantly higher in carotid plaques for the total DC population (311 ± 70 vs. 121 ± 24; p = 0.014), $CD16^+$ moDCs subset (17 ± 5 vs. 5 ± 1; p = 0.019), $CD11b^+$ cDCs subset (197 ± 44 vs. 74 ± 15; p = 0.009), macrophages (50 ± 17 vs. 9 ± 2; p = 0.037), NKT cells (1240 ± 473 vs. 104 ± 30; p = 0.016) and T cells (1125 ± 288 vs. 183 ± 56; p = 0.002). In contrast, the NK cell numbers per gram (1241 ± 201 vs. 917 ± 215; p = 0.277) did not significantly differ between the two locations.

### In advanced human atherosclerosis the number of circulating NKT cells is predictive for plaque inflammation

We investigated whether correlations could be found between the immunological parameters in blood and advanced plaques collected from the same endarterectomy patient. We could find a strong predictive role for NKT cells, which strengthens our observations in mice. Remarkably, the percentage of NKT cells in blood correlate strongly with the percentage of total T cells in the plaque ($\rho$=0.744; p<0.001) (Figure 3.9).

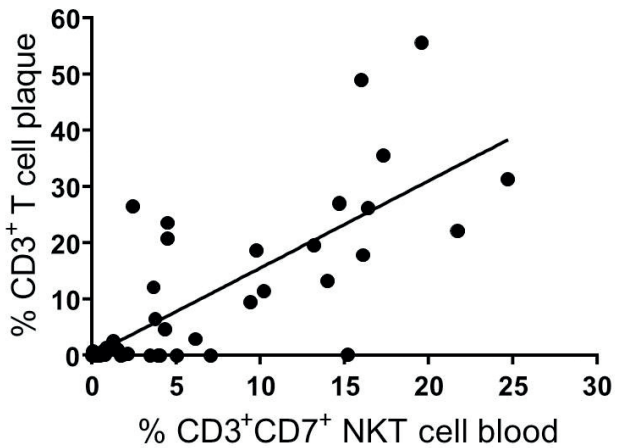


**FIGURE 3.9. Correlation analysis in human plaques and blood.** Spearman rank correlation plot showing the relationship between $CD3^+$ $CD7^+$ NKT cell numbers in blood and the percentage of total $CD3^+$ T cells in plaques obtained from the same endarterectomy patient (n = 57).

A linear regression analysis was performed to explore which of the human plaque variables can be predicted by blood variables. In all models, the logarithm of the plaque variables was entered as outcome variable. Here, we could see that the predictive value of the percentage of NKT cells in blood to predict the T cell (and NKT cell) load in the plaque is very strong ($p<0.001$). This was also the case for the prediction of the NK cell and macrophage load ($p<0.05$). Subsequently, for each of the plaque variables, a multiple linear regression model was fitted with all the risk and blood parameters as independent variables. For these models, the coefficient of determination ($R^2$) was calculated (Table 3.7). This shows that T cells ($R^2 = 0.802104$), NK cells ($R^2 = 0.744789$) and NKT cells ($R^2 = 0.727062$) in plaques are predictable by the combination of risk factors and blood variables.

**TABLE 3.7. COEFFICIENT OF DETERMINATION ($R^2$) VALUES.**

| Plaque variable (%) | $R^2$ |
|---|---|
| Total DCs | 0.484670 |
| $CD11b^+$ cDCs | 0.434602 |
| $CD16^+$ moDCs | 0.534353 |
| Macrophages | 0.500945 |
| T cell | 0.802104 |
| NK cells | 0.744789 |
| NKT cells | 0.727062 |

Coefficient of determination ($R^2$) values for each of the plaque variables calculated from a multiple linear regression model with all the risk factors and blood parameters as independent variables (n=57 plaque and blood samples). This is the amount of variance in the outcome (plaque variable) that can be explained by all the risk factors and blood variables together.

Next, stepwise backward model building was performed, starting with a model including all the plaque variables with an $R^2>0.6$, to obtain multiple regression models with only the most significant predictors for each plaque variable. Strikingly, for both the percentages of T cells and NKT cells in plaques, the NKT cell numbers in blood are the most significant predictors. In addition, partial $R^2$ values were calculated to describe how strongly the cells in blood contribute to the prediction of cells in the plaque, based on all blood parameters and risk factors from a patient. The NKT cell numbers in blood strongly improve the prediction of both the amount of NKT cells (partial $R^2 = 0.36$, $p = 8.9\times10^{-9}$) and T cells (partial $R^2 = 0.20$, $p = 1.1\times10^{-7}$) in plaques, even if all other risk factors are accounted for.

For the prediction of NK cells in plaques, the contribution of NKT cells in blood was not significant (partial $R^2 = 0.006$, $p = 0.34$).

C-reactive protein (hs-CRP) has been endorsed by multiple guidelines as a biomarker of atherosclerotic cardiovascular disease risk.[18,19] However, in this study hs-CRP levels in blood do not correlate with the percentages of NKT cells in blood or plaques from the same patient (data not shown), although this may be due to the fact that there were only few data points available for hs-CRP (n=35).

## DISCUSSION

To date, only a few studies reported the analysis and association of circulating inflammatory cells and advanced atherosclerosis. Most of the existing data comes from subclinical atherosclerosis and asymptomatic patients.[5] The aim of the present study was to analyze the frequency of immune cells in blood, plaque and associated lymphoid tissues (i.e. mouse spleen and aorta-draining LN), and to investigate whether fluctuations in leukocytes are associated with or can be predictive for plaque growth and inflammation.

The most pronounced changes during atherosclerosis in mice occur early in plaque development in cells of the innate immune system. Early atherogenesis is marked by an elevation in plasma cholesterol levels followed by (oxidative) modification of low density lipoproteins, a well-known trigger of inflammation. APCs are needed at this time to clean up these "foreign" antigens, hence more DC are present in the circulation and draining lymph nodes. As atherosclerosis progresses the number of $CD11b^+$ cDCs decline significantly at 12w of WD in all locations investigated, suggestive of massive recruitment to the growing lesions in the aortic wall. Indeed, we observed an increase in the relative expression level of SIRPα in the aorta at the same time, together with a substantial increase in plaque size in the brachiocephalic artery and proximal ascending aorta. Recruitment of immune cells to sites of inflammation, infection or injury is stimulated by chemokines and their receptors.[20-23] CCR5 directs recruitment of immune cells to inflammatory sites like atherosclerotic lesions, while CCR7 can mediate DC and monocyte/macrophage egress from lesions and controls the subsequent migration of immune cells from the plaque to secondary lymphoid organs.[23] We observed a strong inverse correlation between circulating $CD11b^+$ cDCs numbers and CCR5/7 expression in mouse aortic plaques at 12 wk of WD. Accordingly, mice that have a low number of $CD11b^+$ cDCs in their circulation, as is the case at 12 wk of WD, have high expression levels of CCR5/7 in their plaques. This indicates a high degree of leukocyte

trafficking to and from the plaque. In addition, we have also seen an inverse correlation between circulating CD11b$^{+}$ cDCs and the expression levels of T-bet, Vα14Jα18 and Zbtb46 which points to an increased inflammatory status in the plaque.

Additionally, this study revealed that plaque development and inflammation were most pronounced in mice that have a low number of circulating NKT cells at 6 wk of WD. At this time point, expression of inflammation markers, including T-bet, chemokines (CCR5/7) and cDCs (Zbtb46), as well as the degree of stenosis in the proximal ascending aorta correlated with NKT cell numbers in blood, pointing to a very significant role of NKT cells in the initiation of atherosclerosis. In line with these findings, previous research demonstrated that the contribution of NKT cells on atherosclerosis is transient and limited to early fatty streak lesions.[24,25] Similar to the observations made by Aslanian *et al.*, we detected Vα14Jα18 mRNA in early lesions (6 wk of WD) but found no accumulation of Vα14Jα18 after the 6-week time point (12 and 24 wk of WD).[24] Consistent with results from a study by Major *et al.*, we found that NKT cell numbers are low in blood and LN of ApoE$^{-/-}$ mice compared with age-matched wild-type mice at an early stage of atherosclerosis development (6 wk of diet).[26] As the lesion progresses to advanced atherosclerosis, the total DC number increased in the spleen after 12 wk and even more after 24 wk on the WD, due to systemic immune activation.[27] Additionally, this could also be the result of extramedullary hematopoiesis. Of all organs, the spleen is an ideal outsource destination in ApoE$^{-/-}$ mice.[28,29]

Taken together, these data suggest that inflammatory processes, with an emphasis on CD11b$^{+}$ cDCs and NKT cells, are crucial in the early development of atherosclerosis, before any morphological changes (plaque development) are visible. Our correlation analyses revealed that, depending on the target cell, different time points can be interesting for intervention in the atherosclerotic immune response. Specifically, we observed characteristic changes in both innate and adaptive immunity impacting on atherosclerotic lesion formation before and after 12 weeks of WD. Based on these data, we propose that an early intervention, before 12 weeks, is more favorable in order to still be able to trigger changes, especially when assessing the effects of immunomodulatory therapies for preventing the development and progression of atherosclerosis. This is in agreement with Jeon *et al.*, who reported a peak at 12 wk of diet in inflammatory mediators ICAM-1, CCR2, IL-6, IL-12p40, and IL-17.[27] Off course, the likely benefits of intervention before this time point would depend on the nature of the intervention and the degree to which the autoimmune process could be halted or reversed.

Additionally, we observed a predominance of the CD11b$^+$ cDCs subset in human plaques when compared with CD16$^+$ moDCs, while the latter is the main subset in blood. In a recent study, CD11b$^+$ cDCs were described to promote atherosclerosis development by limiting the expansion of Tregs.[30] In consonance with the drop in circulating CD11b$^+$ cDCs in mice, we and others have shown previously that circulating CD11b$^+$/BDCA-1$^+$ cDC numbers are reduced in patients with CAD.[7,31] Here, we only enrolled patients with symptomatic advanced atherosclerosis and were therefore not able to draw a comparison with asymptomatic patients. However, to our knowledge, we are the first to report a direct correlation between NKT cell numbers in blood and the load of T cells (and NKT cells) in the atherosclerotic plaques. Both CD1d-expressing cells and NKT cells were previously shown to be present in advanced human atherosclerotic plaques.[32] Here, we demonstrated that the percentage of NKT cells in blood strongly improves the prediction of both T cells and NKT cells in the plaque, independent of all the other risk factors. In line with these findings, Levula *et al.* applied gene set enrichment analysis and real-time qPCR to human advanced atherosclerotic plaques from carotid and femoral arteries as well as aortas. 26 genes, out of a total of 29 genes, of the NKT pathway were significantly upregulated in atherosclerotic plaques versus non-atherosclerotic controls.[33] Furthermore, in humans, it was reported that circulating NKT cell numbers are reduced in patients who experienced previous cardiovascular events compared with either asymptomatic atherosclerosis patients or young healthy individuals.[32] Unfortunately, data on leukocyte cell numbers in the arterial wall during early atherogenesis are virtually nonexistent as patients mostly present themselves in the clinic when serious blockages are already present. Nevertheless, in the search for better or additional biomarkers that can alert physicians for the presence of inflammatory plaques, circulating NKT cells should be further explored, as also proposed for type 2 diabetes and cancer.[34,35] We need to, however, remain cautious. Even in healthy individuals NKT cell numbers can fluctuate substantially and NKT cell subsets may play different functional roles in atherosclerosis.[36] Future studies, including a higher number of patients and different stages of atherosclerosis, will need to clarify the true potential of NKT cells as biomarkers (or even cellular therapy) for inflammatory, and thus unstable, atherosclerotic plaques.

Finally, we could not find a correlation between circulating hs-CRP levels and the percentages of NKT cells in blood or plaques, although this is most likely due to the small sample size and the associated large variation in hs-CRP levels. CRP is increased in individuals with an overlap to other risk factor pathways such as obesity, low social class and smoking.

Studies on the added value of CRP in risk prediction of cardiovascular disease show that hs-CRP levels can confirm the presence of plaques, but does not provide insight on the degree of stenosis or the inflammation in the plaque.[5]

To date, the value of circulating leukocyte profiles as biomarker of atherosclerosis is underappreciated. Despite the fact that cDCs and NKT cells are quantitatively minor components of the immune system, they do appear to play a major role in modulating the course of the disease. We believe that a profound analysis of circulating leukocytes, in particular $CD11b^{+}$ cDCs and NKT cells, may thus provide a helpful tool to assess the inflammatory and immune status of an atherosclerosis patient.

In conclusion, we provide an extensive quantitative description of systemic and peripheral immune cell dynamics over the entire life span of atherosclerotic lesion development in $ApoE^{-/-}$ mice. Based on the clear turning point in leukocyte trafficking at 12 wk of WD, we propose that a therapeutic intervention, aimed at targeting the dysregulated immune response in atherosclerosis, is to be administered before this time point. Because of its predictive value, the DC-NKT cell axis in atherosclerosis could provide potential as a tool for better patient risk stratification and/or a target for plaque stabilization, especially when determining the optimal timing for therapy.

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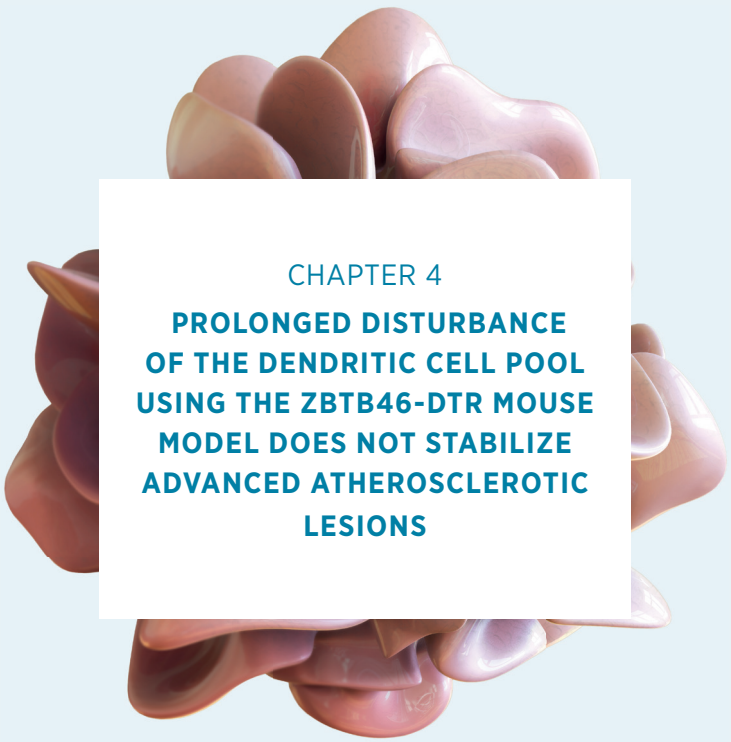

CHAPTER 4

# PROLONGED DISTURBANCE OF THE DENDRITIC CELL POOL USING THE ZBTB46-DTR MOUSE MODEL DOES NOT STABILIZE ADVANCED ATHEROSCLEROTIC LESIONS

ADAPTED FROM

**Rombouts M**, Cools N, Grootaert MOJ, de Bakker F, Van Brussel I, Wouters A, De Meyer GRY, De Winter BY and Schrijvers DM. Prolonged disturbance of the dendritic cell pool using the Zbtb46-DTR mouse model does not stabilize advanced atherosclerotic lesions. *(manuscript submitted)*

## INTRODUCTION

The pathophysiological process of atherosclerosis is marked by chronic inflammation mediated by both innate and adaptive immune responses.[1,2] DCs, the most potent APCs of the body, were found to be central in regulating these immune responses.[3,4] The presence of DCs has been reported in atherosclerotic plaques[5-8], and dyslipidemia associated with atherosclerosis was found to alter DCs activation and migration.[9] In addition, it was shown that vascular DCs play a pivotal role in the earliest accumulation of lipids in the aortic wall[10], which forces us to reconsider the widely held view that monocytes are the earliest immune cells to contribute to lesion development. The majority of murine DCs belong to the cDCs subset. In chapter 3, we described that $CD11b^+$ cDCs are the most predominant subset in human plaques and that circulating numbers of $CD11b^+$ cDCs in mice strongly correlate with inflammation in early plaque development.[11]

The past few years, researchers have attempted to clarify the specific contribution of cDCs in atherosclerotic mice, with mixed or unsatisfactory results due to the lack of selective markers expressed on individual DCs populations.[10,12-14] Recently, a novel and evolutionarily conserved zinc finger transcription factor was identified, Zbtb46 (also known as Btbd4 or zDC), which is exclusively expressed by pre-cDCs, and lymphoid organ- and tissue-resident cDCs, but not monocytes or other immune populations.[15,16] This discovery has led to the development of a new mouse model in which the receptor for diphtheria toxin (DTR) was inserted into the 3' untranslated region of the Zbtb46 locus to serve as an indicator of Zbtb46 expression and as a way to specifically deplete cDCs.[15]

In the present study, we aimed at further elucidating the contribution of cDCs, derived from pre-cDCs, in the context of atherosclerosis. To this extent, lethally irradiated, atherosclerosis-prone, $LDLr^{-/-}$ mice were transplanted with bone marrow from Zbtb46-DTR donor mice, allowing for specific depletion of cDCs following administration of DT. The impact of this depletion was studied by analyzing immune cell distribution in blood and all relevant tissues, including spleen and mediastinal lymph nodes (LN), contributing to the development of atherosclerosis.

## MATERIAL AND METHODS

### Bone marrow transplantation and atherosclerosis induction in mice

Bone marrow chimeras were obtained by means of a bone marrow transplant. Male and female 8-10 weeks old LDLr$^{-/-}$ recipient mice (The Jackson Laboratory; stock number 002207) were exposed to a single dose of 10 Gray total body irradiation using an X-RAD 320 (Precision X-ray) 3 h before reconstitution with $1 \times 10^7$ bone marrow cells from Zbtb46-DTR transgenic mice (The Jackson Laboratory; stock number 019506) via tail vein injection. Drinking water with antibiotics (0.15 mg/ml Baytril®; Bayer) and 5 g/l sucrose (Life Technologies) was introduced one week before irradiation until 3 weeks after transplantation. After a recovery period of 4 weeks, Zbtb46-DTR→LDLr$^{-/-}$ chimeras were fed a WD containing 0.2% cholesterol (4021.90; AB Diets) for 18 weeks.

### Treatment protocol

For transient cDC ablation, mice were injected intraperitoneally (i.p.) with 20 ng diphtheria toxin (DT; Sigma-Aldrich) per gram of body weight (ng/g.bw). To maintain cDCs ablation, Zbtb46-DTR→LDLr$^{-/-}$ chimeras received 4 ng DT/g.bw on the 3rd day after the initial DT injection and every 3rd day thereafter. Control mice were injected with 0.9% sodium chloride solution (Braun). At the end of the experiment, mice were euthanized with sodium pentobarbital (250 mg/kg.bw, i.p.). All animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and had free access to water and food. The animal procedures were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and all experiments were approved by the ethics committee of the University of Antwerp (permit number 2013-68).

### Culture of bone marrow-derived dendritic cells (BMDCs)

Bone marrow cells from wild type C57BL/6J mice (Jackson Laboratory; stock number 000664) and Zbtb46-DTR mice were isolated by flushing femurs and tibias. Cells were passed through a 40 μm cell strainer and subjected to red blood cell lysis (Hybri-Max; Sigma-Aldrich). After two washing steps, $1 \times 10^6$ per ml cells were seeded in complete medium, consisting of RPMI 1640 medium supplemented with GlutaMAX (Life Technologies), 5% heat-inactivated FBS (Sigma-Aldrich), 1% penicillin/streptomycin (Life Technologies), 20 U/ml polymyxin B (Fagron), 1 mM sodium pyruvate (Life Technologies), 50 μM β-mercaptoethanol (Life Technologies) and 10 ng/ml GM-CSF (PeproTech). Cells were cultured at

37°C in a 5% $CO_2$-humidified atmosphere for 6 days. At days 3 and 6, half of the culture medium was refreshed. To study the effect of DT on iDCs, half of the cultured BMDCs did not receive any maturation stimuli, and DT (2000 ng/ml) was administered on day 6. On day 7, the remaining BMDCs were activated by the addition of 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) and 1000 U/ml IFN-γ (PeproTech), together with the addition of DT (2000 ng/ml). Controls were not treated with DT. Twenty-four h after DT administration, cells were harvested for flow cytometric analysis.

### Culture of bone marrow-derived macrophages (Mφ)

Bone marrow cells from C57BL/6 mice were isolated as described above and cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 15% L-cell conditioned medium as a growth factor, at a cell concentration of $0.5 \times 10^6$ cells/ml. At day 3, half of the culture medium was replenished with complete medium. At day 6, 100 ng/ml LPS and 100 U/ml IFN-γ were added to induce differentiation into macrophages (Mφ). Twenty-four h later, cells were harvested for flow cytometric analysis.

### Flow cytometry

Zbtb46-DTR→LDLr$^{-/-}$ chimeras were euthanized and whole blood was obtained by cardiac puncture. The spleen and mediastinal LN were harvested, mechanically disaggregated and passed through a 40 μm cell strainer to obtain single cell suspensions. Red blood cells were lysed, as described above, and leukocytes were counted using a hemocytometer. Cells were resuspended in FACS buffer (PBS supplemented with 0.1% BSA (Sigma-Aldrich) and 0.05% $NaN_3$ (Merck)), preincubated for 10min with Fc blocker (anti-mouse CD16/32 antibody; BioLegend), and then stained with fluorochrome-labeled antibodies (all from BioLegend) for 30 min at 4°C, directed against either CD11c (clone N418), MHC class II (clone KH74), CD11b (clone M1/70), CD103 (clone 2E7), CD3ε (clone 1452C11), CD19 (clone 6D5), NK1.1 (clone PK136), Ly-6C (clone HK1.4), and Gr-1 (clone RB6-8C5). Cells were fixed and permeabilized prior to intracellular staining of heparin-binding epidermal growth factor-like growth factor (HB-EGF) receptor, also known as DTR. A FITC Annexin-V Apoptosis Detection Kit (556547; BD Biosciences) was used for staining of apoptotic cells in *in vitro* cell cultures. Leukocyte subsets were defined as: T cells ($CD3^+NK1.1^-$), NK cells ($CD3^-NK1.1^+$), B cells ($CD19^+$), neutrophils ($CD11b^+Gr\text{-}1^{high}$), monocytes ($Ly\text{-}6C^{low/high}$), and cDCs ($CD11c^+MHCII^+$, either $CD11b^+$ or $CD103^+$).

Immunophenotyping of BMDCs and Mφ was done by immunofluorescence staining using fluorochrome-labeled antibodies directed against CD11c (clone N418), MHC class II (clone KH74), CD115 (clone AFS98), CD169 (clone 3D6.112), CD68 (clone FA-11), and F4/80 (clone BM8; all from BioLegend). All cells were analysed on a BD Accuri C6 cytometer (Becton Dickinson). Debris and dead cells were excluded based on light scatter properties and positive staining for propidium iodide (Invitrogen). Flow cytometric analysis was done using FCS Express 4 software (De Novo Software).

### Total plasma cholesterol

Analysis of total plasma cholesterol was performed by using a colorimetric assay (Randox) according to the manufacturer's instructions.

### Real-time qPCR

Bone marrow transplantation efficiency (*i.e.* determination of chimerism) was assessed as previously described by Kanters *et al.*[17] In brief, qPCR analysis of the LDLr$^{-/-}$ gene was performed on bone marrow cells. Analysis of the household gene P50 was used as a control. Purification of RNA was performed by means of an Absolutely RNA miniprep kit (Agilent technologies). For cDNA synthesis a SuperScript II Reverse Transcriptase kit (Life Technologies) was used. A SensiMix SYBR Hi-ROX kit (Bioline) was used to perform a SYBR Green-based qPCR. A standard curve was generated from a dilution series mix of 100%:0% to 0%:100% LDLr$^{-/-}$:LDLr$^{+/+}$ genomic DNA and obtained by plotting the mean Δ Ct ($Ct_{P50} - Ct_{LDLr-/-}$) against the logarithm of the percentage LDLr$^{-/-}$, followed by the calculation of a regression line. Finally the percentage of LDLr$^{-/-}$ DNA, determined by applying the mean Δ Ct of the sample to the standard curve, was used to calculate the degree of chimerism. RNA from BMDCs and macrophages was purified and reverse transcribed as described above. A SYBR Green-based qPCR was used to determine the expression of Zbtb46, which was normalised to the expression of two household genes (b2m and GAPDH). Used primer pairs are listed in Table 4.1. All samples were assayed in duplicate using a 7300 Real Time PCR System (Applied Biosystems). All data were analyzed with the qBase+ software (Biogazelle).

TABLE 4.1. PRIMER SEQUENCES.

| Gene | Sequence sense | Sequence antisense |
|---|---|---|
| **b2M** | CAGCATGGCTCGCTCGGTGAC | CGTAGCAGTTCAGTATGTTCG |
| **GAPDH** | CCAGTATGACTCCACTCACG | GACTCCACGACATACTCAGC |
| **LDLr$^{-/-}$** | GCTGCAACTCATCCATATGCA | GGAGTTGTTGACCTCGACTCTAGAG |
| **P50** | AACCTGGGAATACTTCATGTGACTAA | GCACCAGAAGTCCAGGATTATAGC |
| **Zbtb46** | TCACATACTGGAGAGCGGC | CCTCATCCTCATCCTCAACC |

Abbreviations: b2M, β-2 microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDLr$^{-/-}$, low-density lipoprotein receptor-deficient; P50, DNA binding subunit of the NF-κB protein complex; Zbtb46, zinc finger and BTB domain containing transcription factor 46.

### Histology and immunohistochemistry

At sacrifice, the aortic root was collected, embedded in Neg-50 (Thermo Scientific) and snap frozen in liquid nitrogen. Atherosclerotic plaque size was analysed on Oil Red O (Sigma-Aldrich) stained cryosections. Collagen content was measured by Sirius red (Sigma-Aldrich) staining. Immunohistochemical stainings were performed for α-smooth muscle actin (α-SMA; Sigma-Aldrich), CD3 (Abcam), and cleaved caspase-3 (Cell Signaling Technology). After incubation with anti-FITC HRP-labeled secondary antibody (for α-SMA) or species-specific HRP-labeled secondary antibodies (for CD3 and cleaved caspase-3), and reactive avidin-biotin complex, tissue sections were stained with 3,3'-diaminobenzidine (for α-SMA) or 3-amino-9-ethylcarbazole (for CD3 and cleaved caspase-3). A shortened version of the Llewellyn protocol was applied to a limited number of spleens before staining with antibodies.[18] First, spleens were fixated in 4% paraformaldehyde for 2 hours at room temperature. Next, tissues were rinsed twice during 10 min in PBS, followed by an overnight incubation in a 20% sucrose solution at 4°C. The next day, tissues were snap-frozen in Neg-50. Cryosections were double stained with rat anti-CD68 (AbD Serotec) and rabbit anti-cleaved caspase-3. Sections were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories) and visualised by fluorescence microscopy. All other images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope and quantified with ImageJ software (National Institutes of Health).

### Statistical analysis

Statistical analysis was performed using SPSS Statistics 23.0 (IBM) by means of a Student t test, One-way ANOVA (followed by Tukey's Multiple Comparison post-hoc test), or Two-way ANOVA (followed by Bonferroni's Multiple Comparison post-hoc test), as appropriate. Univariate analyses were performed for plaque area and composition of aortic root sections. Data that failed the Levene's test of homogeneity of variances were mathematically transformed before statistical analysis was performed. Graphs were created with GraphPad Prism 6 (GraphPad Software). Data are shown as mean ± SEM. Differences were considered significant when $p<0.05$.

## RESULTS

### The Zbtb46-DTR mouse model enables cDC depletion *in vitro* and *in vivo*

To validate the sensitivity of cDCs to DT and the competence of the Zbtb46-DTR mouse model to deplete cDCs, *in vitro* and *in vivo* experiments were performed. First, the selectivity of Zbtb46 for DCs was examined by comparing its expression in *in vitro* cultures of BM-derived DCs and Mφ. As shown in Figure 4.1A, Mφ robustly express signature cell surface markers CD68, CD169, and F4/80, compared to iDCs and mDCs. The M-CSF receptor CD115 is expressed on Mφ as well as on iDCs, but maturation with LPS and IFN-γ induces down-regulation of CD115 expression. It was shown that CD115 is expressed on DCs during differentiation and the down-regulation of CD115 appears to be a critical component in driving DCs (as opposed to Mφ) development.[19] It was reported that Zbtb46 is expressed in $CD11c^+$ DCs generated in BM cultures treated with GM-CSF.[16] Indeed, qPCR analysis confirmed that Zbtb46 is selectively expressed in our BMDCs and not in Mφ (Figure 4.1B).

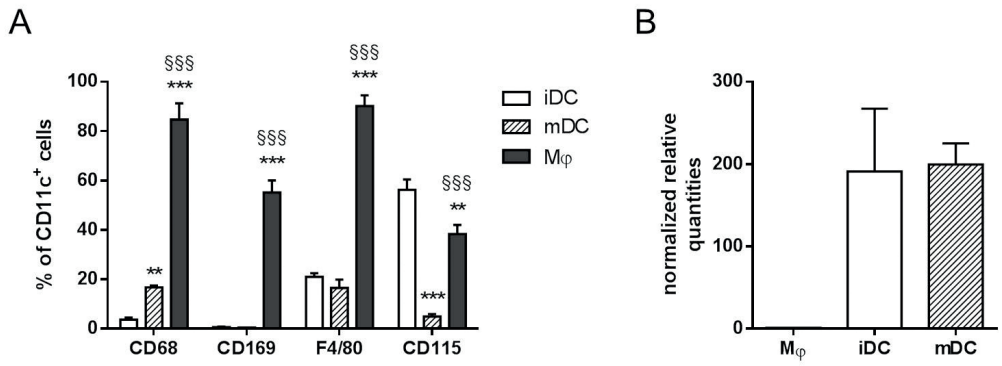


**FIGURE 4.1. Expression of Mφ markers and Zbtb46 in the cultures of wild-type BM-derived iDCs, mDCs and Mφ**. (A) Flow cytometric analysis of CD68, CD169, F4/80 and CD115 expression on iDCs (n=5), mDCs (n=5), and Mφ (n=2-4); **p<0.01, ***p<0.001, significantly different from iDC; §§§p<0.001, significantly different from mDC. (B) The expression of Zbtb46 mRNA in iDCs and mDCs (n=2) measured by real-time qPCR was determined relative to the Zbtb46 mRNA expression in Mφ (n=2) and normalised to the average expression of two household genes: b2M and GAPDH.

Next, treatment of BMDCs of Zbtb46-DTR mice with 2000 ng/ml DT resulted in a significantly lower proportion of immature and mature cDCs (Figures 4.2A,B) that could be recovered from the *in vitro* cell culture. This reduction was accompanied by a concomitant increase in Annexin-V-positive cells (Figure 4.2C). By contrast, immature and mature BMDCs from wild-type mice were insensitive to DT treatment (Figures 4.2D,E), as evidenced by similar proportions of Annexin-V-positive cells following DT treatment (Figure 4.2F).

The *in vivo* experiment was performed following the method described by Meredith *et al.*[15]. In brief, Zbtb46-DTR mice were sacrificed 14h after a single injection with vehicle or 20 ng DT/g.bw. Flow cytometric analysis of splenocytes demonstrated a significant drop (72%) in the percentage of total cDCs 14 h after DT injection (Figures 4.3A,B). As part of the *in vivo* validation of the Zbtb46-DTR model, a cleaved caspase-3 staining was applied to a limited number of spleens. Quantification of the number of cleaved caspase-3-positive cells showed significant difference between the two groups, thereby confirming elevated *in vivo* cell death after DT administration (Figures 4.3C,D). Moreover, the apoptotic cells are not macrophages as CD68 staining did not colocalise with cleaved caspase-3 positive cells (Figure 4.3C).

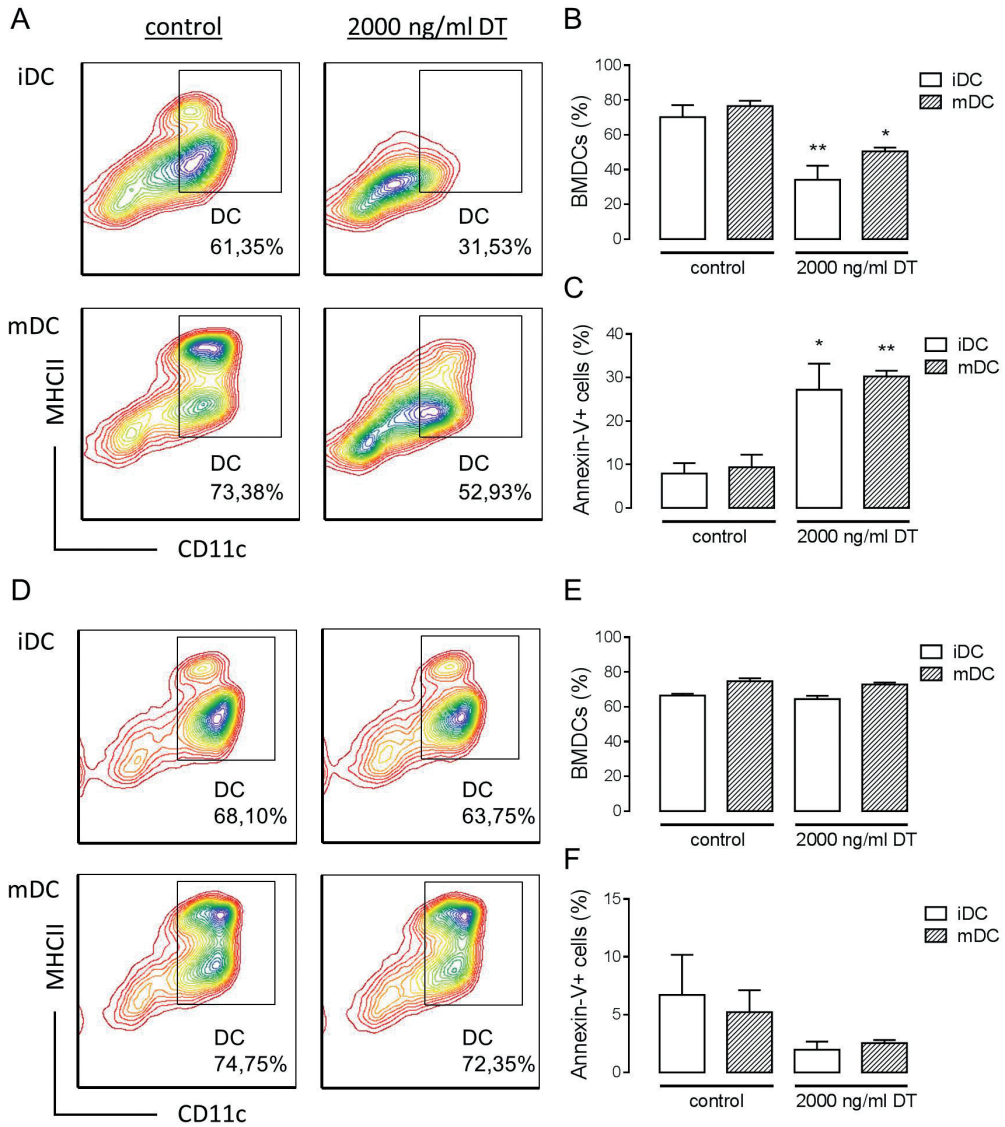


**FIGURE 4.2. *In vitro* validation of the Zbtb46-DTR model to deplete cDCs.** (A,D) Representative contour plots of *in vitro* immature (iDC) and mature (mDC) BMDCs from Zbtb46-DTR (A) or wild-type mice (D) treated with 2000 ng/ml DT for 24 h or left untreated (control); (B,E) Quantification of flow cytometric analysis of BMDCs from Zbtb46-DTR (B) or wild-type mice (E) treated with DT (2000 ng/ml, 24 h) or left untreated (control) (n=2-4); (C,F) Quantification of *in vitro* cultures of BMDCs from Zbtb46-DTR (C) or wild-type mice (F) labeled with FITC Annexin-V for the detection of apoptotic cells after no treatment (control) or treatment with DT (2000 ng/ml, 24 h) (n=2-4); *p<0.05, **p<0.01.

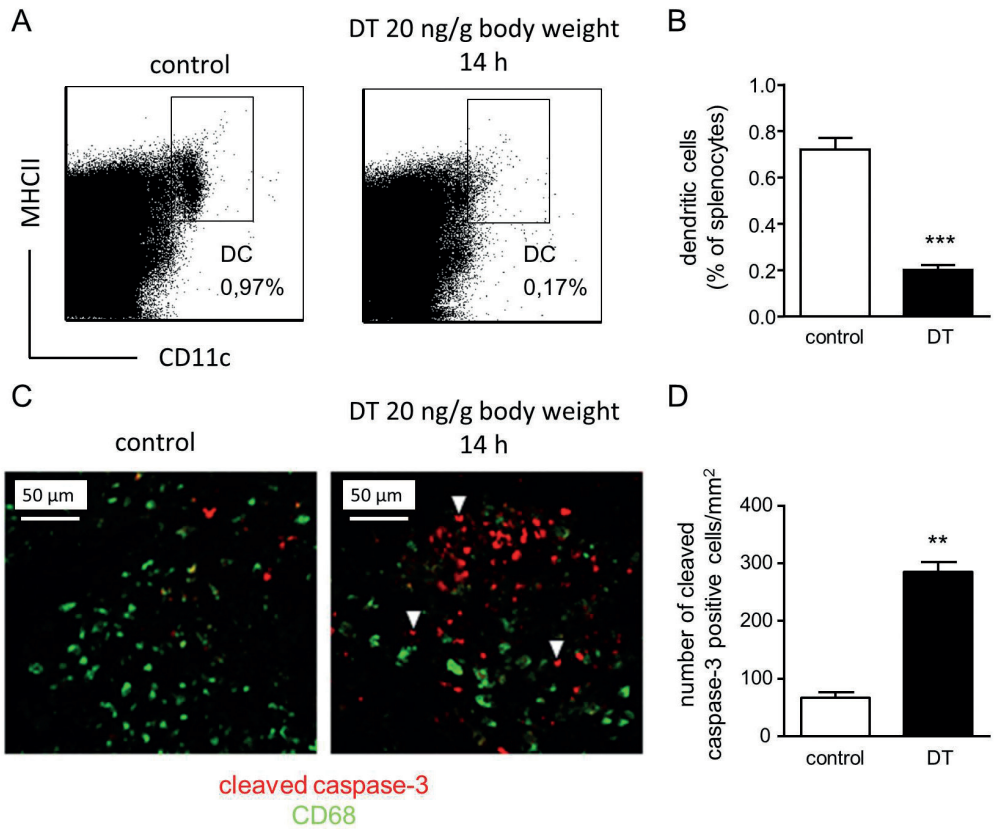


**FIGURE 4.3.** ***In vivo*** **validation of the Zbtb46-DTR model to deplete cDCs.** (A,B) Representative dot plots (A) and flow cytometric analysis (B) of splenocytes of Zbtb46-DTR mice sacrificed 14 h after a single injection with vehicle (control, n=6) or 20 ng DT/g. bw (n=5); (C) Cleaved caspase-3 (red, arrows) and CD68 (green) staining of spleens from Zbtb46-DTR mice treated with vehicle (control) or DT (20 ng/g.bw, 14 h) (scale bar = 50 µm); (D) Quantification of cleaved caspase-3 positivity in spleens (average of 3 measurements per mouse, n=2 mice per group); **p<0.01, ***p<0.001.

**Long-term DT treatment does not affect leukocyte profile in blood, spleen and LN**

Similar to CD11c-DTR mice, it is described that a single injection of DT is lethal in Zbtb46-DTR mice within 24-48h, probably due to the expression of Zbtb46 in committed erythroid progenitors and endothelial cells. Therefore, experiments involving prolonged DT administration necessitate the use of radiation chimeras in which non-hematopoietic cells remain of recipient origin, insensitive to DT.[15,20] In this study, lethally irradiated LDLr$^{-/-}$ mice were grafted with bone marrow cells from Zbtb46-DTR mice to generate Zbtb46-DTR→LDLr$^{-/-}$ chimeras. A degree of chimerism of > 90% was considered successful. On average, a chimerism degree of 94.1 ± 2.4% was achieved.

For the long-term administration of DT (18 weeks), the treatment protocol was followed as described by Meredith *et al.*[15], which consisted of injections of Zbtb46-DTR→LDLr$^{-/-}$ chimeric mice every 3 days with 4 ng/g.bw DT. Upon sacrifice, blood, spleen, and mediastinal LN were collected for flow cytometric analysis. Total leukocyte numbers in blood, spleen, and LN did not differ between the control and treatment group (Figure 4.4A). Total cDCs were significantly reduced from blood and spleens of DT-treated Zbtb46-DTR→LDLr$^{-/-}$ chimeric mice (Figure 4.4B). No differences could be seen in cDCs numbers in the LN, nor in CD11b$^{+}$ and CD103$^{+}$ cDC subsets (Figures 4.4C,D). As leukocytes crucially affect atherosclerosis, we examined the effect of chronic DT treatment of Zbtb46-DTR→LDLr$^{-/-}$ chimeras on other leukocyte subpopulations as well. Flow cytometric analysis of blood, spleen and LN did not reveal any significant differences in the proportion of T cells, B cells, NK cells or neutrophils (Figures 4.4E-H). The percentage of Ly-6C$^{low}$ and Ly-6C$^{high}$ monocytes in blood did not differ between the control and treatment group (Figure 4.4I).

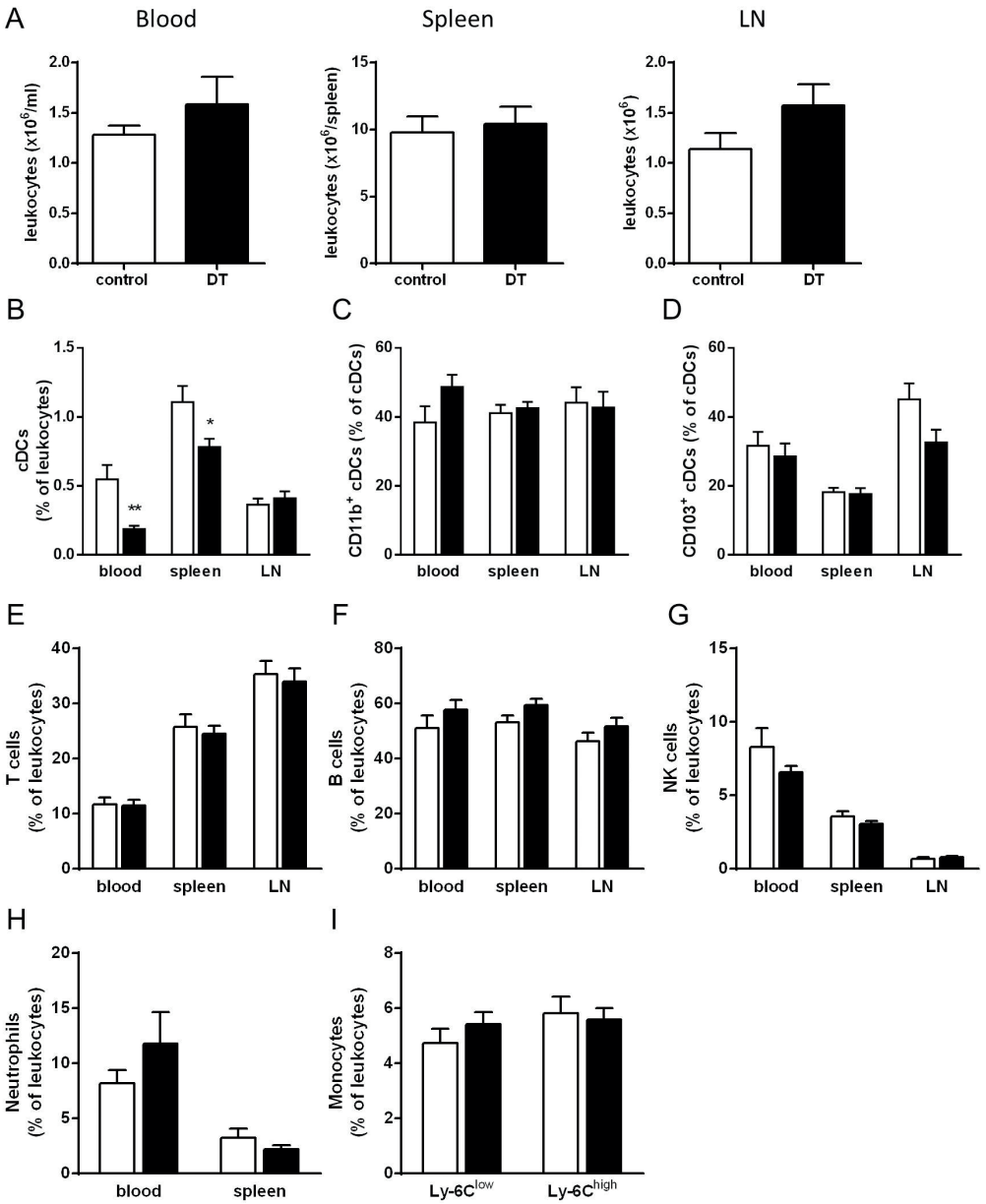


**FIGURE 4.4. Long-term DT treatment of Zbtb46-DTR→LDLr$^{-/-}$ mice does not alter cDCs or leukocyte numbers.** (A) Absolute numbers of leukocytes after 18 weeks of WD feeding in vehicle (control) and DT-treated Zbtb46-DTR→LDLr$^{-/-}$ mice in blood, spleen, and LN (n=17-19); (B-D) Flow cytometric analysis of total cDCs (B) and CD11b$^{+}$ (C) and CD103$^{+}$ (D) cDC subsets in blood, spleen, and LN of control and DT-treated Zbtb46-DTR→LDLr$^{-/-}$ mice (n=14-19); (E-I) Flow cytometric analysis of T cells (E), B cells (F), NK cells (G), neutrophils (H) in blood, spleen and LN, and of Ly-6C$^{low/high}$ monocyte subsets (I) in blood of control and DT-treated Zbtb46-DTR→LDLr$^{-/-}$ mice (n=16-19); *p<0.05; **p<0.01.

### Chronic DT administration does not affect atherosclerosis in Zbtb46-DTR→LDLr$^{-/-}$ mice

In parallel with the flow cytometric analysis, we examined whether long-term DT administration had an impact on atherosclerosis. Therefore, Zbtb46-DTR→LDLr$^{-/-}$ chimeric mice were fed a WD diet while injected with 4 ng DT/g.bw every 3 days for 18 weeks. DT administration was well tolerated, with no differences in body weight between the control and experimental group (Table 4.2). Noteworthy, as a result of the irradiation therapy, the fur of all mice grayed. Analysis of the spleen weight revealed a significant decrease in DT-treated mice, while there was no significant difference for heart weight between the two groups (Table 4.2). Furthermore, plasma cholesterol levels did not differ between the control (733.5 ± 57.2 mg/dl) and DT-treated group (755.1 ± 38.3 mg/dl).

TABLE 4.2. WEIGHT AND PLASMA CHARACTERISTICS.

| | Control | DT |
|---|---|---|
| Body weight (g) | 26±1 | 27±1 |
| Spleen weight (mg) | 133±9 | 108±7* |
| Heart weight (mg) | 130±4 | 116±3 |
| Plasma cholesterol (mg/dl) | 734±57 | 755±38 |

Data from vehicle (control) and DT-treated Zbtb46-DTR→LDLr$^{-/-}$ mice after 18 weeks of WD feeding, mean ± SEM, control n=19, DT n=16-17; *p<0.05.

Oil Red O stained cryosections were used to determine the plaque area. Plaques in the aortic root did not show a difference in size between control and DT-treated chimeras (Figure 4.5A). Features of plaque stability were similar in both groups, as no changes were observed in vascular smooth muscle cell content (Figure 4.5B) and total collagen (Figure 4.5C). In concordance with the observations regarding plaque size or stability, T cell content did not differ between control and DT treated chimeras (Figure 4.5D). DT binds to HB-EGF (which acts as DTR) and, following internalisation, inhibits protein synthesis, which rapidly induces apoptosis.21 Nonetheless, staining of plaques with cleaved caspase-3 antibody did not show a significant increase in plaque apoptosis between control and DT-treated chimeras (Figure 4.5E).

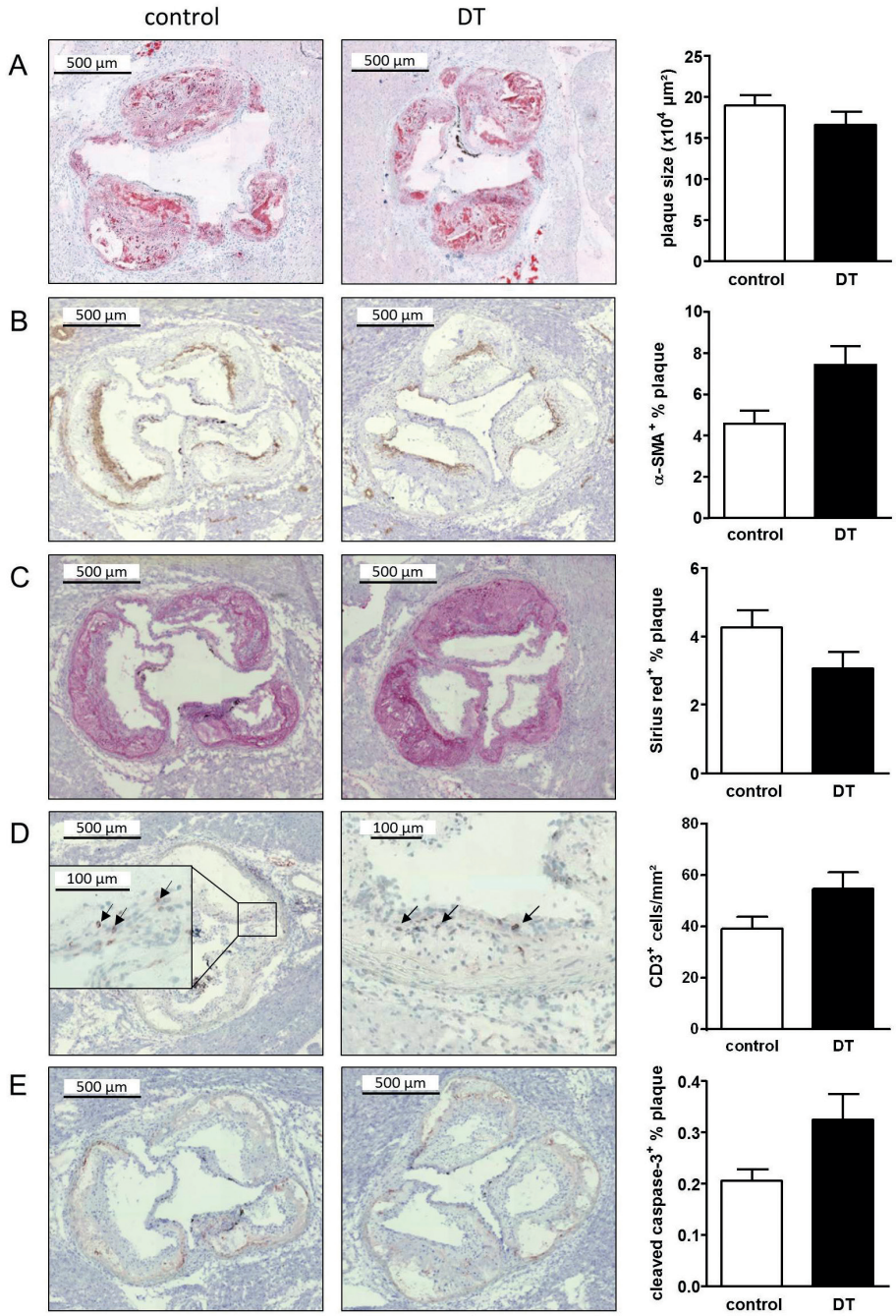


**FIGURE 4.5. Chronic DT administration does not affect atherosclerotic plaque size and composition in Zbtb46-DTR→LDLr$^{-/-}$mice.** Representative images and quantification of (A) atherosclerotic lesion size (Oil Red O+ area), (B) vascular smooth muscle cells (α-SMA staining), (C) collagen (Sirius Red staining), (D) T cells (arrows), and (E) apoptosis (cleaved caspase-3 staining), in aortic root cryosections of control and DT-treated Zbtb46-DTR→LDLr$^{-/-}$ mice (n=15-19).

### Insufficient cell depletion cannot be attributed to loss of DTR expression

Previously, it was described that following continuous DT treatment a cell population lacking DTR expression and hence resistant to further DT-mediated depletion emerged.[22,23] In order to exclude the possibility that the lack of an effect on atherosclerosis development is due to the loss of the DTR on DCs, we compared intracellular DTR expression by flow cytometric analysis between splenic cDCs from Zbtb46-DTR mice before DT treatment and splenic cDCs from Zbtb46-DTR→LDLr$^{-/-}$ chimeras at the end of the study. Splenic cDCs from LDLr$^{-/-}$ mice were used as a control. As shown in Figure 4.6, splenic cDCs from Zbtb46-DTR mice before DT treatment as wells as from Zbtb46-DTR→LDLr$^{-/-}$ chimeras after chronic DT treatment robustly express intracellular DTR.

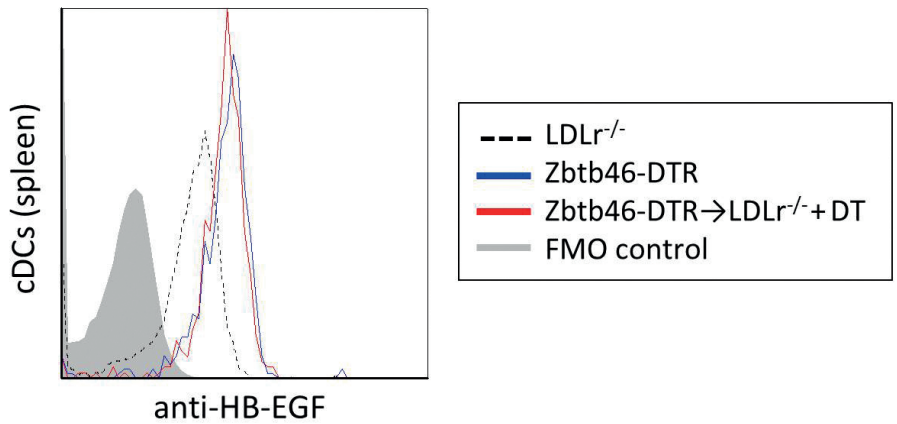


**FIGURE 4.6. Insufficient depletion of cDCs cannot be attributed to emergence of DTR-negative cells.** Representative histogram of intracellular human DTR expression (anti-HB-EGF) assessed by flow cytometry of splenic cDC in LDLr$^{-/-}$ mice (dotted black), Zbtb46-DTR mice (blue) and Zbtb46-DTR→LDLr$^{-/-}$ mice (DT-treated, red) at the end of the study. An FMO sample is used as control (grey).

## DISCUSSION

Up till now, investigating the contribution of cDCs to atherosclerosis was hampered by the fact that there is no selective marker exclusively expressed by cDCs. Nevertheless, depletion of DCs was first achieved by Jung *et al.* who used the murine CD11c promotor for the transgenic expression of human DTR.[12] When bred onto the LDLr$^{-/-}$ background, DT treatment resulted in a marked reduction in intimal CD11c$^{+}$ cells and a 55% decrease in accumulation of lipids during the earliest stages of plaque formation.[10] Prolonging the lifespan of DCs (and CD11c-expressing macrophages) in mice that carry CD11c-specific expression of the anti-apoptotic hBcl2, did not accelerate plaque progression.[14] Moreover, cholesterol levels were reduced in these mice, whereas depletion of DCs resulted in enhanced cholesterolemia, indicative of a potential role for DCs in cholesterol homeostasis.[14] Nevertheless, a major drawback of using the CD11c-DTR mouse model is the depletion of other immune cells besides DCs, also expressing CD11c such as metallophilic and marginal zone macrophages.[24] Depletion of monocytes and macrophages in CD11b-DTR transgenic mice was shown to differentially affect atherosclerosis. Unfortunately, the authors did not investigate whether the CD11b$^{+}$ cDC subpopulation was also depleted.[25] Constitutive deficiency of the chemokine receptor $CX_3CR1$ in ApoE$^{-/-}$ mice resulted in an impaired accumulation of DCs in the aortic wall and reduced atherosclerotic burden at early stages.[26] However, $CX_3CR1$ is also required for monocyte recruitment and survival.

Here, we used the recently developed Zbtb46-DTR mouse model which allows specific depletion of cDCs and their precursors, while sparing other hematopoietic cells.[15,16] Zbtb46 acts as a suppressor of DC activation and MHC class II expression in the immature state.[27] In the present study, we aimed at dissecting the exact role of cDCs in the context of atherosclerosis. A limitation of the Zbtb46-DTR mouse model, however, is the mortality upon repeated injections of DT, most likely due to the expression of Zbtb46 in committed erythroid progenitors and endothelial cells.[15,16] Therefore, lethality was overcome by the generation of bone marrow chimeras, by transplanting LDLr$^{-/-}$ mice with bone marrow from Zbtb46-DTR donor mice, which restricts the expression of Zbtb46-DTR to the hematopoietic compartment.[20]

In analogy to the experiments conducted by Meredith *et al.*[15], our *in vitro* and *in vivo* validation experiments of the Zbtb46-DTR mouse model confirmed that cDCs could be depleted upon injection of DT. However, we were not able to reach the same degree of depletion as reported by Meredith *et al.*[15], who demonstrated a near complete ablation of

cDCs in spleens of DT-treated Zbtb46-DTR mice. On the other hand, even incomplete depletion in CD11c-DTR mice (with 70-80% efficacy) was reported to alter the balance of the immune response[28], and decrease inflammatory cytokine production in atherosclerosis.[29] Here, depletion of cDCs could not be fully sustained for a longer period of time in Zbtb46-DTR→LDLr$^{-/-}$ chimeras, despite continuous DT injections. Except for a partial depletion in blood and spleen, no depletion of cDCs was observed in LN after 18 weeks of DT treatment. In most cases, only short term DT administration is used to evaluate depletion efficiency *in vitro* and *in vivo*. In general, studies examining atherosclerosis in DTR mouse models for more than 2 weeks are scarce and often do not detail on DC numbers in the circulation or associated lymphoid tissues. On the basis of previously published reports regarding the kinetics of DC depletion in DTR transgenic mice, a twice-weekly DT administration regimen was chosen for the long-term depletion of cDCs in Zbtb46-DTR→LDLr$^{-/-}$ chimeras.[12,15] Because DCs are such potent stimulators of T cells, any partial depletion would probably not be sufficient to suppress the development of atherosclerosis in Zbtb46-DTR→LDLr$^{-/-}$ chimeras over a longer period of time. Very recently, an inducible system for cDC depletion was developed by Loschko *et al.*[30] who introduced a loxP-flanked transcriptional Stop element in front of the DTR, and inserted this cassette into the Zbtb46 gene (zDC$^{lslDTR}$). To restrict DTR expression to cDCs, zDC$^{lslDTR}$ mice were crossbred with transgenic mice expressing Cre under the control of the Csf1 receptor gene (Csf1r$^{Cre}$).[30] In Csf1r$^{Cre+}$zDC$^{lslDTR}$ mice expression of DTR under the control of Zbtb46 is restricted to cDCs which bypasses endothelial cells and omits the use of bone marrow chimeras. cDCs can be efficiently depleted for at least 4 weeks in these mice. However, as previously demonstrated[31], the authors reported increased levels of Flt3L upon cDC depletion. Thus, it is unclear whether or not cDC depletion is sustained longer than 4 weeks.

Although in our hands incomplete depletion does not impact atherosclerosis, this is not always the case. Others reported that DT treatment of ApoE$^{-/-}$CD11c-DTR bone marrow chimeras resulted in a significant DC depletion of approximately 78% in the aorta and spleen. As a result, transient depletion reduced oxLDL uptake and cytokine expression in the aortas of DT-injected mice. However, these mice were fed a WD for 12 weeks, of which they were injected with DT during the last 2 weeks, which is a different setup as we started DT treatment before the onset of atherosclerosis followed by a much longer duration of treatment. Hence, no decision can be taken on the possible long-term effects in that study.[29] Nevertheless, it is possible that 18 weeks of DT treatment is too long in order to observe an effect on plaque development, because these mice are already in a more progressive stage of

disease. However, even over a shorter period of time (12 weeks), partial depletion of cDCs had no effect on the development of atherosclerosis (data not shown).

Others recently suggested the emergence of targeted cell populations that have lost DTR expression as an explanation for insufficient cell depletion in DTR models.[22,23] However, our results demonstrate that, even after long-term DT treatment, the receptor for DT is present on splenic cDCs to the same extent as on splenic cDCs from untreated Zbtb46-DTR mice. Zbtb46 expression was shown to be restricted to cDC precursors (pre-cDCs) and lymphoid organ- and tissue-resident cDCs. However, Satpathy *et al*.[16] demonstrated that a major subpopulation of pre-cDCs in the bone marrow expressed the plasmacytoid (p)DC marker SiglecH, and that by inserting a GFP reporter cassette into the Zbtb46 locus, four distinct pre-cDC populations could be defined by Zbtb46 *vs.* SiglecH expression. Importantly, they showed that, although SiglecH is specific for pDCs in peripheral organs, bone marrow progenitors expressing SiglecH but not Zbtb46, retain the potential to develop into cDCs.[16] Hence, although in the Zbtb46-DTR mouse model cDCs and Zbtb46-expressing pre-cDCs are depleted, other pre-cDCs capable to replenish cDCs in the periphery may exist in the bone marrow. In this perspective others demonstrated that Zbtb46 deficiency alters the cDC subset composition in the spleen in favor of $CD8^+$ cDCs.[27] Whether this could have contributed to the immune-mediated pathogenesis seen here should be further examined. Nevertheless, we found no differences in the proportions of $CD11b^+$ and $CD103^+$ cDCs between control and DT-treated mice. Additionally, Zbtb46-DTR mice provide a model to ablate pre-cDC-derived cDCs while sparing other hematopoietic cells.[15] However, as a consequence of hypercholesterolemia, blood monocytes can be recruited into the intima where they can give rise to phenotypically similar monocyte-derived $CD11b^+$ DCs populations making it difficult to observe a relative contribution of cDCs derived from pre-DCs versus those derived from monocytes in the context of atherosclerosis.

Taken together, as a consequence of the unsustained cDC depletion in this study, no clear decision can be taken on the role of cDCs in atherosclerosis. Because cDCs are such potent activators of T cells, any partial depletion would probably not be sufficient to suppress the development of atherosclerosis, which underlines the unsuitability of Zbtb46-DTR→$LDLr^{-/-}$ mice for studying the involvement of cDCs in atherosclerosis, or other models of chronic inflammation, over extended periods of time. Whether or not *bona fide* cDCs significantly contribute to atherosclerotic plaque formation and stability remains to be determined.

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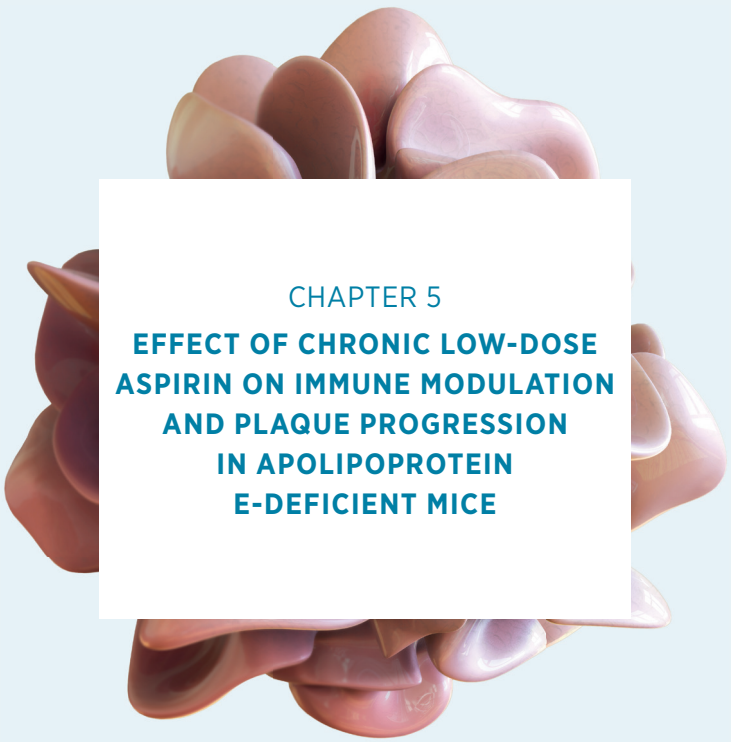

CHAPTER 5

# EFFECT OF CHRONIC LOW-DOSE ASPIRIN ON IMMUNE MODULATION AND PLAQUE PROGRESSION IN APOLIPOPROTEIN E-DEFICIENT MICE

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## INTRODUCTION

The mechanisms of action of aspirin, or acetylsalicylic acid (ASA), and its subsequent biological effects vary with dose. Low doses (80-100 mg/day) are sufficient to irreversibly inhibit cyclooxygenase (COX)-1, resulting in an antithrombotic effect. In line with this, ASA has been used for many years in the treatment and prevention of cardiovascular disease.[1-3] Intermediate doses (650 mg - 4 g/day) inhibit both COX-1 and COX-2, thereby blocking prostaglandin (PG) production, and resulting in analgesic and antipyretic effects. At high doses (4-8 g/day), ASA has anti-inflammatory effects mediated by both PG-dependent and independent pathways.[4] However, the usefulness of ASA at high doses is limited by toxicity, including tinnitus, hearing loss, and gastric intolerance.

Many animal studies have investigated the effect of ASA on atherosclerotic plaque development, though the observed effects vary among the different studies (Table 5.1). In most cases, ASA treatment was started before the onset of atherosclerosis.[5-11] However, it would be clinically more relevant to study the effect of ASA on established atherosclerotic lesions.[12] Some studies showed no effect on lesions after treatment with ASA for 8-12 weeks regardless of the dose.[8,9,13]

A considerable amount of data emerged during the last few years suggesting that ASA can cause immune modulation via several mechanisms. Indeed, *in vitro* studies with human moDCs and mouse BMDCs cultured in the presence of 0.5-2.5 mM ASA revealed a dose-dependent inhibitory effect on DCs.[14-16] Mechanisms of tolerance induction include inhibition of the maturation of DCs, suppression of the NF-κB signaling pathway, impaired IL-12 production, reduced expression of co-stimulatory molecules, and increased expression of immunoglobulin-like transcript-3, a feature of tolerogenic DCs.14-16 Importantly, ASA treatment reduced the allo-stimulatory capacity of DCs, and induced hypo-responsiveness and regulatory activity in responder naive and memory T cells.[15-17]

The goal of the present study was to test whether low-dose ASA, corresponding with the dose used in humans for primary and secondary prevention of cardiovascular disease, reduces plaque progression in ApoE$^{-/-}$ mice with established atherosclerosis mediated by peripheral immune modulation.

TABLE 5.1. OVERVIEW OF THE LITERATURE CONCERNING ASA TREATMENT IN MOUSE MODELS OF ATHEROSCLEROSIS.

| Author & Year | Mouse model | Dose of ASA | Start of ASA treatment | Duration of ASA treatment | Effect on plaques | Summary |
|---|---|---|---|---|---|---|
| Paul *et al.*, 2000 | ApoE$^{-/-}$ | 15 mg/kg/d | start ASA with WD | 10w | Yes | 35% reduction in the size of plaques |
| Cayatte *et al.*, 2000 | ApoE$^{-/-}$ | 30 mg/kg/d | start ASA with WD | 11w | No | no significant effect on atherogenesis or on adhesion molecule levels |
| Cyrus *et al.*, 2002 | LDLr$^{-/-}$ | 5 mg/kg/d | start ASA with WD | 18w | Yes | decreased size and increased stability of plaques; decrease in circulating and vascular levels of adhesion and proinflammatory molecules |
| Tous *et al.*, 2004 | ApoE$^{-/-}$ | 15 mg/kg/d | start ASA with WD | 5w<br>12w<br>16w | Yes<br>No<br>No | time-dependent effects of ASA: smaller lesions (5w), lesions equal in size (12w), larger lesions compared to controls (16w) |
| Cyrus *et al.*, 2006 | LDLr$^{-/-}$ | 5 mg/kg/d | start ASA **after 12w of WD** | 12w | Yes | significant reduction in the progression of atherosclerosis; less vascular inflammation |
| Schulz *et al.*, 2008 | ApoE$^{-/-}$ | 5 mg/kg/d | start ASA with WD | 8-12w | No | no significant effect on atherosclerosis |
| Liu *et al.*, 2010 | ApoE$^{-/-}$ | 58 mg/kg/d | start ASA with WD | 12w | Yes | smaller atherosclerotic lesions; decreased fractalkine expression in the aorta |
| Hoving *et al.*, 2010 | ApoE$^{-/-}$ | 30 and 300 mg/kg/d | start ASA with chow diet | 4w and 30w | Partially | partial inhibition of age-related but not radiation-induced atherosclerosis |
| Yamamoto *et al.*, 2010 | ApoE$^{-/-}$ LDLr$^{-/-}$ | 4 mg/kg/d<br>40 mg/kg/d | start ASA with chow diet | 12w | Yes<br>No | low dose more efficient than high dose; reduced aortic lesions |
| Kraus *et al.*, 2014 | ApoE$^{-/-}$ | 0.1 mg/ml | start ASA with chow diet | 10w | Yes | decreased atherosclerotic root lesion area compared to control mice |
| Yang *et al.*, 2016 | ApoE$^{-/-}$ | 5 mg/kg/d<br>20 mg/kg/d<br>50 mg/kg/d | start ASA 4w after collar-placement | 8w | No<br>Yes<br>Yes | reduced atheroprogression; increased plaque stability in carotid atherosclerotic plaques induced by collar-placement |
| Sorokin *et al.*, 2016 | ApoE$^{-/-}$ | 0.1 g/kg diet/d | start ASA with omega-3 rich diet | 13w | Yes | less aortic lesions and inflammatory lipid mediators in mice on the fish oil diet plus ASA vs. fish oil diet alone |
| Roth *et al.*, (unpublished) | ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ | 5 mg/kg/d | start ASA **after 10w of WD** | 15w | No | no differences in plaque size, vulnerability, incidence of MI and survival; decrease in systolic blood pressure; attenuation of cardiac hypertrophy and coronary fibrosis |

Abbreviations: ASA, acetylsalicylic acid; Fbn1$^{C1039G+/-}$, heterozygous mutation in the fibrillin-1 gene; MI, myocardial infarction; WD, Western-type diet

## MATERIAL AND METHODS

### Mice

Female ApoE$^{-/-}$ mice were fed a WD (4021.90; AB Diets) starting at an age of 6 weeks. The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and had free access to water and food. At the end of the experiment (25 weeks WD), mice were euthanized with sodium pentobarbital (250 mg/kg, i.p.) and blood samples were obtained from the retro-orbital plexus. Analysis of total plasma cholesterol was performed by using a colorimetric assay (Randox) according to the manufacturer's instructions. The animal procedures were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and all experiments were approved by the ethical committee for animal testing of the University of Antwerp.

### Acetylsalicylic acid

At 10 weeks of WD, ApoE$^{-/-}$ mice were randomly divided in 2 groups, receiving either ASA (Aspegic®, 9 mg lysine acetylsalicylate is equivalent to 5 mg ASA; 5 mg/kg/day) added to the drinking water for a period of 15 weeks or plain drinking water as a control. In order to guarantee product stability, the drinking water supplemented with ASA was replenished every day.

### Flow cytometry

Blood samples were collected as described above. Single cell suspensions of the spleen and the para-aortic lymph nodes (LN) were prepared by passage through a 40μm cell strainer. Red blood cells were eliminated by using a red blood cell lysis buffer (Sigma-Aldrich). Staining was performed at 4°C in the presence of CD16/32 Fc-receptor blocker (BioLegend) in FACS buffer (PBS supplemented with 0.1% BSA and 0.05% $NaN_3$). The following antibodies were purchased from BioLegend: CD11c (clone N418), MHC class II (clone KH74), CD3 (clone 145-2C11), NK1.1 (clone PK136), Ly-6C (clone HK1.4), CD11b (clone M1/70), and Gr-1 (clone RB6-8C5). Antibodies for Treg analysis were purchased from Becton Dickinson: CD4 (clone RM4-5), CD25 (clone 7D4), and FoxP3 (clone MF23). For intracellular staining of FoxP3, cells were processed with FoxP3 buffer set (eBioscience). Dead cells were excluded based on forward/side scatter and positive staining for propidium iodide (except for permeabilized cells). The antibody combinations used for the identification of different cell subsets are shown in Table 5.2. The gating strategy for the flow cytometric identification of Tregs in para-aortic LN is depicted in Figure 5.1. Cells were analysed on a BD Accuri C6 cytometer. Data analysis was performed with FCS Express 4.

TABLE 5.2. ANTIBODY COMBINATIONS FOR THE IDENTIFICATION OF LEUKOCYTE SUBSETS BY FLOW CYTOMETRY.

| Cells | Antibody combination |
|---|---|
| **T cells** | $CD3^+NK1.1^-$ |
| **Tregs** | $CD4^+CD25^+FoxP3^+$ |
| **NKT cells** | $CD3^+NK1.1^+$ |
| **neutrophils** | $CD11b^+Gr\text{-}1^{high}$ |
| **monocytes** | $Ly\text{-}6C^{low}$ or $Ly\text{-}6C^{high}$ |
| **DCs** | $CD11c^+MHCII^+$ |

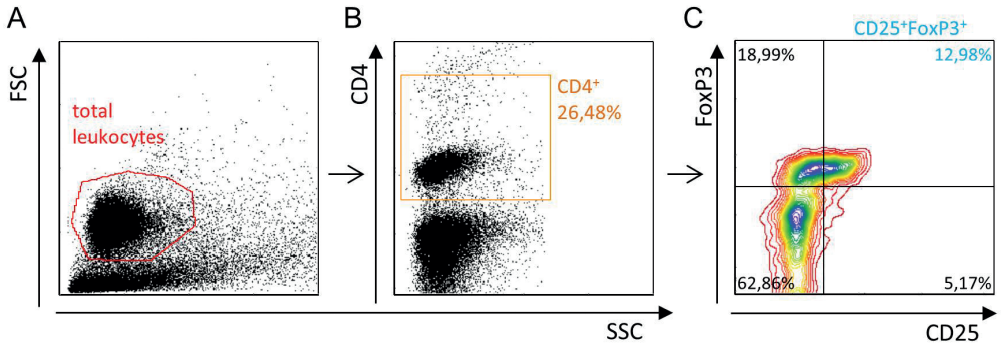


**FIGURE 5.1. Gating strategy for the analysis of Tregs.** (A) Cells were pre-gated on FSC/SSC properties; (B) the gating for CD4 within the total leukocytes gate; (C) Tregs were defined as $CD25^+$ $FoxP3^+$ within the $CD4^+$ population.

## Histology

After sacrifice of $ApoE^{-/-}$ mice, the brachiocephalic artery was collected, fixed in 4% formaldehyde (pH 7.4) for 24h, and embedded in paraffin after overnight dehydration in 60% isopropanol. Serial cross sections of the brachiocephalic artery were prepared for histological analysis. Atherosclerotic plaque size and necrotic core were analysed on H-E stained sections. Collagen content was measured by Sirius red (Sigma-Aldrich) staining. Immunohistochemical staining with a primary antibody against Mac-3 (Pharmingen) was used to determine the percentage of macrophages in the plaques. Fibrous cap thickness was determined as the median value of 10 measurements per atherosclerotic plaque on α-SMA (Sigma-Aldrich) stained sections. All images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope and were quantified with Image J software.

### Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using SPSS software (version 23, SPSS Inc.). Data were analysed by means of a Student t test. Differences were considered significant when $p<0.05$.

## RESULTS

### General characteristics and plaque composition

Mice from the control and the ASA-treated group did not differ in body weight or total plasma cholesterol levels (Table 5.3). The spleen weight of ASA treated mice (91 ± 7 mg) was significantly lower when compared to the control group (117 ± 8 mg). There were no differences in size, necrotic core or fibrous cap thickness of the plaques from the brachiocephalic artery between the control and treatment group (Table 5.3). The collagen content was slightly higher in plaques from ASA-treated mice but this was not significant (Table 5.3; p=0.078). Strikingly, the percentage of macrophages in the plaques was significantly reduced after treatment with ASA (Table 5.3 and Figure 5.2).

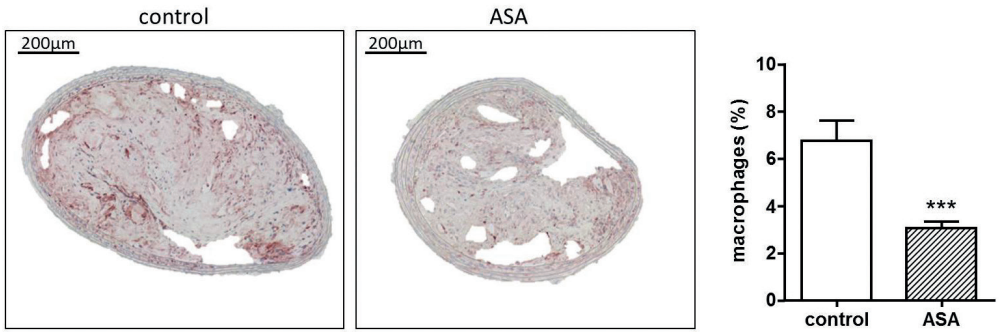


**FIGURE 5.2. Representative images and quantification of macrophage staining in the brachiocephalic artery of control (left) and ASA-treated (right) mice.** Control n=15, ASA n=14; ***p<0.001.

TABLE 5.3. GENERAL CHARACTERISTICS AND ATHEROSCLEROTIC PLAQUE COMPOSITION.

| | Control | ASA |
|---|---|---|
| Body weight (g) | 23.3±0.7 | 22.5±0.7 |
| Cholesterol (mg/dl) | 440.1±26.1 | 413.7±23.5 |
| Spleen weight (mg) | 116.6±8.0 | 91.43±6.9* |
| Plaque size ($\times 10^4$ $\mu m^2$) | 30.79±2.07 | 30.03±1.97 |
| Necrotic core (%) | 11.03±2.31 | 13.59±2.23 |
| Macrophages (%) | 6.78±0.85 | 3.06±0.28*** |
| Fibrous cap thickness (μm) | 12.06±3.11 | 9.94±1.81 |
| Collagen (%) | 6.44±0.85 | 8.53±0.73 |

Data from brachiocephalic artery, control n=9-14, ASA n=10-14; *p<0.05, ***p<0.001.

### Immune cell distribution

It is thought that ASA might play a role in modulating the immune response mediated by different types of immune cells. Therefore, we analysed circulating and peripheral immune cell profiles from control and ASA-treated mice via flow cytometry. There was no difference in the proportion of total T cells between the two groups (Figure 5.3A). The frequency of $CD4^+CD25^+FoxP3^+$ Tregs was increased in LN from ASA-treated mice when compared to control mice (Figure 5.3B). The percentage of neutrophils and NKT cells was decreased in the spleen of ASA-treated mice (Figures 5.3D,E). No changes could be detected in the percentage of DCs or monocytes (Figures 5.3C,F).

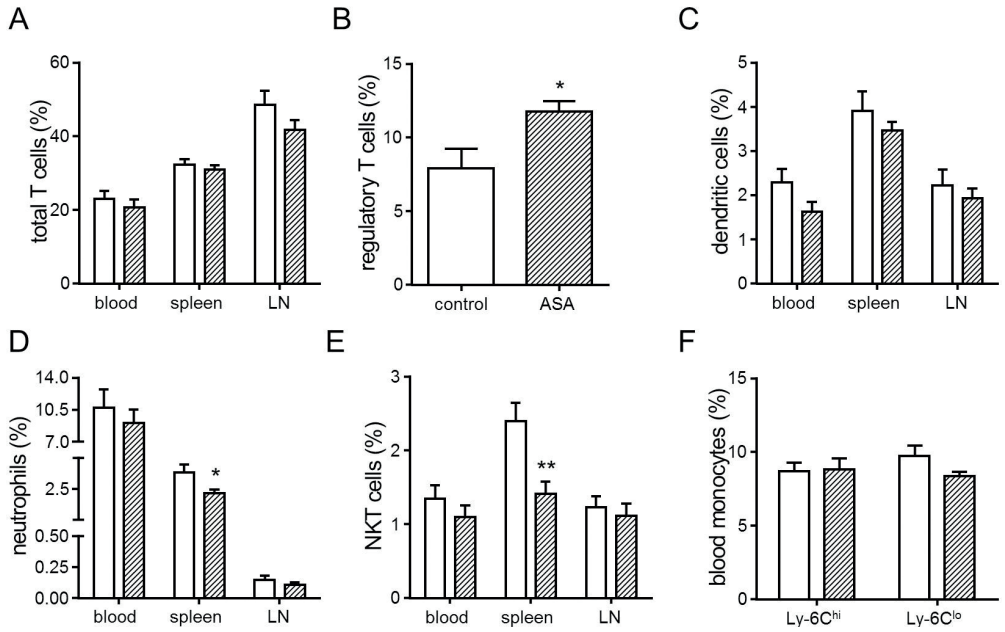


**FIGURE 5.3.** Flow cytometric analysis of total T cells (A), DCs (C), neutrophils (D), and NKT cells (E) in blood, spleen and LN, and of Tregs in LN (B) and monocytes in blood (F) from control mice (white bars, n=10-15) or ASA-treated mice (hatched bars, n=9-14); *p<0.05, **p<0.01.

## DISCUSSION

The past few years, it was suggested that ASA, in addition to its anti-inflammatory effect, may also specifically inhibit autoimmune responses *in vivo*.[15] Results from studies using ASA in mouse models of atherosclerosis are inconsistent, probably due to the use of different animal models, diet, and dose and/or duration of ASA treatment. In most studies, ASA treatment was started along with the onset of atherosclerosis, which often resulted in a decrease in lesion size[5-7,10,18], but this was not always the case.[9,13] Furthermore, Tous *et al*. reported that ASA attenuates the initiation but not the progression of atherosclerosis in ApoE$^{-/-}$ mice.[8] By contrast, Cyrus *et al*. did observe a significant reduction in the progression of atherosclerosis when LDLr$^{-/-}$ mice with established plaques were treated with ASA for 12 weeks, together with reduced signs of vascular inflammation.[12] The latter is clinically more relevant as it studies the role of ASA on established atherosclerotic lesions. Research by our own group investigated the outcome of low-dose ASA treatment in ApoE$^{-/-}$ mice with a heterozygous mutation in the fibrillin-1 gene (Fbn1$^{C1039G+/-}$). These mice develop exacerbated atherosclerosis and spontaneous plaque ruptures, accompanied by MI and

sudden death.[19] However, no differences in plaque vulnerability, the incidence of MI and survival of ASA-treated ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice could be observed. Nevertheless, cardiac hypertrophy and coronary fibrosis were attenuated after ASA treatment, indicating that ASA can reduce cardiac remodelling (unpublished results). Whether or not low-dose ASA influenced systemic or peripheral immune cell profiles was not investigated in these studies.

In the current study, low-dose ASA treatment of ApoE$^{-/-}$ mice with established plaques did not reduce the progression of atherosclerosis. However, plaques of ASA-treated mice appeared more stable, as indicated by the increased collagen content and the significant reduction in the amount of macrophages. Additionally, we observed signs of immune modulation in the periphery. Besides reduced spleen weight, ASA-treated mice displayed a decrease in splenic neutrophils and NKT cells. Neutrophils are among the first responders of host defense against invading microorganisms. In an acute inflammatory setting, low-dose ASA reduced neutrophil and macrophage accumulation in mice and humans.[20] These effects were independent of NF-κB-regulated gene expression or inhibition of PG. In recent years, neutrophils have also gained more attention with respect to chronic inflammatory processes, such as atherosclerosis, because of the multifaceted interactions between neutrophils and other leukocytes.[21,22] Similar to neutrophils, NKT cells respond in an innate-like manner to danger signals and pro-inflammatory cytokines. Over the past years, they have attracted attention as lipid-responsive cells considering that retention of lipids and lipoproteins in the vessel wall are one of the earliest events in the pathogenesis of atherosclerosis. Most experimental data from animal models attribute a proatherogenic role to NKT cells.[23,24] In chapter 3 of this thesis, it is described that the number of NKT cells gradually increases with the progression of the disease. Here, the number of NKT cells is decreased after treatment with ASA, which coincides with an increase in the stability of plaques. In the future, longitudinal monitoring of NKT cells could thus be a valuable method to verify the efficacy of a treatment for atherosclerosis.

Interestingly, we observed, that chronic *in vivo* treatment of ApoE$^{-/-}$ mice with low-dose ASA induced a significant increase in the frequency of Tregs in para-aortic LN. Unfortunately, we do not have data on the number of Tregs in age-matched healthy animals. Previously, it was described that hyperlipidemia diminishes peripheral Treg numbers.[25,26] The amount of Tregs in blood or spleen was not measured in this study. Although further research is warranted, a possible mechanism could be that ASA exerted this indirectly through modulation of DCs, which is in line with *in vitro* studies.[15-17] Noteworthy, the *in vivo* observed Treg increase was mediated by an ASA dose, which is much lower than the one used for

*in vitro* studies (0.5-2.5 mM) or for the treatment of rheumatoid arthritis (average plasma concentrations between 1-3 mM). In line with our observation, intraperitoneal injections of therapeutic doses of ASA (6 mg/kg/d) during 4 weeks significantly enhanced the frequency of $CD4^+CD25^+FoxP3^+$ Tregs in BALB/c mice.[27] Increasing the proportion of Tregs is potentially appealing with respect to the induction of immunological self-tolerance for the treatment of atherosclerosis.[28,29] Due to its diverse effects on immune regulation, ASA continues to be of interest for future investigations.[29]

In the high-risk setting of atherosclerotic CVD or acute MI, the role of ASA in reducing the risk of vascular events and, to a lesser extent, total and cardiovascular mortality, is well established. However, in primary prevention, it is less clear which risk factor profiles benefit from daily ASA intake. This resulted in inconsistent guideline recommendations from various (inter)national organizations.[30] In particular, the balance between cardiovascular risk reduction and the increased risk to bleed from low-dose ASA is difficult to find.[30] Our results reveal immune-modulatory capacities of ASA, in particular by increasing Tregs in para-aortic LN, which may provide further support for the use of daily low-dose ASA in both primary and secondary prevention of CVD.

In summary, chronic low-dose ASA treatment had no influence on the size of atherosclerotic plaques in $ApoE^{-/-}$ mice. By contrast, plaque stability was increased. Moreover, low-dose ASA actively increased Tregs in the aorta-draining LN and reduced key innate immune players in the periphery. Therefore, our data support the use of ASA for the prevention of CVD, alone or as add-on therapy, even in low risk factor profiles not at increased risk for bleeding.

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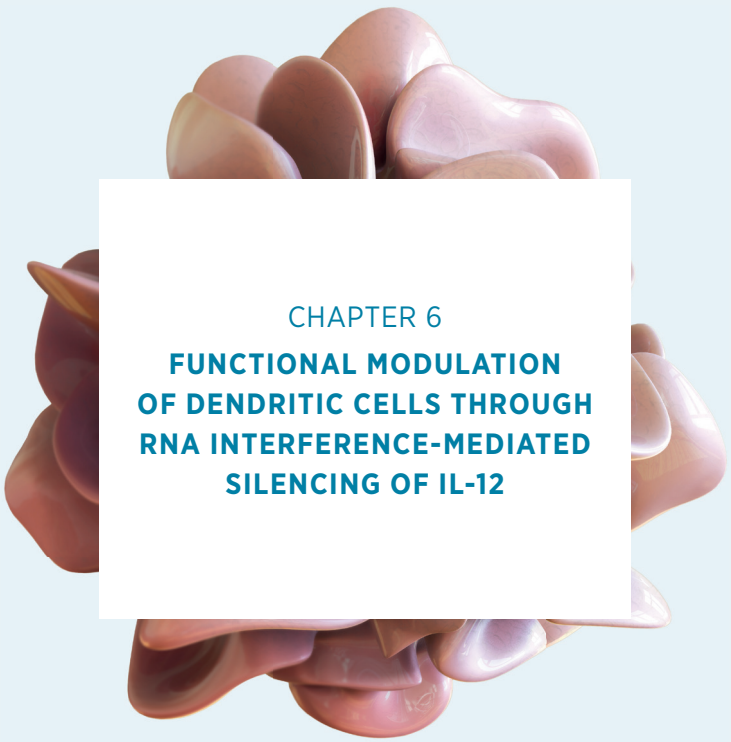

CHAPTER 6

# FUNCTIONAL MODULATION OF DENDRITIC CELLS THROUGH RNA INTERFERENCE-MEDIATED SILENCING OF IL-12

ADAPTED FROM

**Rombouts M***, Nuyts AH*, Derdelinckx J, Peeters K, Van Camp K, De Meyer GRY, Goossens H, Berneman ZN, Van Brussel I, Schrijvers DM**, and Cools N**. Silencing RNA as a tool to overcome the Th1-polarizing capacity of mature dendritic cells. *(manuscript submitted)*

* Both authors contributed equally to this work ** Both senior authors contributed equally to this work

## INTRODUCTION

The use of cells to cure patients is being investigated in a broad range of pathologies including cancer, infections and autoimmune diseases.[1-3] Since DCs are the most efficient APCs and coordinators of the immune response, they are an important target when considering potential therapeutic strategies that can restore the immunological balance in these diseases. One way to modify the immune-stimulatory phenotype of DCs is to prevent the transcription of genes involved in the generation of an immune response by means of selective interference with siRNA. More specifically, interference with the expression of co-stimulatory molecules (e.g. CD80, CD86 and CD40) or the production of pro-inflammatory cytokines (e.g. IL-12p70) by DCs might be instrumental in correcting unwanted immune responses.[1-4] A number of pre-clinical studies supports this notion. For example, targeted silencing of CD80, CD86 and CD40 in murine bone marrow-derived (BM)DCs by using a lipid-based transfection reagent, protected mice from autoimmune arthritis in a collagen-induced arthritis model.[5] Similarly, injection of tolerogenic DCs, generated using anti-sense oligonucleotides targeting CD80, CD86 and CD40 primary transcripts, conferred protection against type I diabetes in the non-obese diabetic mouse strain.[6] Subsequently, results from a phase I clinical trial demonstrated that the administration of human antisense CD40/80/86 oligo-treated DCs is safe and well tolerated in patients with type 1 diabetes.[7]

IL-12p70 was shown to be crucial for the immune-stimulatory function of DCs and the differentiation of Th1 cells.[8] Moreover, previous research by Nuyts *et al.* demonstrated that IL-12p70 secretion, but not CD80 or CD70 costimulation, is mandatory for Th1 polarisation by human DCs (unpublished results). In this chapter, we aimed to (i) establish a culture method for mouse DCs *in vitro*, and (ii) genetically modify DCs *in vitro* by means of siRNA to transiently inhibit the expression of IL-12p35, the unique subunit of the biologically active IL-12p70, in order to provide a reliable way to modify the outcome of T cell polarization. We hypothesize that silencing of IL-12p70 production interferes with the Th1 cell-stimulatory capacity of DCs thereby generating regulatory DCs that ultimately can be used to restore the immunological balance in atherosclerosis and other autoimmune diseases.

## MATERIAL AND METHODS

### Culture of mouse bone marrow-derived (BM)DCs

Bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice (Jackson Laboratory; stock number 000664), passed through a 40 μm cell strainer, washed and resuspended in red blood cell lysing buffer (Sigma-Aldrich). Next, cells were washed and cultured in complete medium, *i.e.* RPMI 1640 medium supplemented with GlutaMAX (Life Technologies), 5% FBS (Sigma-Aldrich), 1% penicillin/streptomycin (Life Technologies), 20 U/mL polymyxin B (Fagron) and 1 mM sodium pyruvate (Life Technologies), at a cell concentration of 1 × 106 cells/ml. Immediately before use, 50 μM β-mercaptoethanol (Life Technologies) and 10 ng/mL GM-CSF (PeproTech) were added. Cells were cultured at 37°C in a 5% CO2-humidified atmosphere for 6 days. At day 3, half of the culture medium was replenished with complete medium. At day 6, non- and loose-adherent cells were harvested for use in silencing experiments (*vide infra*), or were directly stimulated for 24 h with 1 μg/ml LPS (Sigma-Aldrich) and 1000 U/ml IFN-γ (PeproTech) to induce maturation. Alternatively, DCs were left untreated, *i.e.* immature (i)DCs, as a control.

### Flow cytometry

Cells were suspended in FACS buffer (PBS supplemented with 0.1% BSA (Sigma-Aldrich) and 0.05% $NaN_3$ (Merck)) and preincubated for 10 min with Fc blocker (anti-mouse CD16/32 antibody; BioLegend). Immunophenotyping of BMDCs was done by immunofluorescence staining using fluorochrome-labeled antibodies directed against either CD11c (clone N418), MHC class II (clone KH74), CD11b (clone M1/70), CD103 (clone 2E7), CD40 (clone 3/23), and CD80 (clone 16-10A1) (all from BioLegend). Debris and dead cells were excluded based on forward/side scatter and positive staining for propidium iodide (Invitrogen). Samples were measured on a BD Accuri C6 cytometer (BD Biosciences). Analyses were performed using FCS Express 4 software (De Novo Software).

### Cell transfection by siRNA electroporation

DCs were harvested and modified to inhibit the expression of IL-12p70 transiently by means of selective interference using siRNA targeting IL-12p35 (ON-TARGETplus siRNA SMARTpool, Thermo-Fisher Scientific Bioscience). Electroporation with a non-coding ON-TARGET plus siRNA SMARTpool was used as a control for off-target effects of siRNA. Immature BMDCs (10-20 × $10^6$ cells/ml) were electroporated with 2 μg IL-12p35 siRNA/1×$10^6$ cells using a time-constant pulse of 300V for 7-8 milliseconds. Following electroporation and

a 16-32 h resting phase, cells were stimulated with LPS (1 μg/ml) and IFN-γ (1000 U/ml), or left untreated as a control. Twenty-four h later, transfected DCs were harvested for use in further experiments.

### T cell activation assays

Mouse allogeneic naive T cells were isolated from splenic single cell suspensions using a $CD4^+CD62L^+$ T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). The purity of the isolated cells was analysed by flow cytometry with anti-mouse CD4 and CD62L antibodies. In silencing experiments, $3 \times 10^5$ freshly-isolated naive T cells were stimulated with allogeneic electroporated BMDCs at a 10:1 ratio in RPMI medium supplemented with 5% FBS for 5 days.

### Cytokine secretion assays

Secretion of IL-12p70 by BMDCs was determined using an IL-12p70 ELISA (eBioscience). At day 5 of DC:T cell co-culture, cytokine secretion in the supernatant was analysed by means of ELISA for mouse IFN-γ and IL-5 (eBioscience). All assays were used according to the manufacturer's instructions.

### Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad version 5 software (Prism) or SPSS Statistics 23.0 (IBM) by means of Student t-test or Two-way ANOVA (followed by Bonferroni's Multiple Comparison post-hoc test), as appropriate. Differences were considered significant when $p<0.05$.

## RESULTS

### Phenotypical and functional characterization of mouse BMDCs

Several methods for the preparation of mouse BMDCs can be found in literature, all of which differ in the combinations of cytokines used (e.g. GM-CSF, Flt3L, IL4, and IFN-γ).[9-11] Here, we present a simple method for the production of phenotypically and functionally immature and mature BMDCs (see Material and Methods).

After 6 days of culture in the presence of GM-CSF, a population of approximately 70% $CD11c^{high}MHCII^+$ cells (Figures 6.1A,B) was yielded. Our culture method generates a mixed population of DCs, including the $CD103^+$ and $CD11b^+$ DC subsets, of which the latter was

the most predominant. Maturation of BMDCs, using a combination of LPS and IFN-γ, slightly increased the proportion of $CD103^+$ cells whereas the proportion of $CD11b^+$ DCs was somewhat decreased (Figure 6.1C).

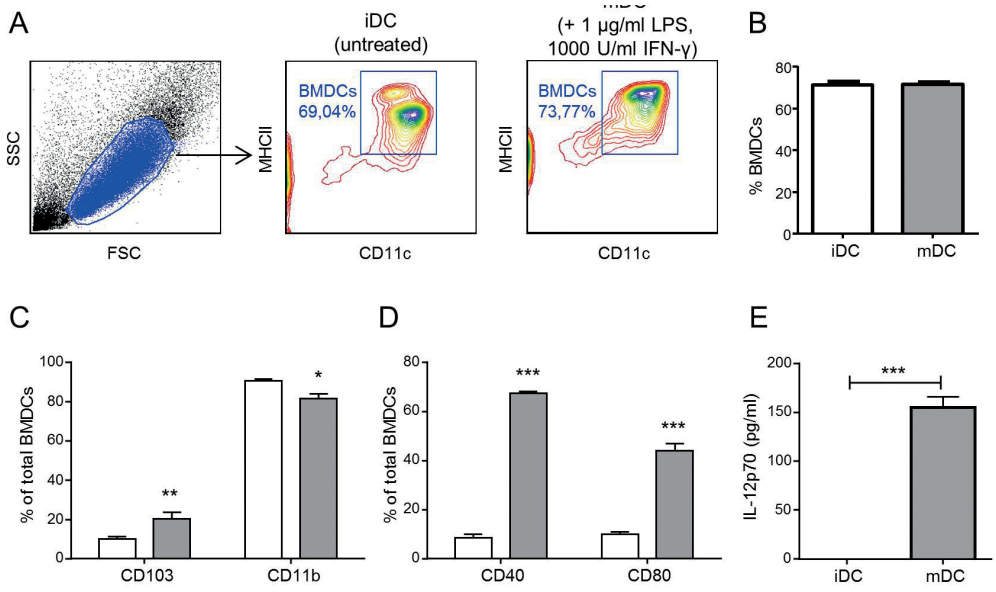


**FIGURE 6.1. Phenotypical and functional characterization of BMDCs.** (A) Dot plot is gated on FSC and SSC to define total cells from debris. The total BMDC population was identified based on the co-expression of CD11c and MHCII (contour plots). (B) After 7 days of culture, a population of approximately 70% immature $CD11c^{high}MHCII$-positive cells was yielded (n=4-5). (C) The BMDC culture contains both $CD103^+$ and $CD11b^+$ DCs. (D) The percentage CD40- and CD80-positive DCs before (iDC) and after stimulation with LPS and IFN-γ (mDC) (n=5); (E) Stimulation of BMDCs with LPS and IFN-γ induces IL-12p70 production by mDCs (n=5); iDC, white bars; mDC, grey bars; *p<0.05, **p<0.01, and ***p<0.001.

Furthermore, maturation resulted in a significant increase in the proportion of CD40- and CD80-expressing mDCs as compared to untreated iDCs (Figure 6.1D). Functionally mature DCs do not only express high levels of co-stimulatory molecules on their cell surface but also produce pro-inflammatory cytokines. As mentioned, IL-12p70 was shown to be crucial for immunogenic DC functions and the differentiation of Th1 cells from naive T cells. As shown in Figure 6.1E, IL-12p70 (154,4 ± 11,5 pg/ml) could be detected in the supernatant of LPS- and IFN-γ-stimulated BMDCs. iDCs on the other hand, did not produce IL-12p70.

### Silencing of IL-12p35 results in reduced IL-12p70 secretion following siRNA electroporation of BMDCs

Previously, Nuyts *et al.* demonstrated that inhibition of IL-12, but not CD70 or CD80, can shift the polarization of allogeneic naive T cells from IFN-γ-producing Th1-like cells towards IL-5-secreting Th2-like cells (unpublished results). Based on this observation, stable knockout of IL-12-secreting function in DCs would provide a valuable approach to generate DCs with immunoregulatory capacities. To this extent, iDCs were electroporated with a mixture of 4 predesigned siRNA targeting the unique subunit of bioactive IL-12p70, namely IL-12p35. Next, siRNA-electroporated DCs were stimulated with LPS and IFN-γ for 24 hours (mDC) or left untreated (iDC). Validation of successful silencing was done at the protein level using an IL-12p70 ELISA. As depicted in Figure 6.2, a significant 62% reduction in the secretion of IL-12p70 was observed after silencing of IL-12p35 by electroporation with siRNA when compared with control siRNA-electroporated BMDCs. In none of the conditions, iDC produced IL-12p70.

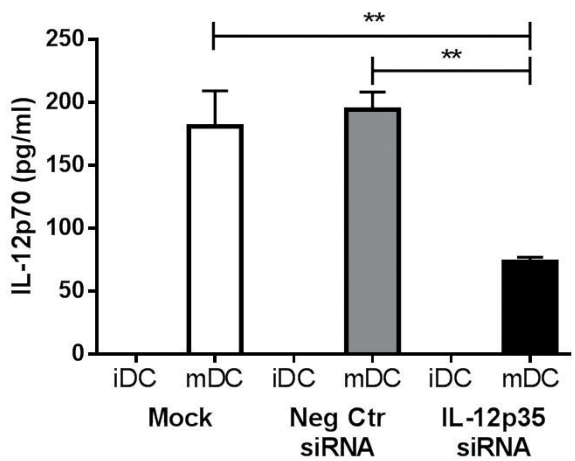


**FIGURE 6.2. IL-12p35 siRNA electroporation results in a reduction of IL-12p70 secretion by DCs.** BMDCs were electroporated with a control pool of non-coding siRNA (grey bars) or with a pool of siRNA targeting the IL-12p35 subunit (black bars). Mock-electroporated DCs were used as a control (white bars). After an overnight resting phase, DCs were stimulated with LPS and IFN-γ for 24h (mDC) or left untreated (iDC). The secretion of IL-12p70 (n=3) was analysed by means of ELISA; **p<0.01.

### Silencing of IL-12p35 reduced the capacity of DCs to promote Th1 cell differentiation

Finally, we evaluated whether silencing of IL-12p35 was sufficient to modulate the T cell-stimulatory capacity of DCs. First, naive T cells were isolated from splenic single cell suspensions with high purity (95,11 ± 0,36% $CD4^+CD62L^+$ naive T cells; Figure 6.3A). Next, IL-12p35-silenced BMDCs were used to stimulate freshly-isolated allogeneic naive $CD4^+$ T cells. Naive $CD4^+$ T cells incubated alone were used as a negative control. Stimulation of allogeneic naive $CD4^+$ T cells with IL-12p35 siRNA-electroporated BMDCs resulted in decreased IFN-γ production, when compared with mock-electroporated DCs or control siRNA-electroporated DCs (Figure 6.3B). No significant changes were observed in the production of IL-5, potentially due to the large variability between the samples (Figure 6.3C).

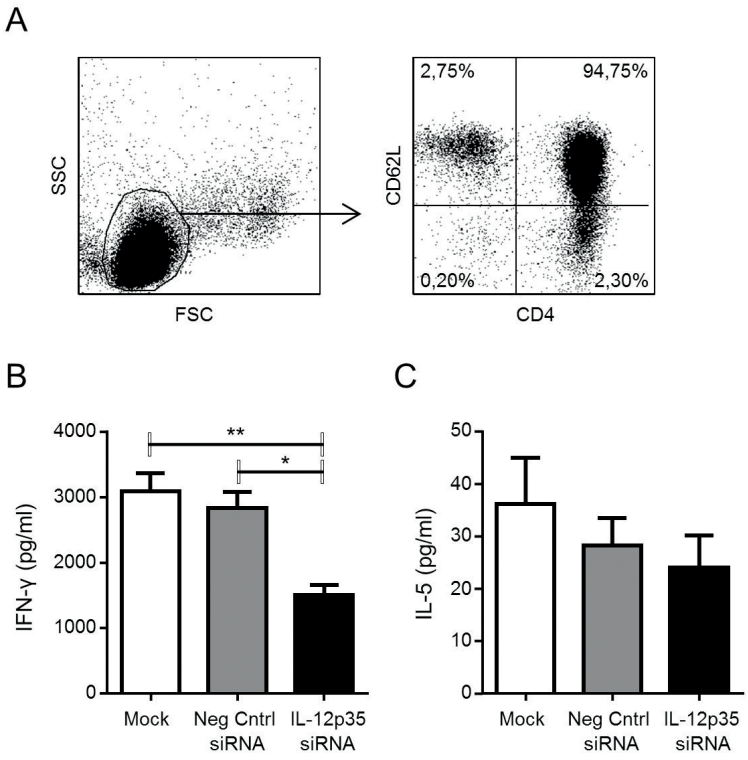


**FIGURE 6.3. IL-12p35 silencing of DCs reduces their Th1-polarizing capacity.**(A) Representative plots of the flow cytometric analysis for the purity of isolated $CD4^+CD62L^+$ T cells; Naive T cells were co-cultured with mock-electroporated DCs (white bars), non-coding siRNA-electroporated DCs (grey bars), or IL-12p35 siRNA-electroporated DCs (black bars). After 5 days, IFN-γ (A) and IL-5 (B) secretion was measured in co-culture supernatant samples by means of ELISA (n=3); *p<0.05 and **p<0.01.

## DISCUSSION

During the last decades, our understanding of the pathogenesis of atherosclerosis has greatly improved and it is currently generally accepted that a disbalance between inflammatory and tolerogenic processes contributes to disease onset and progression. In this respect, it is well known that different subsets of T cells can drive (Th1/Th17) or dampen (Th2/Treg) inflammatory processes.[1] Hence, the induction of Th2/Treg and/or cytokine deviation could provide valuable tools to counteract the immunological imbalance. Given the unique ability of DCs to drive the development of naive T cells into different T helper cells, modulating the immune response at the level of DCs has become an attractive approach in the context of the development of cell-based therapies.[1] The combined expression of costimulatory molecules and cytokines by DCs affects the outcome of DC/T cell interactions. It is well established that the B7-costimulatory pathway (i.e. CD80/CD86-mediated ligation of CD28) together with IL-12 production triggers Th1 polarization. Moreover, IL-12 was shown to be crucial for the induction of IFN-γ secretion by Th1 cells.[8,12] Therefore, selective inhibition of the IL-12 secretion by DCs, by using siRNA, may be an ideal mechanism of immunotherapy for atherosclerosis, as well as other autoimmune diseases.

The two most common techniques to transfect DCs *in vitro* with siRNA are lipofection and electroporation. Previously, it was shown that electroporation is feasible and even superior compared to lipofection for the delivery of mRNA or immune-modulatory agents.[13-15] Here, we investigated the feasibility of electroporation of siRNA in order to generate DCs with modified T cell polarising function. DCs in which IL-12p70 was silenced by means of electroporation with siRNA demonstrate a 62% reduction of the IL-12p70 secretion as compared to control siRNA-electroporated DCs. In addition, we found that IL-12-silenced DCs can reduce the IFN-γ secretion *in vitro* by allogeneic naive T cells. However, we observed no increase in the production of IL-5. Yet, a cell-based therapy that actively shifts the T cell balance away from Th1 polarization and activation could be of significant value in the context of autoimmune diseases. For instance, Hill et al. showed that IL-12p35-silenced bone marrow-derived antigen-loaded DCs induced a Th1 to Th2 shift both *in vitro* and *in vivo*.[16] Furthermore, a single administration of IL-12-silenced DCs using short hairpin RNA has been shown to ameliorate collagen-II-induced arthritis 12 days after antigen priming of the animals.[17]

Nevertheless, silencing the expression of hallmark immune-modulatory molecules entails a number of limitations. A notable concern of cell-based therapies relates to the stability of

their phenotype following *in vivo* administration, especially in a pro-inflammatory environment. With regard to tolerogenic DC-based therapies, this means that a maturation-resistant phenotype is quintessential for their clinical applicability.[18] For instance, *ex vivo* generated tolerogenic DCs by treatment with histone deacetylase inhibitor SAHA were not stable *in vivo* as these DCs regained the ability to upregulate costimulatory molecules in response to LPS. As a consequence, SAHA-generated DCs were not able to alleviate the development of experimental autoimmune encephalomyelitis in mice.[19] While maturation of DCs is a complex process encompassing a number of transcriptional pathways, the downside of silencing RNA is that it focuses on one target only. Hence, it is likely that IL-12p35-silenced DCs can still convert towards a more immune-stimulatory phenotype following *in vivo* activation by proinflammatory stimuli. In order to reduce the risk of *in vivo* maturation, simultaneous targeting of multiple key effector genes, including costimulatory molecules CD80, CD86, and CD40, might offer a solution.[5-7] Even more, cotransfection of multiple siRNAs against several targets showed greater inhibition of individual targets, though the underlying mechanism remains unknown.[20] Even when multiple targeting is applied, practically all studies on siRNA report incomplete inhibition of protein expression, on average 80% or less[21], defining the use of siRNA as a knockdown technique. Whereas this knockdown is mostly transient, siRNA is safer in comparison to other interference methods as it allows one to inhibit a therapeutic target without insertion of the host genome.

Another drawback of using siRNA is the potentially unintended knockdown of genes not being directly targeted, so-called off-target effects. Indeed, Jackson *et al.*[22] used gene expression profiling to characterize the specificity of gene silencing by siRNA and found that siRNA-treated cells show off-target silencing of a large number of genes. Off-targeting may cause unpredictable and/or undesirable side-effects. Therefore, careful monitoring during clinical application is strongly advised.[22-24] The strategy of pooling multiple siRNAs to the same target, which we employed in our study, has been shown to reduce unintended targeting while preserving potent target gene knockdown.[24]

In summary, we were able to develop mature IL-12p35-silenced DCs capable of modulating IFN-γ secretion upon *in vitro* culture with allogeneic naive T cells. Despite these promising results, a number of hurdles will need to be addressed, such as responsiveness to maturation signals, the duration of silencing, and exclusion of off-target effects, when taking this strategy into the clinic. Ultimately, this could provide improved means for antigen-specific immunomodulation in the context of atherosclerosis as well as cancer, infectious diseases and autoimmunity.

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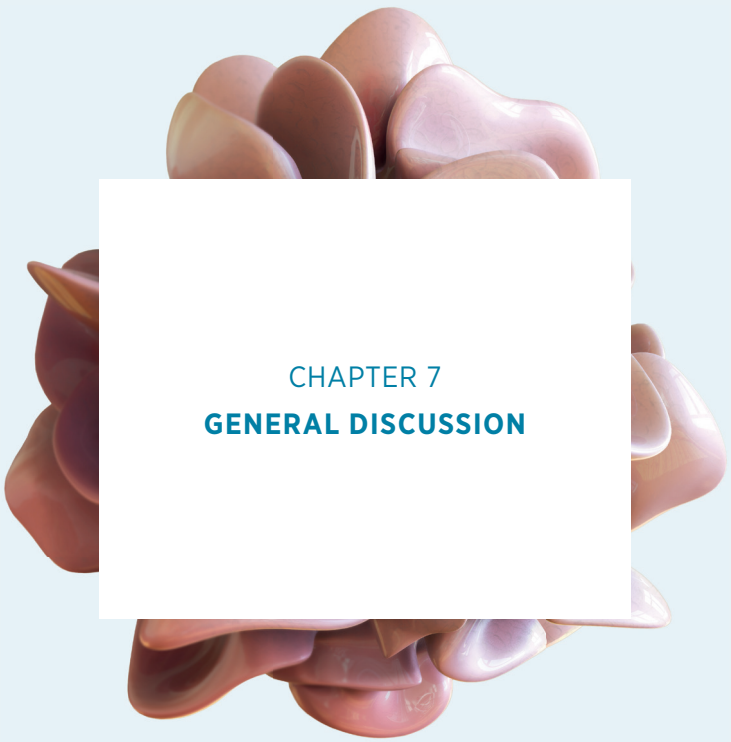

CHAPTER 7

# GENERAL DISCUSSION

Atherosclerosis is a complex multifactorial disease which mainly affects medium to large arteries. Dyslipidaemia and inflammation are believed to be the main underlying cause of induction and progression of inflammation in the vessel wall. The current treatment of atherosclerosis is mainly based on reducing risk factors, such as decreasing hypertension (e.g. beta-blockers) and lowering blood cholesterol levels, with statins as the first-line choice.[1] Recent data suggest that the latter compounds, in addition to their lipid-lowering ability, also have anti-inflammatory properties.[2] For instance, in an *in vitro* model of LPS-induced inflammation, statins significantly decreased the interaction between vascular SMCs and monocytes, and blunted their synergistic production of pro-inflammatory cytokines. Statins also increased the peripheral pool of $CD4^+CD25^+$ Tregs in humans, although in mice, no effect of statins on Tregs was observed. *In vitro* exposure of human DCs to statins may inhibit their maturation and antigen-presenting function, but data remain conflicting.[3] The JUPITER (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) study demonstrated that suppression of low-grade inflammation by statins reduces cardiovascular risk in primary prevention. However, recent clinical trials in high risk groups (e.g. heart failure or chronic kidney disease patients) have failed to document any benefit of statin treatment on clinical outcome.[2] Furthermore, many events continue to occur in patients receiving the best currently available therapy.[1,4] In the Prove-It Trial, 22.4% of patients experienced a coronary event during 2 years of intensive statin therapy.[5] In general, a residual risk of approximately 9% in patients with established CAD remains a reality.[4] Patients with familial hypercholesterolemia and patients who are intolerant or unresponsive to statins might derive no benefit from statin treatment.

Since inflammation plays at least an equally important role in atherosclerosis as dyslipidaemia, anti-inflammatory therapies might provide additional benefit in the treatment of atherosclerosis. So far, the only drugs with anti-inflammatory properties used in CAD are ASA and statins.[6] However, immunomodulatory or immuno-suppressive therapies, some of which are already being used in other immune-related diseases, may have benefits in atherosclerotic disease. Currently, two clinical trials evaluating anti-inflammatory agents will address for the first time whether targeting inflammation itself will reduce cardiovascular events and risks. We especially look forward to the outcomes of the Cardiovascular Inflammation Reduction Trial (CIRT; NCT01594333), and the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS; NCT01327846). CIRT will evaluate the effect of low-dose methotrexate, routinely and safely used as an anti-inflammatory regimen for the treatment of rheumatoid arthritis (RA), in the secondary prevention of

cardiovascular disease.[7] In phase II of CANTOS, canakinumab (an anti-human IL-1β monoclonal antibody) reduced the levels of CRP and IL-6 in patients with diabetes at high risk of cardiovascular events. Currently, they are recruiting participants for phase III of CANTOS, which will evaluate the effects of canakinumab in preventing MI, stroke and cardiovascular death.[8] If both or either of the two clinical trials are successful, they would support the inflammatory hypothesis of atherosclerosis.[6,9,10] Inhibition of TNF-α signalling, either with humanized blocking antibodies such as infliximab and adalimumab, or with the recombinant fusion protein etanercept, which targets the TNF-α receptor, reduced the incidence of acute cardiovascular events in RA patients.[11] This indicates that TNF-α is an important proinflammatory factor in atherosclerotic CVD and suggests that TNF-α blockade might be useful for cardiovascular prevention. However, in view of the adverse effects described in patients receiving TNF-α blockade (e.g. injection site reactions, neutropenia and infections), this treatment should probably be reserved for high-risk individuals such as those with autoimmune diseases. Additional studies are needed to confirm a potential atheroprotective effect of TNF-α blockade. An alternative to TNF-α blockers that can be used in the treatment of RA is tocilizumab. This IL-6 receptor blocker was described to have anti-inflammatory effects, but also elevates LDL, HDL and triglycerides, thereby limiting its potential use for CVD.[6,12] Nevertheless, the blockade of other potential targets such as CCR2 (MLN1202) and CD20 (rituximab), could provide benefit in atherosclerotic disease.[6] However, because of the many side effects associated with these treatments, the need for more specific treatments is high. Therefore, in order to achieve long-lasting and drug-free disease control, more knowledge regarding the contribution of detrimental autoreactive responses to the (immune)pathogenesis of atherosclerosis is warranted. Ultimately, this may result in the identification of new therapeutic strategies tackling atherosclerosis.[13]

## DENDRITIC CELLS: PATHOGENIC PLAYERS IN ATHEROSCLEROSIS

The past few years, investigators have postulated an autoimmune nature for atherosclerosis, according to which the first stage of the disease consists of an autoimmune reaction against self-proteins (autoantigens) that are released when the body is stressed by classical atherosclerosis risk factors. Due to their critical role in effector T cell differentiation from naive T cells, it is not surprising that the (dys)regulation of immune responses by DCs against plaque-associated antigens is considered a key event in a cascade of processes occurring during the development and progression of atherosclerosis.[14,15] Indeed, as professional APCs of the immune system, DCs bridge the innate immune system with the adaptive immune

response, and are imperative both in induction of T cell immunity, as well as tolerance.[14,16] Therefore, research into the disease mechanisms underlying atherosclerosis and the contribution of DCs herein might open the door for the development of better treatments that interfere with or enhance DC subset differentiation and functions.[17]

The origin and function of distinct DC subsets that potentially control atherogenesis is still not fully understood. To assess the functional significance of a cell in a certain disease, the cell itself or one of its main functions can be deleted, either via constitutive deficiency (knockout models) or inducible depletion (antibodies and *DTR* transgenic mice) (Table 7.1). A popular method used to deplete cDCs in atherosclerosis is the use of a strain of mice that expresses DTR under control of the CD11c promoter (CD11c-DTR mice)[18] (Table 7.1). Although CD11c is commonly accepted as a pan-DC marker, CD11c expression is actually not restricted to DCs. Indeed, CD11c is also found on some macrophages, activated T cells, NK cells, and Ly-6C$^{low}$ monocytes.[18,19] Moreover, CD11c-DTR mice have also been used as a tool to deplete macrophages.[20]

TABLE 7.1. OVERVIEW OF CONDITIONAL AND INDUCIBLE DC DEPLETION MODELS IN ATHEROSCLEROSIS.

| Authors & Year | Mouse model | Target/Aim | Treatment protocol | Lesions | Summary |
|---|---|---|---|---|---|
| Shaposhnik *et al.*, 2007 | GM-CSF$^{-/-}$ LDLr$^{-/-}$ | GM-CSF deficiency | 12w WD | ↓ | 60% decrease in CD11c$^{+}$ DCs in aortic lesions but no change in total monocyte-derived cells; 20-50% decrease in aortic lesion size |
| Liu *et al.*, 2008 | $CX_3CR1^{-/-}$ ApoE$^{-/-}$ | Arterial DC accumulation | 10w chow diet | ↓ | Fewer intimal CD11c$^{+}$ DCs and fewer early stage lesions |
| Gautier *et al.*, 2009 | DC-hBcl-2→LDLr$^{-/-}$; DC-hBcl-2 ApoE$^{-/-}$ | Prolonged DC survival | 12w WD; 8w WD | = | Expanded DC population; enhanced T cell activation; Th1/Th17 cytokine expression profile; decreased plasma cholesterol levels |
| Gautier *et al.*, 2009 | CD11c-DTR→LDLr$^{-/-}$; CD11c-DTR ApoE$^{-/-}$ | CD11c$^{+}$ cDC depletion | 2w WD, 1x DT, harvest after 24u | ? | Increased plasma cholesterol levels compared to control mice |
| Paulson *et al.*, 2010 | CD11c-DTR LDLr$^{-/-}$ | DCs in early atherosclerosis | 1x DT followed by WD for 5d | ↓ | 55% reduction in intimal lipid area |
| Weber *et al.*, 2011 | CCL17$^{-/-}$ ApoE$^{-/-}$; anti-CCL17 antibody | CCL17$^{+}$ DC depletion | 6m chow diet; 4w WD followed by 4w depletion | ↓ | CCL17 deficiency reduced atherosclerosis; anti-CCL17 blocking antibody expanded Tregs and reduced atheroprogression |
| Choi *et al.*, 2011 | Flt3$^{-/-}$ LDLr$^{-/-}$ | CD103$^{+}$ cDC depletion | 12w WD | ↑ | Increased lesion size; decreased % of Tregs in aorta; increased relative pro-inflammatory cytokine expression in aorta |
| Daissormont *et al.*, 2011 | anti-120G8 antibody in LDLr$^{-/-}$ (carotid collar) | pDC depletion | 4x/w for 3w<br>- starting with collar placement<br>- 4w after collar placement | ↑ | Increased lesion development and progression; T cell accumulation and proliferation; increased IFN-γ plasma level; IDO increased |
| Koltsova *et al.*, 2012 | CD11c-DTR ApoE$^{-/-}$→ApoE$^{-/-}$ | DC depletion | 12w WD, DT every 2d from 10w of WD | ↓ | Decrease in CD11c$^{+}$CD11b$^{-}$ cells in aorta en spleen; reduced expression IL6 en IL12p35 |

| Authors & Year | Mouse model | Target/Aim | Treatment protocol | Lesions | Summary |
|---|---|---|---|---|---|
| MacRitchie *et al.*, 2012 | anti-PDCA-1 antibody in ApoE$^{-/-}$ | pDC depletion | 28d WD, injections every 5$^{th}$ day | ↓ | Reduced atherosclerosis formation in the root; IL12 serum levels decreased; plaque stabilization |
| Döring *et al.*, 2012 | anti-PDCA-1 antibody in ApoE$^{-/-}$ | pDC depletion | 4w WD, injections at day 1 and day 7 after the start of WD | ↓ | Reduction in plaque size and lesional macrophage frequencies |
| Lievens *et al.*, 2013 | CD11cDNR ApoE$^{-/-}$ | TGF-β defect in CD11c$^{+}$ DCs | 20w chow diet | ↑ | Two-fold increase in plaque area; increased T cell content; increased TNF-α and IL-12 production; strong induction of T cell proliferation/activation |
| Subramanian *et al.*, 2013 | CD11cCre Myd88$^{fl/fl}$→LDLr$^{-/-}$ | Block TLR signaling in CD11c$^{+}$ DCs | 10w WD | ↑ | Increased atherosclerotic lesion size; increased lesional monocyte recruitment associated with loss of Treg-mediated suppression of MCP-1 |
| Sage *et al.*, 2014 | μMT:*pIII+IV*$^{-/-}$→LDLr$^{-/-}$ | selective MHC II deficiency on pDCs | 6w WD | ↓ | Reduction in aortic root lesion size; decreased T cell infiltration in lesions; reduced IFN-γ |
| Sage *et al.*, 2014 | CD11cCre Tcf4$^{-/fl}$→LDLr$^{-/-}$ | pDC deficiency | 8w WD | ↓ | Significantly reduced lesion development; reduction in plaque T cell accumulation |
| Mandl *et al.*, 2015 | BDCA2-DTR ApoE$^{-/-}$ | pDC depletion | 4w WD, DT 3x/w | = | No change in lesion size or stability; altered levels of inflammatory cytokines; loss of DTR on reoccurring pDCs |
| Legein *et al.*, 2015 | Batf3$^{-/-}$→LDLr$^{-/-}$ | CD8α$^{+}$ DC depletion | 10w WD | = | 80% decrease in CD8α$^{+}$ DC numbers in spleen and LN; reduced cross-priming capacity; no change in lesion size or stability |

Abbreviations: Batf3, basic leucine zipper ATF-like transcription factor 3; BDCA-2, blood dendritic cell antigen-2; CCL17, CC chemokine ligand 17; CD11cDNR, functional inactivation of TGFβ receptor II signalling in CD11c$^{+}$ cells; CX3CR1, CX3C chemokine receptor 1; Flt3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; hBcl-2, anti-apoptotic B-cell lymphoma 2; Myd88, myeloid differentiation factor 88; PDCA-1, plasmacytoid dendritic cell antigen-1; Tcf4, transcription factor 4 (also basic helix-loop-helix transcription factor (E protein) E2-2); μMT:*pIII+IV*, selective MHC class II deficiency on pDCs.

Thus, a clear identification of the role of cDCs in atherosclerosis is still limited by the lack of an unambiguous marker. In this dissertation, we used a recently developed mouse model in which a DTR transgene is inserted into the Zbtb46 gene (Zbtb46-DTR mice). In the immune system, Zbtb46 was identified to be expressed specifically by cDCs and their immediate precursors, by certain activated monocytes, but not by pDCs, macrophages or other immune cells. By implementing the Zbtb46-DTR mouse model in our research work, we aimed to provide a first understanding of the role of cDCs in atherosclerosis (**chapter 4**). For this, lethally irradiated, $LDLr^{-/-}$ mice were transplanted with bone marrow from Zbtb46-DTR donor mice. However, the outcome of our study was not what we had anticipated. The depletion efficiency was not as high as predicted and cDC depletion could not be sustained over a longer period of time. For this, several explanations may be put forward, such as the existence of other sources for cDCs or the long duration of the study, which are commented on below. However, other possible explanations for insufficient depletion that could also be further explored include: genetic alterations in the transgene, an increasingly efficient DT metabolism in the recipients, or immune reactions to DT (e.g. DT neutralizing antibodies).

All DCs and other mononuclear myeloid cells are derived from bone marrow progenitors that undergo progressive lineage restriction (Figure 7.1). Initially, it was suggested that DCs can arise from either lymphoid-restricted progenitors (CLPs) or myeloid-restricted progenitors (CMPs), as long as they expressed Flt3. Later, a common macrophage-DC progenitor (MDP) was suggested to be the first precursor downstream of CMP, which has the potential to generate DCs and monocyte-derived cells. The existence of MDPs and whether MDPs truly represents a bi-potential progenitor for DCs and monocytes is still a subject of debate. Moreover, as MDPs do not always exhibit pDC potential, they might not always be developmental precursors of pDCs.[21] In the classical model of DC development, the MDP generates the common DC progenitor (CDP), a key cellular stage of DC development. The CDP can give rise to both pDCs and cDCs but no other cell types.[22] Some even suggest that the existence of a common precursor for cDCs and pDCs is also debatable and that lineage divergence among myeloid cell types might occur upstream of MDPs, known as the lymphoid-primed multipotent progenitor (LMPP).[21] In this respect, others have suggested an overlap model, whereby progenitors can commit to DC subtypes at many points along the hematopoietic pathway. This could imply that DCs branch from yet earlier progenitors, upstream of the myeloid and lymphoid split.[23] This being said, most researchers can agree on the fact that there are cDC-restricted progenitors (pre-cDCs) that can be distinguished

from pDC-specific progenitors (pre-pDCs), which are restricted to their respective lineage but not yet fully mature. pDCs mature in the bone marrow, but immature CCR9$^-$ pDCs can differentiate into cDCs *in vitro* and *in vivo*. Thus pDC development could be redirected until the terminal stage of maturation.[24] Furthermore, the currently used definitions of DC progenitors in bone marrow appear incomplete.[22,24] Using a GFP knockin reporter for Zbtb46 expression, Satpathy *et al*. combined Zbtb46-GFP expression with analysis of additional markers such as SiglecH.[25] On the basis of the expression of Zbtb46-GFP and SiglecH, CDPs and especially pre-DCs can be divided into four populations. In particular, they showed that, although SiglecH is specific for pDCs in peripheral organs, bone marrow progenitors expressing SiglecH actually retain potential for cDC development. This suggests that the CDP/pre-cDC stage is more heterogeneous than initially thought.[22,24,25] While Zbtb46-GFP$^+$ progenitors are restricted to become cDCs, SiglecH$^+$Zbtb46-GFP$^-$ progenitors are not committed to the pDC lineage and can also retain the potential to generate cDCs. Whether the latter population overlaps with CCR9$^-$ pDC-like precursors is not yet explored. Although the phenotypic definitions of cDC progenitors used by Satpathy *et al*. may need some refinement, the fact that DC progenitors likely represent heterogeneous populations indicates that we are still not fully aware of which progenitor cells precede the distinct DC subtypes and where the different branching points in the hematopoietic tree that lead to functional differences are.

In Zbtb46-DTR mice, DTR was shown to be absent on Lin$^-$CD11c$^-$CD11b$^+$CD115$^+$ monocytes. Consequently, monocytes are not depleted upon DT administration.[26] However, like cDCs, activated monocytes can present antigen *in vitro* and *in vivo*.[27,28] Furthermore, monocyte levels are elevated under hypercholesterolemic conditions and they can give rise to moDCs during inflammation. For this, one hypothesis arguing our observations is that monocytes could have replenished the cDC pool driving the differentiation of moDCs, as suggested by an absence of change in the proportions of CD11b$^+$ DCs in DT-treated mice in our study. Thus, under inflammatory conditions, additional pathways may exist along which the cDC pool can be maintained. In this respect, we cannot rule out the possibility that cDC depletion might have had an effect in the early developmental stages of atherosclerosis. However, after 18 weeks on the WD, which corresponds to an advanced stage of the disease, other mechanisms could have surpassed the function of DCs. Generally, results from cDC depletion studies (Table 7.1) are mainly obtained in much shorter time span.

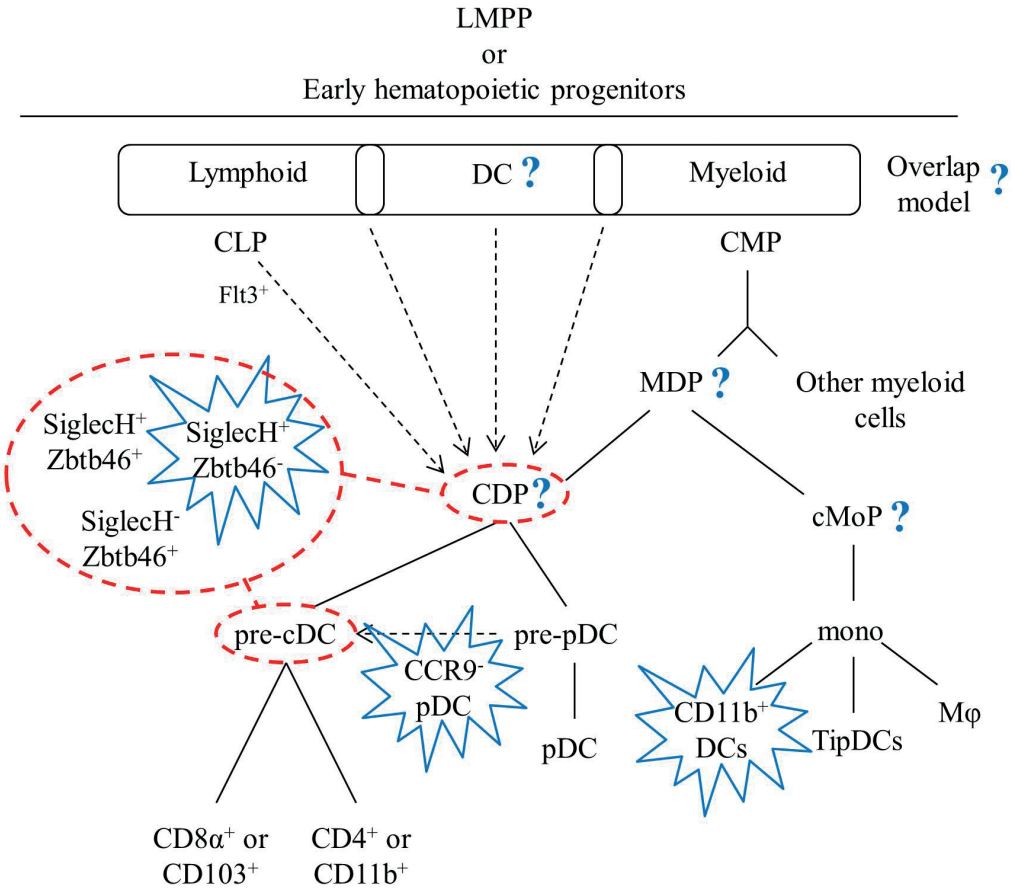


**FIGURE 7.1. Overview of the existing and suggested pathways of DC development in the mouse.** In a revised model of DC development it is suggested that a putative DC program may involve overlapping of the myeloid and lymphoid programs in the earliest hematopoietic progenitors (overlap model; dashed arrows). In this way, DCs represent a separate lineage from the existing myeloid and lymphoid lineages. The known and plausible (question marks) progenitor cells are shown. The existance of MDP or a common precursor for cDCs and pDCs (CDP) is debatable. The theoretical heterogeneity of DC progenitor stages is depicted by red dotted circles. Solid lines represent the current model of the developmental pathways of DCs. Both in steady state as well as under inflammatory conditions, additional pathways may exist along which the cDC pool can be maintained (star shaped). Abbreviations: CDP, common DC progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; cMoP, common monocyte progenitor; LMPP, lymphoid-primed multipotent progenitor; MDP, common macrophage-DC progenitor; Mφ, macrophage; TipDCs, TNF/iNOS-producing DCs.

## CHARACTERIZATION OF LOCAL AND SYSTEMIC IMMUNE PROFILES IN ATHEROSCLEROSIS AND POTENTIAL AS A BIOMARKER OF ATHEROSCLEROTIC DISEASE

A biomarker is a measurable indicator that represents, either directly or indirectly, biologic processes in an organism at a given time. It can therefore be used to measure the severity or presence of some disease state.[29] Besides indicating pathological changes and reflecting the severity of the disease, an ideal biomarker should have the following characteristics: standardised, reproducible, reliable and cost effective; high sensitivity and specificity; safe and easy to measure; consistent across gender and ethnic groups; and modifiable with treatment. In atherosclerosis, novel biomarkers should be able to predict cardiovascular risk independently of conventional risk factors.[30] Biomarkers may be used alone or in combination (panel) for diagnosis, monitoring treatment, or prognosis of a disease.

Atherosclerosis in its early stages is often asymptomatic. The identification of atherosclerosis itself is not routine in clinical practice but involves non-invasive and invasive imaging techniques. For example, ultrasonic imaging is the most frequently used method in clinical practice to evaluate carotid plaques. Currently, the degree of carotid artery stenosis is used to predict the risk of future cerebrovascular events. Imaging of vessels can be used in primary prevention in those deemed to be at high risk of undergoing cardiovascular events, or for secondary prevention in patients already undergoing catheterization.[31] Consequently, most clinical decision making is based on symptomatic status and the degree of stenosis detected. Even though imaging the extent of atherosclerosis in vessels can be a predictor of destabilisation, sampling of peripheral blood to assess vulnerability has the advantages of ease of use and low cost.[29,31] The role of biomarkers in detecting vulnerable plaques is a field of great interest due to its potential to aid in the prevention of cardiovascular events.[29] Firstly, the determination of biomarkers related to vulnerable atherosclerotic plaques in blood would enhance primary prevention by identifying individuals in need of further diagnostic testing, and it may aid in the planning of intervention and in choosing the best medical treatment. Secondly, biomarkers would improve secondary prevention by serving as surrogate markers of drug efficacy in patients known to have atherosclerotic disease. Moreover, the utilization of biomarker panels could increase the accuracy of predicting the transformation of the atherosclerotic plaque into a vulnerable one.[29,31]

Since atherosclerosis is regarded as a form of chronic vascular inflammation, numerous molecules associated with inflammation have been identified as possible novel biomarkers. CRP,

one of the acute-phase proteins primarily produced in the liver, is one of the most actively studied. However, CRP response can be triggered by many disorders unrelated to atherosclerosis such as infections and other chronic inflammatory diseases.[32,33] Various cytokines have also been shown to be involved in atherosclerosis. For example, in an observational study, blood IL-6 concentrations were significantly elevated in individuals who developed MI as compared to those who did not.[34] However, as is the case with CRP, cytokines itself are not specific to the development or progression of atherosclerosis. Furthermore, several enzymes, including matrix-degrading enzymes, lipoprotein-associated phospholipase A2, and myeloperoxidase, have been investigated as possible inflammatory markers considering their role in fibrous cap thinning and eventually plaque rupture. More recently, clinical studies detected significant changes in the blood vessels of certain microRNA molecules in patients with CVD.[33] These, and many others, were shown to be associated with increased risk of cardiovascular events, including MI or death, but their relevance to atherosclerosis progression is less established. Moreover, the lack of correlation between CRP and plaque inflammation was recently confirmed by immune-pathological analysis of carotid endarterectomy specimens.[35]

Thus, there is a strong demand for more disease-specific biomarkers for atherosclerosis and CVD. Considering that white blood cells constitute the effector arm of the immune system and provide a prompt response to tissue damage, the identification of alterations in white blood cell profiles may provide a valuable tool to evaluate the inflammatory and immune status of the patient. In this regard, circulating immune cells might serve as biomarkers of atherosclerosis. Until now, only few studies analysed the association between circulating immune cells and advanced carotid atherosclerotic disease.[32] In this thesis we have shown that, by conducting a longitudinal study in ApoE$^{-/-}$ mice (**chapter 3**), after 12 weeks on a WD, a clear turning point in immune cell dynamics occurs. In particular, a significant drop of the CD11b$^{+}$ cDC subset was seen at all peripheral lymphoid locations. This time point coincides with a substantial increase in the size and area of atherosclerotic plaques, and correlation analyses revealed a strong association between the percentage of circulating CD11b$^{+}$ cDCs and signs of inflammation in the plaque. Furthermore, our observations confirm the previous findings by others that NKT cells are important early in plaque development in atherosclerosis-prone mice.[36,37] We also observed that the proportion of NKT cells increases progressively with the disease. Later in this discussion, it is described that treatment of ApoE$^{-/-}$ mice with ASA reduced NKT levels (**chapter 5**). At the same time, an increase was observed in plaque stability. This confirms the possibility that NKT cells may

be an important biomarker in the future. However, additional research is still required. For example, different subsets of NKT cells may play different functional roles in atherosclerosis and other autoimmune diseases. For instance, in humans, the relative ratio of NKT cell subsets may influence susceptibility vs. resistance to immune-mediated diseases.[38,39] Although we have not performed a subset analysis, we are the first to demonstrate that, in advanced human atherosclerosis, the number of circulating NKT cells explains a large part of the variance in both the T cells and NKT cells in the plaque, independent of all the other risk factors. In the future, longitudinal studies including a higher number of healthy individuals and patients with disease, coupled with more detailed NKT cell analysis, might allow certain characteristics of NKT cells to be identified as biomarkers (or even cellular therapy) for inflammatory, and thus unstable, atherosclerotic plaques.[39]

Some caution is required if we want to apply these findings to the human clinical situation. It has to be noted that immune cell numbers in mice differ from humans. Whether differences in cell numbers are also reflected in differences in function needs further study. Despite these differences, initial atherosclerosis development is similar. In this respect, it was recently reported that gene expression patterns in mice closely correlate with genomic responses in human inflammatory conditions, strongly arguing for the use of mice as a relevant model system of human disorders.[40]

## MODULATION OF cDCS AND IMMUNE CELL PROFILES IN ORDER TO MOVE TOWARD NOVEL THERAPEUTIC APPROACHES FOR PLAQUE STABILISATION

DCs are endowed with the supreme capacity to capture, process, and present antigens to T cells. Based on this prominent feature, they may constitute an essential component of vaccination. Indeed, several laboratories have pursued DC-based vaccination strategies as a novel method to either improve or reduce immunity in a variety of diseases. For example, a DC-based cancer vaccine may consist of *ex vivo*-generated tumour antigen-loaded DCs that are injected back into patients.[41] Over the past few years, DC-based stimulatory immunotherapies were proven to be safe and effective for the boosting of immunity in cancer[42] and infectious diseases[43]. Moreover, the US Food and Drug Administration (FDA) has approved DC therapy for the treatment of prostate cancer.[44] In contrast to cancer vaccines, the goal of a DC vaccine for autoimmune disorders is to induce a specific tolerogenic response (tolDCs).[14] The therapeutic potential of this strategy was already demonstrated in experimental animal models of different autoimmune disorders such as RA, multiple

sclerosis, inflammatory bowel disease, type 1 diabetes and atherosclerosis.[14] To date, the safety of tolDCs has been demonstrated for type 1 diabetes, RA and Crohn's disease, and more phase I tolDC trials are underway in MS, neuromyelitis optica, type 1 diabetes, and kidney transplantation. However, the clinical efficacy of tolDCs remains to be determined. In this respect, a phase II trial with tolDCs in type 1 diabetes will start to recruit patients.[45]

Our general hypothesis is that in the context of atherosclerosis an imbalance exists between pro-inflammatory Th1/Th17 cells and anti-inflammatory Th2/Tregs in response to (altered) self-antigens, leading to immune responses responsible for plaque development and progression. Restoration of this imbalance could beneficially affect atherosclerosis. At the moment, no treatment that directly modulates the inflammatory response has reached the clinic. In this thesis, modulation of the immune response was assessed in two ways: (i) by *in vitro* modification of the T cell stimulatory capacity of DCs to directly induce a tolerogenic phenotype, and (ii) by *in vivo* administration of ASA, a drug known to induce tolerogenic DC responses.

As described above, a powerful strategy to achieve modulation of inflammatory responses is vaccination. For many diseases, vaccination is used as a preventive therapy. One of the first vaccination approaches to modulate the immune response in experimental animal models of atherosclerosis was via immunisation with proteins both related and unrelated to LDL, but the outcomes of these studies varied greatly.[46-48] Moreover, this approach has some limitations. The antigen composition of the particles is difficult to standardise and they may also contain harmful antigens. Furthermore, immunomodulatory components (adjuvants) need to be added, which might induce a risk for side effects. Lastly, the mechanism of action is poorly understood.[48] Therefore, vaccination with tolDCs against immunogenic antigens offers a promising, more targeted strategy to prevent or treat atherosclerosis. The use of RNA interference provides an attractive tool to silence expression of immune stimulatory pathways and induce tolerance in DCs. In this thesis we performed an *in vitro* proof of concept study (**chapter 6**) whereby DCs where genetically modified by means of electroporation with siRNA to transiently inhibit the expression of IL-12p35, the unique subunit of the biologically active IL-12p70. By using this technique, we demonstrated that the production of IL-12 in mouse BMDCs could be efficiently silenced. Furthermore, IL-12 silencing modified the T cell stimulatory capacity of DCs, as shown by a significant decreased IFN-γ production by allogeneic T cells in co-culture experiments.

To further strengthen these results, other Th2 cell cytokines (e.g. IL-10, IL-13), could be measured as well to further document Th1/Th2 polarisation. In addition, extension of these data with an intracellular cytokine staining for T cell transcription factors (T-bet, GATA-3, FoxP3 and RORγt) would help to get more insight into the polarisation and the amount of cytokine produced per T cell. Additionally, tolDCs may be loaded with relevant antigens. The expression of MHC class II on tolDCs and the ability to migrate to secondary lymphoid tissue (e.g. through CCR7), and to process and present the antigens, should be investigated.

Besides the use of RNA interference, DCs can be converted into tolDCs by addition of various immunomodulating agents, as reviewed extensively by Van Brussel *et al.*[14] These include (but are not limited to) pharmacological agents (e.g. vitamin D3, dexamethasone, and ASA) and biologicals (e.g. IL-10, TGF-β, and apoptotic cells).[14] Because of its widespread use, we explored the potential of ASA in affecting atherosclerosis. Indeed, ASA has been suggested to potentially induce immunological self-tolerance through induction of tolerogenic properties in DCs which in turn initiate regulatory activity in responder T cells. However, the concentrations of ASA that are used in *in vitro* experiments may exert toxic effects upon administration *in vivo*. Therefore, we tested whether chronic low-dose ASA treatment could modulate inflammatory responses in $ApoE^{-/-}$ mice and consequently reduce atherosclerotic lesion development. Our results in **chapter 5** demonstrate that chronic low-dose ASA does not reduce the size of lesions, but it ameliorates lesion stability as shown by a reduction in plaque macrophages and increased collagen content. Importantly, ASA treated mice displayed signs of peripheral immune modulation, such as lower levels splenic neutrophils and NKT cells. As mentioned earlier, **chapter 3** of this thesis reports that the number of NKT cells gradually increases with the progression of the disease. ASA treatment reduced the levels of NKT cells, while the stability of plaques was increased (**chapter 5**). This reinforces our theory that, in the future, longitudinal monitoring of NKT cells may be a valuable method to verify the efficacy of a treatment for atherosclerosis.

Of particular interest is the increased proportion of LN Tregs in ASA treated mice. Although a disturbed Th1/Th2 balance has long been suggested as the underlying cause of the auto-inflammatory pathology in atherosclerosis, this theory has lately been challenged by a new hypothesis in which Tregs play a pivotal role.[49] Indeed, in experimental animal models, targeting Tregs by deletion of co-stimulatory molecules, CD25 neutralising antibody, or with DCs transfected with FoxP3 encoding mRNA resulted in increased atherosclerotic lesions.[50,51] Clinical studies also showed that the number of Tregs in peripheral blood from patients with acute coronary syndrome or aortic aneurysm was reduced.[52,53] Similarly,

patients with low baseline levels of $CD4^+FoxP3^+$ T cells have an increased risk to develop acute MI, which suggest that these patients may have the propensity to develop a higher burden of atherosclerosis.[54] Thus, strategies focused on expanding the potency of Tregs, and restoring or increasing the amount of Tregs, could be considered as a new therapeutic approach to inhibit pro-inflammatory immune responses in atherosclerosis.[55-57] In a more indirect approach, improvement or enhancement of Tregs can be obtained via induction of tolerogenic DCs. Modulation of DCs *in vitro* towards a more tolerogenic phenotype can result in induction of Tregs upon transfer *in vivo*.[58-60] However, whether or not chronic low-dose ASA resulted in a tolerant DC phenotype was not investigated in our study.

## FUTURE PERSPECTIVES

By applying a principal component analysis (PCA) to our mouse data in **chapter 3**, we took a first step in the direction towards a different way of searching for profiles that match a particular disease phenotype. The main purposes of a PCA is to reduce a complex data set in order to identify sometimes hidden patterns. The disadvantage of this technique is that it requires complete data, thus values that are excluded from the analysis (e.g. outliers) may greatly reduce the number of data points on the score plot.

Similarly, we provide a complete subset analysis of multiple human cell types in plaque and blood at the same time. The next step would be to compare the profile of immune cell subsets in blood of patients undergoing endarterectomy with the peripheral blood profile of healthy controls to determine the extent to which the immune activation in the blood is reflected in the local inflammation in the plaque.

Others have also attempted to link immune cell (subsets) to plaque instability. Via immunohistochemistry, it was demonstrated that a change in the inflammatory fingerprint occurs before and during plaque destabilisation.[61] Mapping and network analyses of differentially expressed genes in peripheral blood and endarterectomy samples from patients with symptomatic and asymptomatic carotid stenosis was performed to identify pathways linked to plaque instability.[62] Ultimately, techniques such as flow cytometry and transcriptomics, should be combined with intravital imaging to fully understand cellular dynamics in living tissue.[63]

In addition to the profiling of immune cells, gene expression profiling coupled with protein analysis of isolated DC and NKT cell subsets may provide a valuable tool for the identifi-

cation of specific types of inflammatory responses associated with plaque progression or vulnerability. By comparing the functional characteristics of DC and NKT cell subsets in inflamed tissues from patients with atherosclerosis, with their circulating counterparts in disease and in health, cell-specific markers may be found that are not present in the blood, which could be used for specific targeting of inflammatory tissue-infiltrating DCs or NKT cells. Furthermore, sorted cells can also be used to determine functional characteristics *in vitro*. The crosstalk of DC populations with other cell types (e.g. T cells, NKT cells, B cells, macrophages and mast cells) could be studied. It is hypothesised that a disrupted cross-talk underlies the disturbed immunological homeostasis in atherosclerosis. This approach would allow us to determine the consequences of modulation of signaling pathways on DC activation and the resulting immune responses. An impressive new technique to map interactions at the single cell level are the so-called "nanoarenas" for cell attacks, using microfluidics, to find out how individual immune cells cooperate with each other.[64]

Despite the promising outcomes in **chapter 6**, there are many questions and obstacles that must be solved before tolDCs can be translated into the clinic and used as a treatment for atherosclerosis. First, atherosclerosis is a complex multifactorial disease, which is dependent on the interplay between lipid metabolism, cellular reactions and inflammation. Hence, one of the main obstacles is the identification of the primary target antigen(s) that can be used to load tolDCs. For instance, in other autoimmune diseases such as multiple sclerosis and RA, tolDCs may be loaded with synovial fluid or with citrullinated peptides, respectively.[14,65] However, in a phase IIa clinical trial in MS, some patients developed exacerbations of the disease when treated with an HLA class II-restricted altered peptide ligand of myelin basic protein.[66] Similarly in atherosclerosis, vaccination with oxLDL-pulsed LPS-stimulated DCs reduced lesion development[67], while MDA-LDL-pulsed LPS-stimulated DCs were found to aggravate atherosclerosis.[68] However, those DCs were not made tolerogenic prior to vaccination. Indeed, a single treatment of "humanised" mice with ApoB100-loaded DCs that were made tolerogenic by the addition of IL-10 reduced the plaque burden in $LDLr^{-/-}$ mice by approximately 70%.[69] In atherosclerosis, the best strategy would probably be to use a cocktail of different antigens to mimic as closely as possible the situation *in vivo*.[70] However, in the study by Giannoukakis *et al.*, which used DCs treated *ex vivo* with a mixture of antisense oligonucleotides against CD40, CD80, and CD86, for the treatment of type 1 diabetes, no antigen was used.[71] This raises the question whether it is necessary to load tolDCs with relevant disease-associated antigens.

Second, administration of tolDCs into an inflammatory microenvironment may entail the risk of *in vivo* conversion to immunisation. It is therefore highly necessary to preserve a tolerogenic DC phenotype. Thus, *in vitro* washout experiments should be performed to ascertain that IL-12-silenced DCs retain a tolerogenic phenotype upon encounter with a pro-inflammatory environment.

Third, atherosclerosis is a slow, progressive disease that may begin as early as childhood. However, by the time symptoms occur, atherosclerosis is advanced and serious obstructions may already be present. Therefore, therapy timing and the immunological status of a patient are additional critical issues that need to be considered carefully when designing proper clinical applications.[72,73] The identification of suitable biomarkers could provide a useful adjunctive criterion to ensure better risk stratification of patients. According to our findings presented in **chapter 3**, immune cell profiling might be a valuable tool to assess the risk profile of a patient. A qualitative and quantitative assessment of immune responses in patients, i.e. the identification of surrogate end-points for tolerance-inducing trials, needs to be established.

Once these hurdles are addressed, other numerous questions still remain in view of advancing this concept to the human arena. These include questions regarding: vaccine safety; proper selection of patient populations for testing; the dose, route and frequency of administration. Moreover, the effect of immunisation with tolDCs should be durable, preferably permanent or at least for years following intervention. This may require a number of repetitive injections with tolDCs. Therefore, cryopreservation of tolDCs in ready-to-use aliquots for clinical application would significantly improve the feasibility of consecutive injections and facilitate the routine use of DC trials. Recently, it was shown that cryopreserved tolDCs have similar phenotypic and functional properties as compared to freshly prepared DCs, which demonstrates the feasibility of cryopreservation of these tolDCs.[74,75] Lastly, the economic cost of such personalised medicine should be assessed. However, if we can get the right treatment to the right patient at the right dose through the use of suitable biomarker tests and targeted therapies, substantial savings can be accomplished in the long-term costs associated with individual patients, such as reductions in subsequent hospital admissions.

## CONCLUSION

Atherosclerosis is a complex multifactorial disease of the arterial wall, involving both innate and adaptive immune responses. Over recent years, the contribution of DCs herein has become more apparent. DCs are found in healthy arteries and accumulate in atherosclerotic lesions and engage in diverse pathogenic and protective mechanisms during atherogenesis. Accordingly, targeting DCs may be a valuable approach for the development of (cell-based) immunotherapies for atherosclerosis. In this dissertation, we aimed to gain more knowledge regarding the contribution of DCs to the (immune)pathogenesis of atherosclerosis with the intention of identifiyng new therapeutic strategies to tackle this disease (Figure 7.2).

We tried for the first time to elucidate the true role of cDCs in atherosclerosis by using the Zbtb46-DTR mouse model. However, cDC depletion could not be sustained for a long period of time, making this mouse model unsuitable to examine effects of long-term depletion of cDCs. Moreover, the questionable sharpness of the boundaries between monocytes and DCs (*i.e.* cell plasticity), together with the unappreciated heterogeneity in (pre-)DCs, ensures that we cannot make a statement about the true potential function of DCs in atherosclerosis based on our results. Understanding DC ontogeny and refining cellular identification is still a work in progress. Eventually, this may lead to more selective depletion models that can assist in clarifying the exact role of DC subsets in atherosclerosis.

Nevertheless, based on the results from our longitudinal study, we believe that it would be particularly interesting to do more in-depth research on the role of $CD11b^+$ cDCs and NKT cells during atherogenesis. Moreover, we hypothesize that a careful analysis of circulating leukocytes may provide a valuable tool to evaluate the inflammatory and immune status of the patient. Using this approach DCs, NKT cells, or other leukocytes, may well serve as biomarkers. In particular, longitudinal monitoring of NKT cells may be a valuable method to verify the efficacy of a treatment for atherosclerosis.

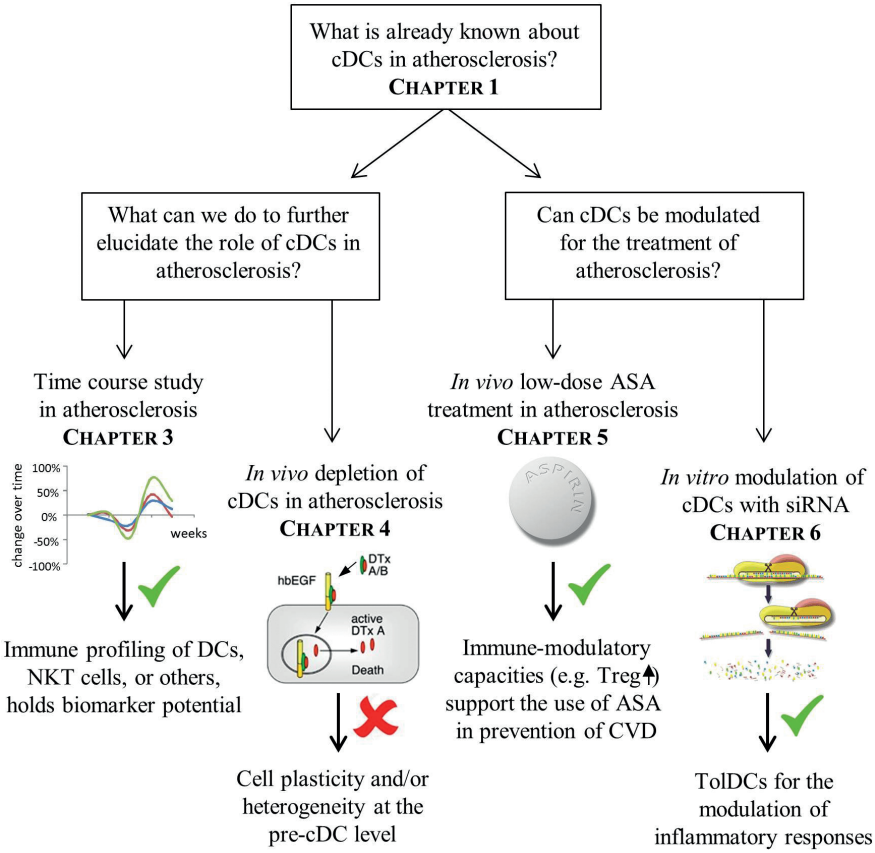


**FIGURE 7.2. Schematic outline of the thesis and the main results.**

Furthermore, we showed that the pathogenic immune response in atherosclerosis can be modulated via treatment with low-dose ASA, and that tolDCs can be generated *in vitro* using IL-12p35 siRNA. Because the patient's own DCs can be harvested, transduced with siRNA and injected back into the patient, this technique raises the possibility for individualized therapy of atherosclerosis. A major benefit of the use of cellular therapy is the potential to induce antigen-specific tolerance. This approach would ensure a more targeted therapy and leave other immune responses unaffected.

However, the complexity and heterogeneity of atherosclerosis may require integration of several tolerance induction mechanisms to fully control the disease. For example, a combination of IL-12-silenced DCs together with low-dose ASA treatment may be used in order to target different pathways. Ultimately, because of the intricate interplay between immunity, inflammation and dyslipidemia in atherosclerosis, the treatment of atherosclerosis will most likely consist of a combination of lipid-lowering drugs and immune modulating agents.

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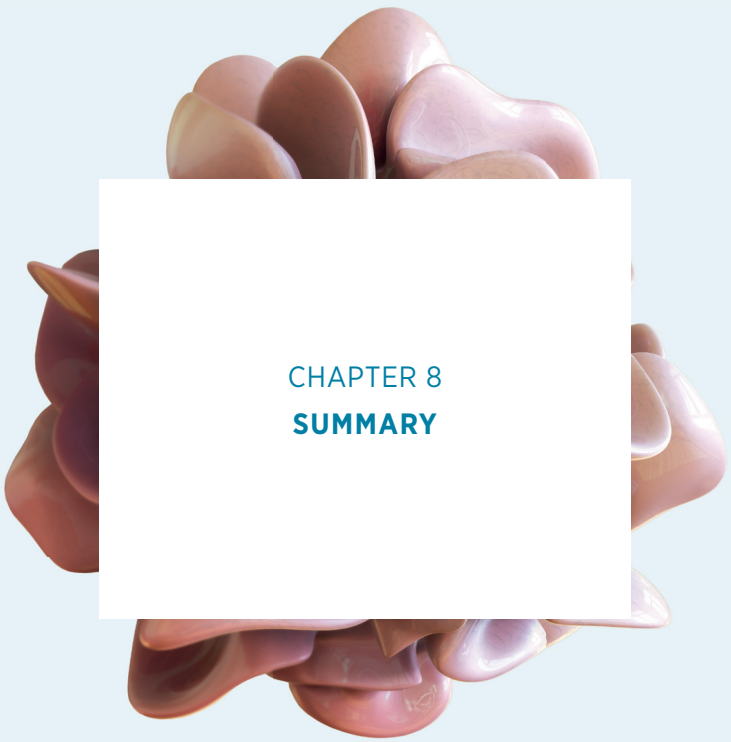


CHAPTER 8

# SUMMARY

Atherosclerosis, the dominant cause of cardiovascular disease (CVD), is a chronic inflammatory condition of middle sized and large arteries, characterized by the gradual build-up of plaques within vessel walls. Even though the build-up of plaque per se could decrease blood flow through stenosis, the major cause of clinical complications appears to be thrombus formation as a result of rupture of the plaque surface through the effects of proinflammatory cytokines and chemokines on the fibrous cap. Subsequently, inhibition of blood flow by thrombi can lead to complications such as myocardial infarction and stroke. CVD remains the leading cause of death and a considerable cause of morbidity worldwide. The treatment of atherosclerosis is currently based on reducing risk factors, such as lowering lipids (e.g. statins) and decreasing hypertension (e.g. beta-blockers). While statins are considered to be highly effective in preventing atherosclerotic events, a substantial portion of treated patients still progress to overt CVD. Hence, there is a high unmet need for continued research for novel and enhanced therapeutic strategies for atherosclerosis.

Because there is increasing evidence for a role of disturbed immune processes underlying the disease mechanism in atherosclerosis, it has become even more important to examine the feasibility and applicability of immunomodulatory treatments for atherosclerosis. Dendritic cells (DCs) are professional antigen-presenting cells that bridge the innate immune system with the adaptive immune response, orchestrating both induction of T cell immunity, as well as immune tolerance. In this dissertation, we aimed to unravel the contribution of conventional DCs (cDCs) in the pathogenesis of atherosclerosis and to investigate if cDCs can be targeted for the treatment of atherosclerosis.

First, we aimed to characterize cDC subsets and effector cells during atherosclerosis in mice and men. In addition, we assessed whether changes in immunological cell profiles correlate with the progression or severity of atherosclerotic disease (**chapter 3**). For this, we conducted a longitudinal study in which immune cell distributions in blood and peripheral lymphoid organs of apolipoprotein-E ($ApoE^{-/-}$) deficient mice were analysed at several time points during atherosclerosis development. We demonstrated that, after 12 weeks on a western-type diet (WD), a clear turning point in immune cell dynamics occurs. In particular, a significant drop of the $CD11b^+$ cDC subset was seen at all locations. This time point coincides with a substantial increase in the size of atherosclerotic plaques. Additionally, strong correlations could be found between circulating natural killer T (NKT) cells and $CD11b^+$ cDCs with signs of plaque inflammation at 6 weeks and 12 weeks of WD, respectively. We observed a predominance of $CD11b^+$ cDCs in plaques from patients undergoing endarterectomy, compared to the $CD16^+$ moDCs subset. Remarkably, the number

of circulating NKT cells in these patients was predictive for the number of T cells and NKT cells in advanced plaques, independent of all other risk factors. These results in both mouse and human atherosclerosis underline the importance of the role of CD11b$^{+}$ cDCs and NKT cells during atherosclerotic plaque development. It is our opinion that further in-depth analysis of circulating leukocytes, in particular DCs and NKT cells, may provide a valuable tool to evaluate the inflammatory and immune status of the plaque and may well serve as biomarkers. Future studies, including a higher number of patients and different stages of atherosclerosis, are needed to strengthen and fine-tune our results.

The origin and function of distinct DC subsets that potentially control atherogenesis is still not fully understood. Up untill now, a clear identification of the role of cDCs in atherosclerosis was lacking due to the complexity in identifying an unambiguous marker for cDCs. Recently, Zbtb46 was identified to be expressed specifically by cDCs and their immediate precursors. In this dissertation, we used a recently developed mouse model in which a diphtheria toxin receptor (DTR) transgene is inserted into the Zbtb46 gene (Zbtb46-DTR mice). By using this mouse model, we aimed to elucidate the true role of cDCs in atherosclerosis (**chapter 4**). For this, lethally irradiated low-density lipoprotein receptor-deficient (LDLr$^{-/-}$) mice were transplanted with bone marrow from Zbtb46-DTR donor mice. Subsequently, Zbtb46-DTR→LDLr$^{-/-}$ chimeras were fed a WD for 18 weeks. During this time, cDCs were specifically depleted by administering DT to these mice. We confirmed that cDCs from Zbtb46-DTR mice could be depleted *in vitro* and *in vivo*. However, at the end of the atherosclerosis study, analysis of chimeras showed that depletion of cDCs could not be sustained. Accordingly, advanced atherosclerotic plaque size and composition was not altered in these mice. For this, several explanations may be put forward. First, it is possible that additional cDC subsets (Zbtb46 negative) may exist allowing the cDC pool to be maintained. DC progenitors likely represent heterogeneous populations indicating that we are still not fully aware of which progenitor cells precede the distinct DC subtypes. Second, under inflammatory conditions, blood monocytes can be recruited into the plaque where they can give rise to monocyte-derived DC populations, phenotypically similar to CD11b$^{+}$ cDCs. Thus, it is possible that cDC depletion might have had an effect in the early developmental stages of atherosclerosis, but that 18 weeks of DT treatment might be too long in order to observe an effect on plaque development, because these mice are already in a more progressive stage of disease. Taken together, as a consequence of the unsustained cDC depletion, no clear conclusions can be drawn on the role of cDCs in atherosclerosis. Importantly, cDCs are very potent activators of T cells, thus any partial depletion would

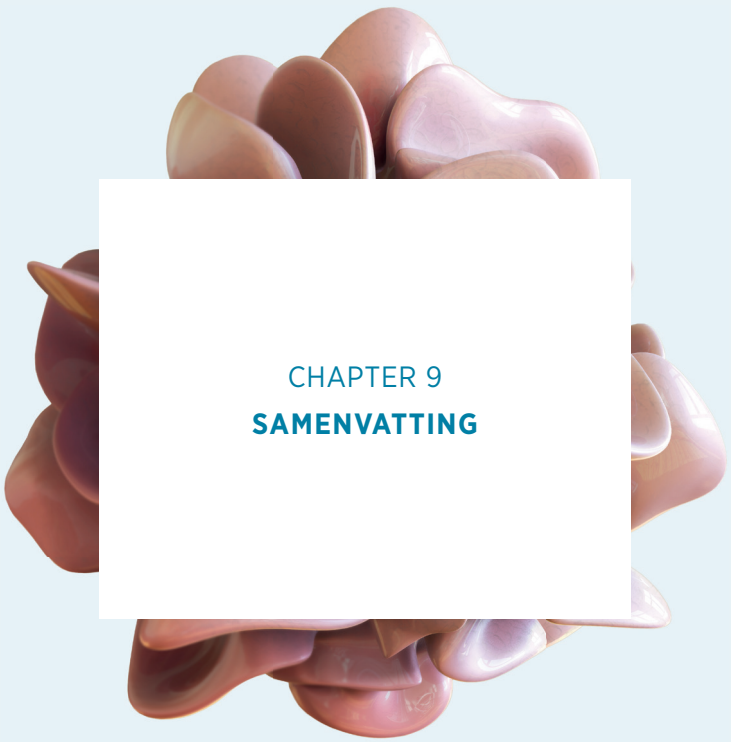
not be sufficient to suppress the development of atherosclerosis which further confirms the unsuitability of Zbtb46-DTR→LDLr$^{-/-}$ mice for studying the involvement of cDCs in atherosclerosis over extended periods of time. Whether or not *bona fide* cDCs significantly contribute to atherosclerotic plaque formation and stability remains to be determined. A better understanding of DC ontogeny and a refinement of cellular identification may eventually lead to more selective depletion models that can assist in clarifying the exact role of DCs in atherosclerosis.

DCs are endowed with the supreme capacity to capture, process, and present antigens to T cells. Based on this prominent feature, they may constitute an essential component of an immunomodulatory therapy. In the second part of this thesis, strategies to modulate immune cell profiles and DC function were explored in order to move toward novel therapeutic approaches for plaque stabilisation.

In **chapter 5**, we investigated the outcome of low-dose aspirin (ASA) on immune modulation and plaque progression in ApoE$^{-/-}$ mice. ASA is an irreversible inhibitor of the enzyme cyclooxygenase and inhibits platelet aggregation at low dose, preventing the formation of an occlusive thrombus after plaque rupture. Hence, ASA has been used for many years in the treatment and prevention of CVD. At high doses, ASA was shown to have anti-inflammatory effects *in vivo*, and immune modulatory effects on DCs *in vitro*. However, the usefulness of ASA at high doses is limited by toxicity. We administrated low-dose ASA (5 mg/kg/day) to the drinking water of ApoE$^{-/-}$ mice from 10 to 25 weeks of WD. ASA treatment of ApoE$^{-/-}$ mice with established plaques did not reduce the progression of atherosclerosis, but plaques appeared more stable. Additionally, we observed signs of immune modulation in the periphery such as a decrease in splenic weight and in the total number of splenic neutrophils and NKT cells. These results, combined with our observations on NKT cells in chapter 3, suggest that longitudinal monitoring of NKT cells could be a valuable method to verify the efficacy of a treatment for atherosclerosis. Interestingly, treatment of ApoE$^{-/-}$ mice with low-dose ASA induced a significant increase in the frequency of regulatory T cells (Tregs) in aorta-draining lymph nodes. Although further research is warranted, it could be that ASA exerted this effect indirectly through modulation of DCs, which is in line with *in vitro* studies. Increasing the proportion of Tregs is potentially appealing with respect to the induction of immunological self-tolerance for the treatment of atherosclerosis. Therefore, our data support the use of ASA for the prevention of CVD, alone or as add-on therapy, even in low risk factor profiles not at increased risk for bleeding.

Another powerful strategy to achieve modulation of inflammatory responses is vaccination. Since DCs are unique in their capacity to drive naive T cells to effector T cells, they may constitute an essential component of vaccination. Several laboratories have pursued DC-based vaccination strategies as a novel method to either improve or reduce immunity in a variety of diseases (e.g. cancer, infectious diseases). The goal of a DC vaccine for atherosclerosis is to induce a specific tolerogenic response (tolDCs). In **chapter 6**, we investigated the feasibility and consequences of modulation of the immune-polarising capacity of DCs *in vitro*. *In vitro* cultivated bone marrow-derived DCs were genetically modified by means of electroporation with silencing (si)RNA to transiently inhibit the expression of IL-12p35, the unique subunit of the biologically active IL-12p70. This cytokine was shown to be crucial for immune-stimulatory DC functions and the differentiation of T cells towards pro-inflammatory Th1 cells. By using this technique, we demonstrated that the production of IL-12 in DCs could be efficiently silenced. IL-12 silencing modified the T cell stimulatory capacity of DCs, as shown by a significantly decreased IFN-γ production by allogeneic T cells in co-culture experiments. Despite these encouraging outcomes, there are many questions and obstacles that must be solved before tolDCs can be translated into the clinic and used as a treatment for atherosclerosis. These include but are not limited to: the identification of the target antigen(s) that can be used to load tolDCs; the stability of tolDCs upon administration *in vivo*; therapy timing; vaccine safety; proper selection of patient populations; the dose, route and frequency of administration. Nevertheless, we believe that tolDC therapy constitutes a valuable approach for the treatment of autoimmune disorders such as atherosclerosis.

In conclusion, the data presented in this dissertation confirm that DCs are a worthwhile cell population to investigate, not only from an immunopathogenic point of view, but also from a therapeutic perspective in atherosclerosis. Our results prompt the need for more in-depth research into the role of $CD11b^+$ cDCs and NKT cells in atherosclerosis. Additionally, a careful analysis of circulating leukocytes may provide a valuable tool to evaluate the inflammatory and immune status of the plaque. Furthermore, we showed that the pathogenic immune response in atherosclerosis can be modulated via treatment with low-dose ASA, and that tolDCs can be generated *in vitro* using IL-12p35 siRNA which ultimately can be used to restore the immunological balance in atherosclerosis and other autoimmune diseases. It is likely that the future treatment regimen for CVD will consist of a combination of a lipid-lowering drug (e.g. statin) and an immunomodulatory therapy. Future studies will need to establish the clinical benefits of using such combined therapy.



CHAPTER 9

# SAMENVATTING

Atherosclerose, de belangrijkste oorzaak van het ontstaan van hart- en vaatziekten (HVZ), is een traag progressieve, inflammatoire aandoening die gekenmerkt wordt door de vorming van atherosclerotische plaques in de arteriële bloedvatwand door de plaatselijke ophoping van vetten. Het is mogelijk dat een plaque zo sterk aangroeit dat dit kan leiden tot de afsluiting van een bloedvat (i.e. stenose). Echter, de frequentste klinische complicaties, een hartinfarct of beroerte, ontstaan door trombusvorming als gevolg van het ruptureren van de plaque. HVZ vormen de belangrijkste doodsoorzaak in de huidige Westerse maatschappij en een belangrijke oorzaak van morbiditeit wereldwijd. De huidige behandeling van atherosclerose is voornamelijk gericht op het verminderen van risicofactoren, zoals het verlagen van lipiden (bv. statines) en het behandelen van hypertensie (bv. bètablokkers). Hoewel statines zeer doeltreffend blijken in het verlagen van het cardiovasculair risico, toch blijft bij een aanzienlijk deel van de behandelde patiënten de kans op een nieuwe cardiovasculaire complicatie hoog. Daarom is er nood aan aanvullende, doelgerichte therapieën voor atherosclerose.

Omdat er steeds meer aanwijzingen zijn dat verstoorde immunologische processen ten grondslag liggen aan het ziekteproces in atherosclerose, is het prioritair geworden om de haalbaarheid en toepasbaarheid van immunomodulerende behandelingen voor atherosclerose te onderzoeken. Dendritische cellen (DCs) zijn de krachtigste antigen-presenterende cellen en vormen de verbinding tussen de aangeboren en adaptieve immuunrespons. DCs zijn essentieel voor het goed functioneren van ons immuunsysteem en spelen een cruciale rol in het bewaren van de balans tussen immuniteit en tolerantie. Het doel van dit proefschrift was om de bijdrage van conventionele (c)DCs in de pathogenese van atherosclerose verder te ontrafelen en te onderzoeken of cDCs aangewend kunnen worden voor de behandeling van atherosclerose.

Allereerst beoogden we immuuncel subsets te karakteriseren tijdens de ontwikkeling van atherosclerose in muizen en mensen. Vervolgens hebben we onderzocht of dynamische veranderingen in het profiel van immunologische cellen correleren met de progressie of de ernst van atherosclerose (**hoofdstuk 3**). Hiervoor werd een longitudinale studie uitgevoerd waarbij de immuuncel distributie in perifeer bloed en lymfatische organen van $ApoE^{-/-}$ muizen werd geanalyseerd op verschillende tijdstippen tijdens de ontwikkeling van atherosclerose. We toonden aan dat, na 12 weken op een Westers dieet (WD), een duidelijk keerpunt in de immuuncel dynamiek optreedt. In het bijzonder werd een aanzienlijke daling van de $CD11b^{+}$ cDC subset waargenomen op alle onderzochte locaties. Dit tijdspunt komt overeen met een forse toename in de grootte van de atherosclerotische plaques. Bovendien

werden sterke correlaties waargenomen tussen circulerende *natural killer* T (NKT) cellen en CD11b$^+$ cDCs met tekenen van plaque inflammatie op respectievelijk 6 en 12 weken WD. Ook plaques van patiënten die een endarterectomie ondergingen bleken meer CD11b$^+$ cDCs te bevatten in vergelijking tot de CD16$^+$ monocyt-afgeleide (mo)DCs. Een opmerkelijke bevinding was dat het aantal NKT cellen in het perifeer bloed bij deze patiënten voorspellend was voor het aantal T cellen en NKT cellen in hun gevorderde plaques, en dit onafhankelijk van andere risicofactoren. Deze resultaten onderstrepen het belang van de rol van CD11b$^+$ cDCs en NKT cellen tijdens de ontwikkeling van plaques. We zijn van mening dat een verdere, grondige analyse van circulerende leukocyten, in het bijzonder van DCs en NKT cellen, een waardevol hulpmiddel kan zijn om de immuunactiviteit van plaques te evalueren. Dergelijke analyse zou ons eveneens in staat stellen atherosclerose-specifieke biomerkers en therapeutische doelwitten te identificeren. Toekomstige onderzoek, met een groter aantal patiënten en verschillende stadia van atherosclerose, is nodig om onze resultaten te versterken en te verfijnen.

De oorsprong en functie van de verschillende DC subsets die bijdragen tot atherosclerose is nog steeds niet volledig gekend. De rol van cDCs in atherosclerose werd tot op heden bestudeerd met niet-selectieve depletiemodellen wegens gebrek aan een selectieve merker voor cDCs. Recentelijk werd een nieuwe transcriptiefactor geïdentificeerd, namelijk Zbtb46, die selectief tot expressie wordt gebracht door cDCs en hun onmiddellijke voorlopercellen, maar niet door andere immuuncellen. In dit proefschrift werd daarom gebruik gemaakt van een recent ontwikkeld muismodel waarbij een transgen dat codeert voor de humane difterietoxine receptor (DTR) in het Zbtb46 gen werd geïntroduceerd (Zbtb46-DTR muizen), wat toelaat om specifiek cDCs uit te schakelen. Om de exacte rol van cDCs in atherosclerose te onderzoeken werd een beenmergtransplantatie uitgevoerd, waarbij LDLr$^{-/-}$ muizen getransplanteerd werden met beenmerg van Zbtb46-DTR donor muizen (**hoofdstuk 4**). Vervolgens werden Zbtb46-DTR→LDLr$^{-/-}$ chimeren gedurende 18 weken op een WD geplaatst. Gedurende deze tijd werden cDCs specifiek geëlimineerd door toediening van DT. Door middel van korte *in vitro* en *in vivo* experimenten bevestigden we dat cDCs van Zbtb46-DTR muizen geëlimineerd kunnen worden met behulp van DT. Echter, aan het eind van de atherosclerosestudie wees een analyse van de chimeren uit dat depletie van cDCs niet in stand kon worden gehouden. Bijgevolg was er geen verandering in de grootte en samenstelling van de geavanceerde plaques in DT behandelde muizen. Hiervoor kunnen verschillende verklaringen naar voren worden gebracht. Ten eerste is het mogelijk dat er bijkomende cDC subsets bestaan, negatief voor Zbtb46, van waaruit cDC aantallen kunnen

worden onderhouden. We vermoeden dat de voorlopercellen van DCs een heterogene populatie betreft, wat aangeeft dat we nog steeds niet volledig weten welke stamcellen juist voorafgaan aan de verschillende DC subtypes. Ten tweede, tijdens inflammatie kunnen monocyten uit het bloed worden aangetrokken tot in de plaque, waar ze aanleiding kunnen geven tot een monocyt-afgeleide DC populatie dat qua fenotype vergelijkbaar is met $CD11b^{+}$ cDCs. Het is ook mogelijk dat de depletie van cDCs een effect zou kunnen hebben in de eerste stadia van atherosclerose, maar dat een DT behandeling van 18 weken te lang is om een effect op plaque ontwikkeling te kunnen waarnemen, omdat deze muizen zich reeds in een progressieve fase van de ziekte bevinden. Vermits de depletie van cDCs niet in stand kon worden gehouden, kan geen definitief besluit genomen worden over de rol van cDCs in atherosclerose. Vermits cDCs zulke krachtige T cel stimulatoren zijn, is een gedeeltelijke depletie waarschijnlijk onvoldoende om de ontwikkeling van atherosclerose te remmen. Dit beklemtoont dat Zbtb46-DTR→$LDLr^{-/-}$ muizen ongeschikt zijn voor het bestuderen van de rol van cDCs in atherosclerose gedurende langere tijd. Of cDCs al dan niet significant bijdragen aan plaquevorming en -stabiliteit moet nog worden bepaald. Een beter kennis van de ontogenese van DCs en een verfijning van de identificatie van DC subpopulaties kan uiteindelijk leiden tot meer selectieve depletiemodellen die kunnen helpen bij het ontrafelen van de precieze rol van DCs in atherosclerose.

DCs kunnen zeer efficiënt antigenen opnemen, verwerken en presenteren aan T cellen, wat maakt dat zij een essentieel onderdeel kunnen uitmaken van immunomodulerende therapieën. In het tweede deel van dit proefschrift werden strategieën onderzocht om immuuncel profielen en DC functie te moduleren met het oog op nieuwe therapeutische behandelingen voor plaque stabilisatie.

In **hoofdstuk 5** onderzochten we het effect van een lage dosis aspirine (ASA) op de modulatie van het immuunsysteem en de progressie van plaques in $ApoE^{-/-}$ muizen. ASA is een irreversibele inhibitor van het enzym cyclooxygenase en remt de bloedplaatjesaggregatie, zelfs wanneer het wordt toegediend in een lage dosis. Het verhindert de vorming van een occlusieve trombus na ruptuur van een plaque en wordt daarom al jaren gebruikt in de preventie van HVZ. Bij hoge doseringen, bleek ASA anti-inflammatoire effecten *in vivo* en immuun modulerende effecten op DCs *in vitro* te hebben. De bruikbaarheid van ASA bij hoge dosis is echter beperkt vanwege toxiciteit. We hebben een lage dosis ASA (5 mg/kg/dag) toegevoegd aan het drinkwater van $ApoE^{-/-}$ muizen tussen 10 en 25 weken WD. Deze behandeling resulteerde niet in een remming van de groei van bestaande atherosclerotische plaques, maar ze waren wel stabieler. Daarnaast zagen we tekens van immuun modulatie

in de periferie, zoals een afname in milt gewicht en het aantal neutrofielen en NKT cellen in de milt. Deze resultaten, gecombineerd met onze waarnemingen over NKT cellen in **hoofdstuk 3**, suggereren dat het longitudinaal opvolgen van NKT cellen een manier kan zijn om de doeltreffendheid van een behandeling voor atherosclerose na te gaan. Daarnaast resulteerde de behandeling in een aanzienlijke toename in de frequentie van regulatoire T cellen (Tregs) in aortadrainerende lymfeknopen. Hoewel meer onderzoek nodig is, is het mogelijk dat dit een indirect effect is van ASA door middel van modulatie van DCs, wat in lijn is met *in vitro* studies. Het indirect verhogen van het niveau van Tregs is een aantrekkelijke strategie voor de inductie van immunologische zelftolerantie voor de behandeling van atherosclerose. Onze resultaten ondersteunen daarom het gebruik van ASA voor de preventie van HVZ, alleen of als *add-on* therapie, zelfs bij laag risico profielen die geen verhoogd risico hebben op bloedingen.

Een andere strategie om de inflammatie in atherosclerose te moduleren is vaccinatie. Aangezien DCs uniek zijn in hun vermogen om naïeve T cellen te differentiëren tot effector T cellen, hebben verschillende laboratoria DC vaccinatie reeds toegepast als een nieuwe methode om het immuunsysteem te stimuleren of te onderdrukken in verschillende ziekten (bv. kanker, infectieziekten). Het doel van een DC vaccin voor atherosclerose is het moduleren van DCs naar een tolerogeen fenotype (tolDCs). In **hoofdstuk 6** was het onze bedoeling om beenmerg-afgeleide DCs *in vitro* te moduleren via de inhibitie van de transcriptie van genen betrokken bij de vorming van een immuunreactie met behulp van selectieve interferentie. Hiertoe hebben we DCs genetisch gemodificeerd met behulp van siRNA elektroporatie om de expressie van IL-12p35, de unieke subeenheid van het biologisch actieve IL-12p70, te verhinderen. Dit cytokine is noodzakelijk voor de immunogene functies van DCs en de differentiatie van T cellen richting proinflammatoire Th1 cellen. We hebben aangetoond dat de productie van IL-12p70 door DCs via deze techniek efficiënt kan worden onderdrukt. Stimulatie van allogene naïeve T cellen met deze IL-12p35-geremde DCs resulteerde in een verminderde interferon-γ secretie door de T cellen. Niettegenstaande, zijn er nog veel vragen en obstakels die moeten worden opgelost alvorens tolDCs in de kliniek kunnen worden gebruikt als een behandeling voor atherosclerose, zoals: de identificatie van het antigeen om tolDCs te laden; de stabiliteit van tolDCs na toediening *in vivo*; therapie timing; veiligheid; juiste keuze van patiëntenpopulaties; de dosis, route en frequentie van toediening. Desalniettemin zijn wij van mening dat tolDC therapie een waardevolle strategie vormt voor de behandeling van auto-immuunziekten zoals atherosclerose.

We kunnen besluiten dat DCs een veelbelovende celpopulatie vormen voor atherosclerose onderzoek, niet enkel vanuit een immunopathologisch perspectief maar ook vanuit een therapeutisch perspectief. Onze resultaten beklemtonen de nood aan meer diepgaand onderzoek naar de rol van $CD11b^+$ cDCs en NKT cellen in atherosclerose. Bovendien kan een zorgvuldige analyse van circulerende leukocyten een waardevol hulpmiddel zijn om de ontstekings- en immuunstatus van de plaque te evalueren. We hebben aangetoond dat de pathogene immuunrespons bij atherosclerose kan worden gemoduleerd via behandeling met een lage dosis ASA, en dat tolDCs *in vitro* kunnen worden gegenereerd door gebruik van IL-12p35 siRNA. Deze kunnen uiteindelijk gebruikt worden om de immunologische balans in atherosclerose en andere auto-immuunziekten te herstellen. We denken dat een toekomstig behandelingsregime voor HVZ zal bestaan uit een combinatie van lipiden verlagende geneesmiddelen (bv. statine) en een immunomodulerende therapie. Toekomstig onderzoek zal nodig zijn om de klinische voordelen van het gebruik van een dergelijke gecombineerde therapie vast te stellen.

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THESISWISDOM


motivation is like toothpaste...
at the end of the tube you
have to squeeze harder