

A fatal attraction: macrophage recruitment to the atherosclerotic plaque

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A fatal attraction:

**Macrophage recruitment to the
atherosclerotic plaque**

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A fatal attraction:

Macrophage recruitment to the atherosclerotic plaque

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In het openbaar te verdedigen
op woensdag 15 februari 2012 om 16:00

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Pieter Goossens

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CONTENTS

Chapter 1	General introduction	7
Chapter 2	Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions	39
Chapter 3	Myeloid I κ B α deficiency promotes atherogenesis by enhancing leukocyte recruitment to the plaques	61
Chapter 4	A survivin-targeted lentiviral vaccination strategy reduces murine atherogenesis	77
Chapter 5	Macrophage heterogeneity: relevance and functional implications in atherosclerosis	89
Chapter 6	General discussion	123
	Supplemental figures	143
	Summary	153
	Samenvatting	159
	List of abbreviations	165
	Dankwoord	171
	Curriculum vitae	179

Chapter 1

General Introduction

Atherosclerosis

Clinical implications

The life expectancy and disease profile of human societies are known to be correlated with the level of their economic development and social organization. Upon industrialization, the major cause of death shifted from nutritional deficiencies and infectious diseases towards degenerative diseases including cardiovascular diseases (CVD), cancer and diabetes¹. With 1.92 million people dying each year of coronary heart disease and 1.42 million of stroke, CVD include the first and the second most common cause of death in Europe^{2,3}. Both diseases are characterized by the disturbance of blood flow in coronary or cerebral arteries respectively, in the majority of the cases a result of a progressing or rupturing atherosclerotic plaque. This makes the atherosclerotic plaque an extremely important phenomenon to study.

Atherosclerosis literally means hardening of the arteries (“sclerosis”) due to the accumulation of lipids, cells and connective tissue (“atheroma”). It is a progressive disease characterized by the thickening and loss of elasticity of the walls of large and medium sized arteries. Most probably almost everyone in western society suffers from this disease but its progression usually occurs without clinical symptoms because the artery compensates for the narrowing lumen by remodeling and thereby enlarging its diameter⁴. Though a chronic disease, clinical symptoms are often acute and occur either upon (partial) occlusion of an artery by a growing atherosclerotic lesion beyond the ability to compensate for it or upon rupture of an instable lesion with the formation of a thrombus. Depending on the location of the plaque, blood flow in the downstream organ will be disturbed resulting in a wide variety of symptoms. Atherosclerosis in the coronary arteries can cause angina pectoris or myocardial infarction whereas in the carotid arteries it can result in transient cerebral events or stroke. In the peripheral circulation it might cause many different manifestations including acute limb ischemia, gangrene, ischemic renal failure, hypertension, mesenteric ischemia and bowel infarction⁵.

Several risk factors determine the progression of an atherosclerotic plaque, most of them well-known like a Western-type diet, smoking, hypertension, obesity, a lack of physical exercise, diabetes and age, others less known like infectious agents and elevated homocysteine levels⁶⁻⁸. Up to now, therapy strategies focus on the reduction of these risk factors, managing lipid levels with statins⁹ or lowering blood pressure with ACE inhibitors and β -blockers¹⁰. A better knowledge of the cellular and molecular mechanisms involved in the atherogenesis and its development into a clinically relevant risk however might lead to more targeted and more efficient therapies.

Atherogenesis

Atherosclerosis is a chronic inflammatory disease¹¹⁻¹⁴ in which macrophages play a key role but also other cell types and immune mechanisms are involved^{12,15-17}. It initiates when the permeability of the inner layer of the blood vessel is altered, for example by elevated levels of (modified) LDL, locally disturbed laminar blood flow or changes in shear stress. Because of this endothelial dysfunction, the vessel wall, that is only selectively permeable, suddenly becomes permeable for the LDL particles and they accumulate in the intima¹⁸, the matrix between the endothelial and the media layer of the blood vessel, where they are trapped by an interaction with the matrix proteoglycans¹⁹. Another consequence of the vessel wall damage is the activation of the endothelial cells, causing them to upregulate the expression and presentation of adhesion molecules, including E-selectin, vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM). In combination with the upregulation of chemokines such as monocyte chemoattractant protein-1 (MCP-1) they stimulate monocytes to adhere to and migrate through the endothelial layer, forming a so-called fatty streak. In this subendothelial space, the monocytes proliferate and differentiate into macrophages under the influence of monocyte colony stimulating factor (M-CSF), which is also produced by the activated endothelial cells. These macrophages use their scavenger receptors, such as CD36 and scavenger receptor A (SR-A), to take up the (modified) LDL particles²⁰. Macrophages that are filled with lipids, called foam cells, get activated which induces them to start producing cytokines and chemokines, creating an inflammatory milieu and attracting more macrophages and other immune cells including T cells, dendritic cells and neutrophils to the vessel wall. This influx of new cells results in a complex atherosclerotic lesion that gains in size^{16,21}.

As the plaque progresses, the vascular smooth muscle cells (SMC) residing in the media underneath it will start to proliferate, triggered by foam cell produced growth factors. They migrate from the media to the endothelial layer that covers the luminal side of the plaque, where they will also produce extracellular matrix constituents such as collagen and proteoglycans. This results in a thick fibrous cap covering the plaque, which reduces the risk of plaque rupture and thus is a very important aspect in the concept of plaque stability^{16,21,22}.

Contrary to this plaque stabilization, other processes increase the risk of plaque rupture, the most important being the death of lipid-laden macrophages and the resulting necrotic core. Foam cell apoptosis in advanced atherosclerotic plaques can be explained through combinations of several mechanisms^{23,24}. Besides a lack of sufficient growth factors, there is an abundance of cytotoxic agents such as oxygen radicals, modified lipids and specific cytokines²⁵. They are all able to induce caspase activation and thereby apoptosis. Another mechanism however has gained in attention. Here, excessive lipoprotein-derived cholesterol accumulation in the cells and thus also in the membrane of the endoplasmic reticulum (ER) can induce an unfolded protein response

(UPR). This ER stress can, alone²⁶ or in combination with a secondary signal such as pattern recognition receptor (PRR) signaling^{27,28}, lead to a release of Ca^{2+} from the ER lumen^{26,29}, a mechanism shown *in vivo* to be strongly correlated to foam cell apoptosis³⁰. The remains of the apoptotic cells are cleared by the macrophages in the early stages of atherogenesis. In later stages however, the macrophages are already overfilled and unable to phagocytose these particles efficiently. The uncleared apoptotic bodies go into secondary necrosis, leading to the accumulation of cell debris and thereby the formation of a necrotic core²⁰. While cell death in early lesions is considered beneficial, a growing necrotic core in more advanced plaques is associated with increased lesion instability and thus increased risk of clinical implications³¹. An additional risk comes from the tissue factor (TF) released by the necrotic cells, which can stimulate thrombus formation³². Other factors in the necrotic core induce neovascularization, branching microvessels from the vasa vasorum. This new microvasculature is often leaky and thereby initiator of yet another plaque destabilizing event, intraplaque hemorrhage³³.

In the end, the ratio of fibrosis over necrosis will be a strong determinant of plaque stability, so besides influencing plaque size, the shift of this ratio might be a second option for future therapeutic strategies²².

Animal models of atherosclerosis

History

While experimental intervention possibilities in humans are limited and mostly ethically unacceptable, atherogenesis appears to be too complex for *in vitro* studies because of the many different interacting cell types in a confined, inflammatory and lipid-rich milieu. *In vitro* studies can never mimic the physiological situation accurately enough and can therefore merely be considered indicative. Therefore, the use of animal models is inevitable. During the early years, rabbits and to a lesser extent pigs and non-human primates were used to reveal the cellular background of atherogenesis³⁴. Like in other biomedical research topics however, the use of mice gained in popularity³⁵.

Mouse models

Mice have some undeniable advantages when used in atherosclerosis and other research. First of all, mice are small, easy to handle and quick to breed. Since atherosclerosis experiments have a relatively high variability they require large groups, making the ability to house them in relatively small facilities important. Second, the mice' genetic background is well studied and many transgenic models with over-expression or deletion of specific genes exist and continue being made, which can be applied in atherosclerosis studies³⁶.

Mice however also have disadvantages. Because of their size, the amount of materials collected during the experiment and post-mortem, like blood and organs, are limited while the study of small tissues can be challenging. Second, however their lesions generally develop much like human lesions and contain the same cell types, some features of the human plaque still lack in mice. Since murine blood vessels are small, oxygen can diffuse more freely to all cells without the need of vasa vasorum. Therefore, their plaque hypoxia differs significantly from human lesions, also limiting its relevance in necrotic core formation and plaque neovascuogenesis³⁷. Moreover, the shear stress within the vessel remains limited so reliable models for lesion rupture are not available yet³⁸. Third and most important, mice don't spontaneously develop hypercholesterolemia and therefore no atherosclerosis. To overcome this, mice were initially given an atherosclerosis inducing diet containing high saturated fat, cholesterol and cholic acid salts (cholate). This cholate however can induce an inflammatory response, an enormous drawback especially when studying the role of inflammation in atherosclerosis³⁹. Therefore, transgenic mice have become the model of choice.

Most transgenic atherosclerosis mouse models are developed in C57BL/6 mice because they have a relative high susceptibility for atherogenesis compared to other mouse strains⁴⁰. The two most common models have a deletion of either the *apoe* gene encoding apolipoprotein E (ApoE) or the *ldlr* gene encoding the LDL receptor (LDLR), while other models have an overexpression of ApoB or mutant forms of ApoE, like human ApoE2⁴¹ or ApoE*3-Leiden^{42,43}. All these genetic modifications serve the purpose of raising the cholesterol levels in these mice and are most often combined with a period in which the mice are fed a diet containing high percentages of fat and cholesterol.

In an effort to speed up atherogenesis for short-term experiments or for simulation of high shear-stress, arteries can be surgically narrowed. An example is the tightening of a small silastic collar around the carotid arteries, creating a turbulent blood flow that results in downstream plaque formation⁴⁴.

In order to combine atherosclerosis proneness with other gene deficiencies, these mouse models can be crossed with mice that lack the gene of interest, creating double KO mice. When specifically studying bone marrow derived cells however, a quicker method is the bone marrow transplantation. In this case, *ldlr*^{-/-} mice are irradiated in order to deplete their bone marrow cells. Subsequently, their production of new blood cells is reconstituted by injection of bone marrow from a full or cell-type restricted KO model for the gene of interest. This creates a hybrid mouse that will still develop atherosclerotic plaques but lacks the gene of interest in all or a selection of its bone marrow derived cells⁴⁵. A different transplantation method can be used for studying changes in genetics of the vasculature during atherogenesis. Surgical transplantation of the aortic arch from a KO mouse model to an atherosclerosis prone mouse or vice versa creates hybrid mice in which the plaque and its surrounding vessel have a different genetic background from the blood and the newly infiltrating leukocytes^{46,47}.

Even within one mouse model, there are different locations where atherosclerosis can be quantified (**Figure 1.1**). In one very common method, sections are cut from the aortic root. This is the location where the aorta leaves the left ventricle of the heart, immediately downstream of the tricuspid valves. Because of the very turbulent and pulsatile blood flow in this part of the aorta, atherosclerotic plaques develop relatively rapid in this location. Therefore, it is the recommended location to study when performing short-term experiments. Moreover, the growth and phenotype of these plaques is well described in literature, making it easy to evaluate effects of the experiment on these criteria^{45,48}. In a similar way, sections can be cut from the aortic arch or the innominate artery, where advanced atherosclerotic plaques will appear after longer periods of high-fat diet⁴⁹. Another common method is the *en face* atherosclerosis assessment, in which the complete aorta is isolated, stained and pinned down⁵⁰. This provides a clear overview of plaque size and distribution throughout the aorta but should ideally be combined with aortic root analysis since it only provides basic information on the composition of the plaques, as the immunohistochemical analysis that can be performed is limited.

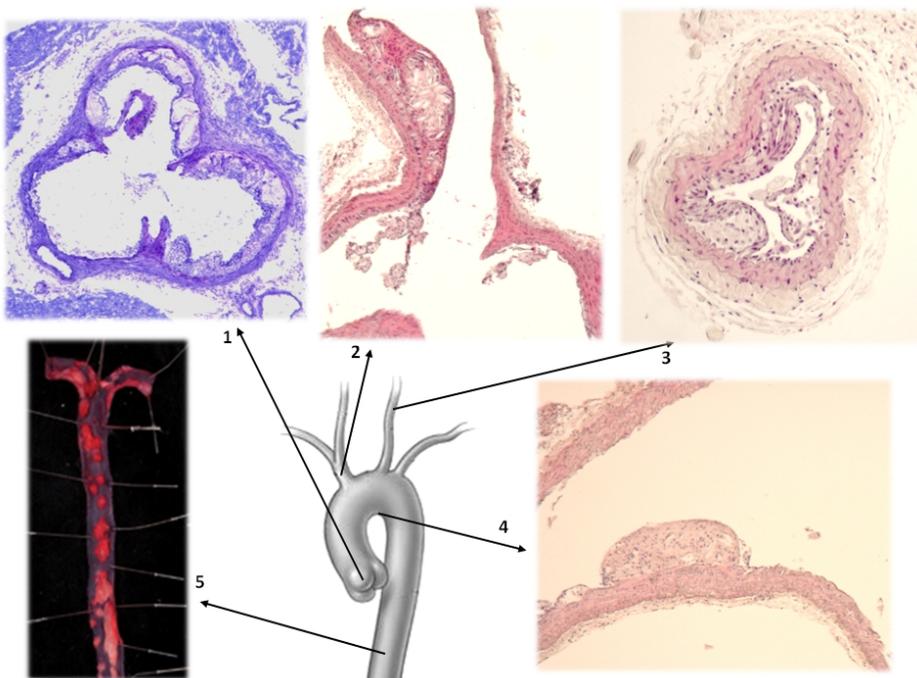


Figure 1.1: Different locations to study atherogenesis in mouse models. **1:** The aortic root (Toluidine blue). **2:** The innominate artery (HE). **3:** The carotid artery, proximal of a collar (HE). **4:** The aortic arch (HE). **5:** The descending aorta *en face* (Oil Red O, image © Charles River).

To study the gene expression within the atherosclerotic plaque, whole sections of the aorta can be lysed for RNA isolation. The results will however be disturbed by the abundant presence of RNA from the vessel wall, making the plaque-specific RNA levels in each sample dependent on the relative size of the plaque compared to the total section size. This can be solved by correcting each sample for the expression of a constant, plaque-specific gene, like the macrophage marker CD68⁵¹. A more precise and sensitive method however is laser capture microdissection, which makes it possible to isolate the plaques or even specific parts or cells in the plaques from histological sections, however providing a very limited RNA yield⁵².

Recently, new methods emerged to study the atherosclerotic plaque composition *in vivo*, including intravital microscopy^{53,54}, magnetic resonance imaging (MRI)⁵⁵, fluorescence imaging (e.g. GFP or luciferase reporter systems)⁵⁶, bioluminescence imaging⁵⁷, ultrasound imaging⁵⁸ and nuclear imaging (PET and SPECT)⁵⁹. These methods are often non- to mildly invasive and can monitor the trends of several parameters over time. Their major drawback however remains the resolution, which is at present often still inadequate to fully characterize murine plaques⁶⁰.

Macrophages

Macrophages are the most abundant and versatile cell type in atherosclerotic plaques. These immune cells with an unusual broad range of functions are formed when the bone marrow derived and in the blood circulating monocytes extravasate from the blood through the endothelial layer into the tissues, where they differentiate under the influence of macrophage- or granulocyte/macrophage colony stimulating factor (M-CSF or GM-CSF). Depending on the tissue the macrophage resides in, it may be known under a different name, with for example microglia in neural tissue, Kupffer cells in the liver, osteoclasts in the bone and sinusoidal cells in the spleen. However these macrophages usually preserve their broad range of functions independently of their location, they may as well exhibit a more specialized phenotype^{61,62}.

Probably the most important function of the macrophage is guarding and patrolling the tissues in search for pathogens. Once such a pathogen is detected, a series of actions follows in an attempt to clear them from the tissue. The macrophage will start to produce oxygen radicals to actively kill the pathogens and will engulf and digest the debris by phagocytosis, hence the name macrophage. At the same time, it will produce and secrete a set of cytokines and chemokines that bind to their respective receptors on the surrounding cells as a warning of the possible danger and as a trigger for the migration of other immune cells towards the site of infection. These cells include on the one hand myeloid “innate immune” cells (macrophages and granulocytes) that will assist in killing and digesting the intruders and on the other hand the antigen presenting cells (macrophages and dendritic cells) and lymphoid “adaptive immune”

cells (T- and B-cells) that will create a memory of the pathogenic patterns, which will allow the host to react more quickly and adequately upon a next encounter with the same pathogen. Upon engulfment of the pathogen, the phagosome will fuse with the lysosomes in which the pathogen is digested and antigens are formed that can be presented through MHCII molecules to the adaptive immune cells⁶³.

Remarkably however, the macrophage not only induces the accumulation of immune cells and an inflammatory environment, it is also responsible for the resolution of this inflammation once the pathogens are cleared. Indeed, in a second wave of cell infiltration the macrophages secrete anti-inflammatory cytokines that neutralize the inflammatory milieu and inactivate the inflammatory cells in order to protect the tissue from damage and to avoid exaggerated, systemic inflammation. Both the inability of the macrophages to raise an inflammatory response upon infection or to neutralize this response once the infection is cleared can be detrimental to the host's survival⁶⁴, as explained in chapter 5.

Similar to the phagocytosis of pathogens, also endogenous material like modified molecules, apoptotic bodies and debris of necrotic cells are cleared from the tissues by the macrophage. This way, it attempts to avoid the unnecessary inflammation that could be caused by the presence of mostly harmless agents⁶³.

Role of macrophages in atherosclerosis

Due to their function as scavengers, macrophages play an important role in the first phase of atherogenesis. Although patrolling already the vascular wall of healthy blood vessels, additional macrophages infiltrate the subendothelial space upon the presence of modified LDL particles⁶⁵, which they ingest using the scavenger receptors on their cell surface⁶⁶. While this process is initially beneficial, the inability to process the particles results in lipid-laden foam cells¹¹. As described above, these highly activated cells are responsible for the attraction of new macrophages and other cell types to the plaque through the production of chemokines and inflammatory cytokines but they also temper the local inflammation through the simultaneous production of anti-inflammatory cytokines. The importance of macrophages in early atherogenesis is illustrated best by experiments with mice that lack the M-CSF growth factor. These mice developed virtually no atherosclerotic plaques, as described later in this chapter⁶⁷.

In the later phases of atherogenesis, apoptosis and secondary necrosis of foam cells is causing the formation of a plaque destabilizing necrotic core while the remaining foam cells rather induce plaque stabilizing factors such as the formation of extracellular matrix and smooth muscle cell proliferation and migration²¹.

Both balances between pro- and anti-inflammatory cytokine production and between plaque stability and instability are therefore highly dependent on the stimulation of the macrophage by its local environment, as further discussed in chapter 5⁶². This diversity in macrophage function however is not unique to atherosclerotic plaques but has also

been described in macrophages involved in other pathologies and physiological processes such as tumour associated macrophages (TAMs)⁶⁸, adipose tissue macrophages (ATMs)⁶⁹ and the earlier mentioned macrophages reacting to infectious diseases. It led to the concept of macrophage heterogeneity.

Macrophage heterogeneity

Recently, this macrophage heterogeneity gained a lot of attention and attempts were made to classify the different macrophage subsets into a comprehensive framework. The nomenclature makes a distinction between the pro-inflammatory, classically activated M1 and the alternatively activated, anti-inflammatory M2 phenotype, in analogy to the T helper cells which are also subdivided into Th1 and Th2 cells^{70,71}. Macrophages are skewed towards an M1 phenotype by the Th1 cytokines IFN γ , TNF and/or microbial molecules such as lipopolysaccharide (LPS). They are characterized by the production of pro-inflammatory cytokines and chemokines such as IL-1 β , IL-6 and TNF and anti-microbial effector molecules such as reactive oxygen and nitrogen intermediates (ROI and RNI). The M2 macrophages appeared in further study to be representing a collection of at least three different subtypes, dependent on the stimulus that induces them⁷². They are named M2a, which are induced by Th2 cytokines such as IL-4 and IL-13, M2b which are skewed by immune complexes in combination with LPS or IL-1 β and M2c which are rather deactivated by cytokines such as IL-10 and transforming growth factor β (TGF- β) or by glucocorticoids.

It has shown to be difficult to find appropriate markers to identify the different macrophages, mainly because most markers described so far are not exclusively expressed but rather overexpressed in a certain subtype. Moreover, this framework is merely an oversimplified model and actual macrophages rarely display these extreme

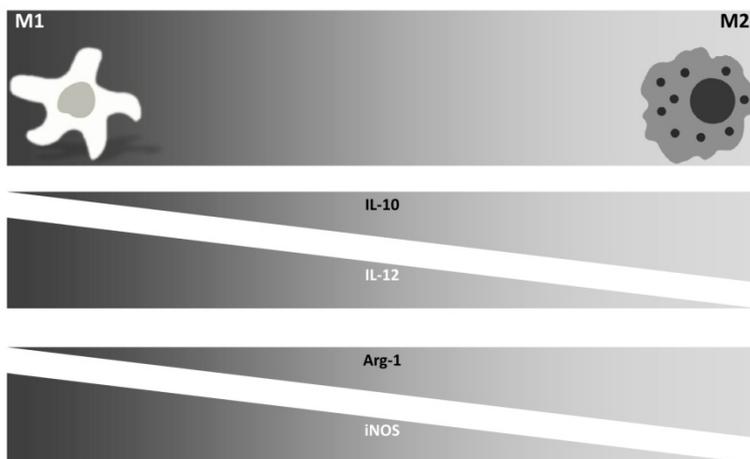


Figure 1.2: The IL-10/IL-12 and iNOS/arginase balances are two methods for determining the macrophage phenotype within a simplified model of heterogeneity.

differences in phenotype. To be able to make a quick and easy distinction between M1 and M2 macrophages however, two different characterizations are described (**Figure 1.2**). The first one is based on the inflammatory status of the macrophages and compares the expression of the pro-inflammatory cytokine IL-12, which is typically expressed in M1 macrophages, to that of the anti-inflammatory and thus M2 indicating IL-10. A second balance looks at the L-arginine metabolism and shows that this is tipped towards the expression and production of inducible nitric oxide synthase (iNOS) in M1 macrophages while the differentiation towards M2's correlates with an induction of arginase 1 (Arg-1)⁷³.

Different methods to induce macrophage heterogeneity in *in vitro* cell culture have been described. In the most common one, skewing cytokines (e.g. IFN γ for M1 and IL-4 for M2) are added to the culture of a macrophage cell line or macrophages obtained through the differentiation of bone marrow cells or monocytes by M-CSF. Others however differentiate bone marrow cells into distinct phenotypes by culturing them either with M-CSF or GM-CSF⁷⁴.

Other leukocytes in the plaque

While this thesis mainly focuses on macrophages, a range of other blood cell types are described to be present in the atherosclerotic plaque^{75,76}. Most of them, and especially their numerous interactions, contribute to plaque progression, inflammatory phenotype and stability¹⁴.

T lymphocytes

T lymphocytes, or simply T cells, develop through strict selection procedures from bone marrow derived thymic and peripheral hematopoietic progenitor cells and are historically subdivided into two major subtypes, primarily based on the presence of cluster of differentiation molecules CD4 and CD8 on their cell membrane. More recent studies however revealed a far more complex subdivision, including T helper cells (T_H), cytotoxic T cells (CTL), regulatory T cells (T_{reg}), natural killer T cells (NKT), memory T cells and gamma delta T cells ($\gamma\delta$ T). This extensive heterogeneity allows them, comparable to the different macrophage subsets, to possess both pro- and anti-atherogenic properties. Especially within the T_H cell subtype, the most abundant subset within the atherosclerotic lesion which can be further subdivided into T_H1 and T_H2 and other minor subtypes, this dual role is striking⁷⁷. While T_H1 cells promote atherogenesis by the production of the cytokine IFN γ , stimulation of SMC proliferation and a stimulus for the antigen presenting cells to upregulate their major histocompatibility complex type II (MHC-II)¹⁷, the T_H2 cells rather produce anti-atherogenic cytokines like IL-4 and IL-10 and induce the production of antibodies directed against oxLDL^{78,79}. Indeed, Th1 cells

account for most of the T lymphocytes in early lesions⁸⁰ and the absence of all T cell subsets through a Rag-1 KO mainly attenuates early atherogenesis⁸¹. In later phases of atherogenesis however, the T_H1/ T_H2 balance can be seen as a good indicator for T cell mediated plaque progression and stability⁸².

Also the other T cell subsets are shown to be present in the atherosclerotic plaque and are overall believed to promote atherosclerosis. Their specific roles in plaque progression and stability remain however somewhat elusive and demand more focused and detailed research^{14,83,84}.

B lymphocytes

B lymphocytes, or B cells, develop in the bone marrow and are mainly responsible for the production of antibodies. They constantly circulate the body and can therefore also be found in the vessel wall of healthy arteries. Upon atherogenesis however, they can start proliferating in the adventitia at the base of the plaque and can migrate into the plaque from there⁸⁵⁻⁸⁷. Their antibody production against oxLDL associated antigens seems to coincide with a reduction in atherogenesis and T cell infiltration into the plaque⁸⁸. Indeed, B cell deficiency through bone marrow transplantation into *Id1r*^{-/-} mice promotes atherogenesis⁸⁹. Depletion of mature B cell by a CD20-specific monoclonal antibody however results in reduced atherosclerosis, indicating that B cells can not be simply regarded as athero-protective cells⁹⁰.

Granulocytes

Granulocytes are also covering a collection of different cell types, all derived from myeloblast progenitor cells and characterized by the presence of cytoplasmic granules. While the role of eosinophils and basophils in atherogenesis is still quite unknown⁷⁵, more information on the role of neutrophils emerges. They are constantly attracted to the inflammatory regions at the luminal side of the plaque and in the adventitia lining the base of the lesion site⁹¹. Here they produce pro-inflammatory cytokines, which promote atherosclerosis and plaque instability, and myeloperoxidase (MPO), which might induce endothelial cell apoptosis^{91,92}. Furthermore, they secrete mediators that promote the attraction of additional monocytes to the lesion⁹³.

At sites of plaque rupture, another type of granulocytes named mast cells can be found⁹⁴. By degrading the athero-protective high-density lipoproteins (HDL) and impairing cholesterol efflux, these cells promote lipid accumulation and thereby plaque progression⁹⁵. Moreover, they destabilize the plaque by apoptosis of the collagen-secreting smooth muscle cells⁹⁵ and by activation of matrix metalloproteinases (MMP)^{96,97}. Furthermore, they are shown to be involved in angiotensin activation, intraplaque hemorrhage, macrophage apoptosis, vascular permeability and leukocyte recruitment to the plaques^{98,99}.

Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells bridging the innate with the adaptive immunity. They are formed from myeloid precursors after which they circulate the body as immature cells and finally mature upon uptake of antigens, resulting in the migration towards a lymph node and subsequent presentation of these antigens to T cells.

In atherosclerosis, vascular DC's accumulate in a GM-CSF dependent way in the intima beneath the plaque and in the shoulder regions of the plaque itself^{100,101}. Here they promote plaque growth, inflammation, the attraction of T cells and their activation through antigen presentation^{101,102}. Upon uptake of an antigen, they may also migrate out of the plaque towards secondary lymph nodes, where they can activate antigen specific T cells¹⁰³.

Co-localizing with the conventional DCs, a second DC phenotype can be found in the plaque shoulder regions. These plasmacytoid DCs (pDC) are not as capable in antigen endocytosis and T cell stimulation but rather react to debris from apoptotic and necrotic cells in the plaque by secreting type I interferons and stimulating CD4⁺ T cells in an antigen independent manner to produce IFN γ and TNF-related apoptosis-inducing ligand (TRAIL), which can induce smooth muscle cell apoptosis¹⁰⁴⁻¹⁰⁶. Moreover, they seem able to induce T_{reg} differentiation¹⁰⁷.

Signal transduction

An atherosclerotic plaque is an actively changing and highly inflammatory region containing an abundance of triggers that can induce a response in the residing cells, including modified lipids, signaling molecules, oxygen radicals and debris of apoptotic and necrotic cells. The response of the cells to these changes in their environment ranges from proliferation to cell death, from lipid uptake to -efflux and from pro- to anti-inflammatory cytokine secretion, depending highly on the way the signals are transduced towards a gene expression pattern. Many different signal transduction pathways are known to be playing a role in atherogenesis and plaque stability, and their interplay offers the advantages of signal amplification, precise fine-tuning and effective feedback loops. Indeed, the availability, abundance and combination of different receptors and signaling molecules often determines the eventual response but differs between cell types or even within the heterogeneous cell types described above, explaining the multifunctionality of these cells within the plaque.

Inflammatory signaling

An interesting example of the complex signal transduction cascades that is also very relevant to this thesis is the way in which different signals within the plaque can induce

and modify an inflammatory response. In contrast to the inflammation usually seen as a reaction to pathogen-associated molecular patterns (PAMPs), atherosclerosis is considered an example of so-called sterile inflammation. Though reports of microbiological origins of atherosclerosis exist^{108,109}, macrophage activation within the plaque is above all triggered by autologous damage-associated molecular patterns (DAMPs), including modified lipoproteins, cholesterol crystals, cytokines and debris of necrotic or apoptotic cells¹¹⁰⁻¹¹².

A key mediator that regulates the expression of several pro- and anti-inflammatory genes in atherosclerosis is NF- κ B^{45,113,114}. This transcription factor, which in fact represents a family of different homo- or heterodimeric combinations of five different NF- κ B/Rel proteins, is shown to be activated in atherosclerotic plaques, while it is usually inactive in healthy blood vessels^{115,116}. In order to induce the transcription of over 160 genes, NF- κ B binds to a specific consensus DNA sequence. In non-activated cells however, this interaction is impeded through tight association of an inhibitor to cytoplasmic NF- κ B, preventing it to enter the nucleus. The five different proteins that make up for the inhibitor of NF- κ B (I κ B) family are I κ B α , I κ B β , I κ B γ , I κ B ϵ and Bcl-3. They are NF- κ B target genes themselves, implicating a feedback loop of self-inactivation. Upon phosphorylation of the I κ B inhibitors by the I κ B kinase complex (IKK1, IKK2 and NEMO) followed by poly-ubiquitination and subsequent proteasomal degradation, NF- κ B can freely migrate to the nucleus and influence the gene expression¹¹⁷.

Within an atherosclerotic plaque, several signals can result in the activation of NF- κ B and thereby evoke an inflammatory response that influences the plaque progression. The CD36 scavenger receptor can, upon oxLDL binding, heterotrimerize with Toll-like receptors (TLR) 4 and 6, initiating the same NF- κ B activating pathway that can be found upon detection of viral or bacterial components by the TLR receptors^{118,119}. Besides oxLDL, also the heat-shock protein HSP60 and fragments of fibronectins are TLR activating factors present in the plaque, and a similar pathway can be found downstream of the receptor for the cytokine IL-1 β ¹²⁰. Indeed, deficiency of these receptors or their downstream adaptor molecule MyD88 attenuates plaque growth in murine atherosclerosis models¹²¹⁻¹²³. Through a different signaling pathway, the binding of the inflammatory cytokine tumor necrosis factor (TNF) to its receptor (TNFR) converges with the above-mentioned pathways in the activation of the IKK complex and promotes atherosclerosis¹²⁴⁻¹²⁷. Also CD40, a receptor from the same family as TNFR, will bind CD40 ligand (CD40L), resulting in a similar pro-atherogenic effect¹²⁸⁻¹³¹. Finally, the intracellular accumulation of cholesterol and subsequent ER stress response in foam cells was found to activate NF- κ B¹³², while foam cells undergoing apoptosis can induce NF- κ B activation in the neighboring phagocytes^{133,134}.

Worth mentioning here is the strong inflammatory and pro-atherogenic cytokine IL-1 β , which is produced upon NF- κ B signaling in its inactive precursor form IL-1. Recently it was shown that accumulation of cholesterol crystals within the plaque macrophages stimulates the NLRP3 inflammasome complex and thereby promotes caspase-1

dependent IL-1 processing to IL-1 β ^{135,136}. Moreover, inflammasomal caspase activation might be yet another way in which NF- κ B can be activated in plaque macrophages¹³⁷.

Cytokines and chemokines

Cytokines are a family of proteins that can be produced and secreted by many different cell types upon their acute or chronic activation or are produced constitutively at low levels. The family can be divided into different classes: interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), colony stimulating factors (CSF), transforming growth factors (TGF) and chemokines¹³⁸. Unlike hormones, they mainly act autocrine and paracrine through binding to their specific receptors, which initiates signaling pathways that lead to the activation of factors including interferon regulatory factors (IRF), janus kinase & signal transducers and activators of transcription (JAK/STAT), mitogen-activated protein (MAP) and NF- κ B. While chemokines regulate attraction of new cells by creating a gradient that affects the motility and adhesion capacity of their targets and colony stimulating factors initiate maturation and proliferation of specific cell types, the other cytokines are rather messengers of the inflammatory and immune responses. Noteworthy, there are both pro- and anti-inflammatory cytokines and their balance determines the net inflammatory status of the environment. This also implicates that the different T_H- and macrophage phenotypes that have been described above can be discriminated through their profile of cytokine production.

In an atherosclerotic plaque, the co-localization of many different immune cell types combined with the presence of several stimuli like modified lipoproteins, oxygen radicals and necrotic cell remnants can induce cytokine production in the foam cells as well as in the other immune cells, the endothelial cells and the smooth muscle cells. Thereby, these cells cooperate in the creation of an inflammatory milieu that attracts and activates even more cells from the blood stream and the adventitia.

During the last 20 years, many animal experiments involving over-expression, inhibition or knock out of one of these cytokines or their receptors have proven their capital role in atherogenesis¹³⁸. Their specific effects on atherogenesis range from altering the inflammatory and cellular balance of the lesion to plaque growth and alteration of plaque stability. These murine data can be assumed to be relevant in humans as well since similar cytokine production and cytokine receptor presence was also already shown in human atherosclerotic plaques.

Inflammatory cytokines in atherosclerosis

The inflammatory cytokine families can be sub-divided into members that mediate pro-inflammatory or anti-inflammatory effects. Their production and secretion is mainly triggered by stimuli activating the cell and influenced by a pro- or anti-inflammatory skewing of the cell. They are able to mediate both the inflammatory reaction of the

cells as well as its resolution through binding to specific receptors, initiating a range of different signaling pathways.

Because atherosclerosis is known to be a chronic inflammatory disease with many cell types mediating inflammation through the production and secretion of cytokines, their role in atherogenesis is well studied. Roughly, one could summarize that pro-inflammatory cytokines promote atherogenesis (TNF^{126,139}, IL-1¹⁴⁰⁻¹⁴², IL-2¹⁴³, IL-12^{144,145}, ...) while anti-inflammatory cytokines are anti-atherogenic (IL-5¹⁴⁶, IL-10¹⁴⁷⁻¹⁴⁹, IL-33¹⁵⁰, TGFβ¹⁵¹⁻¹⁵⁵, ...) and others have ambiguous effects (IL-4¹⁵⁶⁻¹⁵⁸, IL-6¹⁵⁹⁻¹⁶¹). Therefore, their balance within the lesion, which is most probably dependent on the presence and activation of the different macrophage- and T helper cell subsets, is largely determining plaque growth and –stabilization. Knowledge on this balance and the specific roles of the cytokines in the plaque might reveal therapeutic possibilities, however site and/or cell type specific targeting of the therapy remains the biggest challenge.

Chemokines in atherosclerosis

Chemokines are small cytokines specialized in the chemotaxis of new cells to the site of inflammation. They share a typical three dimensional structure stabilized by disulfide bonds between two pairs of cysteines. These can be either adjacent (the CCL family) or separated by one or three other amino acids (the CXCL and CX₃CL family respectively)¹⁶². They play a role in the embryonic development and later in the homeostasis of tissues but their release is also often triggered by inflammatory stimuli, creating a concentration gradient able to attract specific cell subsets. Upon binding a family of G-protein coupled receptors, they induce the activation of phospholipase C (PLC). This results in the creation of the molecules inositol triphosphate (IP₃) and diacylglycerol (DAG). These mediate the effects of the chemokine binding through activation of protein kinase C (PKC), the MAP kinase (MAPK) pathway and an altered intracellular Ca homeostasis. These effects include degranulation, an enhanced cell motility and a more efficient binding of the cell's integrins to adhesion molecules^{162,163}.

Several interactions between chemokines and their receptors influence atherogenesis^{164,165}, the first one discovered being the CCL2-CCR2 axis^{166,167}. Later however it was shown that the attraction of new cells to the site of plaque formation mainly depends on three different and non-redundant interactions. These are CCL2 (MCP-1), CCL5 (RANTES) and CX3CL1 (Fractalkine) and their binding to the respective receptors CCR2, CCR5 and CX3CR1. Their importance was demonstrated by a simultaneous inhibition of CCL2 and the receptors CCR5 and CX3CR1, which almost completely abolished atherogenesis¹⁶⁸, a far more pronounced effect than the inhibition of individual chemokines or other combinations¹⁶⁹⁻¹⁷².

More recently, the focus has changed from a net effect of chemokines on atherosclerosis to a more detailed image where chemokine production differs between

early and advanced lesions and chemokine receptors are often specific to monocyte or T cell subsets. This implicates that changes in chemokine production not only affects lesion size but can also affect the attracted cell types or –subsets and thereby the plaque composition and inflammatory status^{173,174}.

Interferons in atherosclerosis

Interferons were the first known cytokines, discovered as the product of virus-infected cells that caused resistance to infection with the same or other viruses¹⁷⁵. They are produced by lymphocytes upon contact with pathogens or tumor cells in order to alert neighbouring cells of the potential danger. Interferons are divided into two families, called type I interferons and type II interferons. They have distinct secretion profiles and functions, bind to different receptors but both signal through Jak/STAT pathways. Recent papers show a crosstalk between the two families through their effect on and use of STAT1¹⁷⁶.

Type I interferons include IFN α , IFN β , IFN ζ and IFN ω , which all bind to a heterodimer of interferon- α receptors IFNAR1 and IFNAR2¹⁷⁷. They are best known for their ability to “interfere” with viral infection. Because of this, they have been used for decades in the treatment of certain virus related diseases like Hepatitis C, often in combination with the antiviral ribavirin^{178,179}. More recently however, their anti-proliferative and pro-apoptotic effects proved to be useful in the treatment of certain tumors^{177,180} while their immunomodulatory properties made it the most commonly used treatment for the autoimmune disorder multiple sclerosis (MS)^{181,182}.

Type II interferons only include IFN γ , which binds the heterodimeric interferon- γ receptor (IFNGR1 and IFNGR2). Next to limited antiviral and antitumoral effects they mainly activate leukocytes, contributing to an inflammatory milieu.

While the pro-atherogenic role of IFN γ is well studied¹⁸³⁻¹⁸⁶, type I interferons in atherosclerosis were yet poorly documented. Moreover, the few studies published failed to determine their effect on atherogenesis and clarify the mechanisms behind this^{104,187,188}. Chapter 2 focuses on determining and further clarifying the effect of type I interferon signaling on mouse models for atherosclerosis and the possibility of translating these results to human atherosclerosis⁵¹.

Colony stimulating factors in atherosclerosis

Growth factors are a family of cytokines each responsible for the differentiation and proliferation of a certain cell type. In the atherosclerotic setting, M-CSF is produced by the endothelial and smooth muscle cells and mainly ensures the differentiation of monocytes into macrophages and further macrophage proliferation¹⁸⁹. Its importance in atherosclerosis was shown in mice with osteopetrotic (op) mutations. Both homozygous mice with the naturally occurring *Csfm*^{op}/*Csfm*^{op} (op/op) mutation, that

lack functional M-CSF, and mice with a heterozygous *op* mutation shown significantly reduced atherogenesis^{67,190-192}.

Another growth factor playing a role in atherosclerosis is GM-CSF, with both treatment and deletion approaches resulting in increased plaque size^{193,194}. The exact mechanism however is yet to be revealed. Besides recruiting endothelial progenitor cells to the plaque and promoting vasa vasorum neovascularization¹⁹⁴⁻¹⁹⁷, it regulates dendritic cell formation and proliferation in the lesions^{101,198} and reduces macrophage PPAR γ activity and ABCA1 expression¹⁹³.

Cell recruitment to the atherosclerotic plaque

The growth of the early atherosclerotic plaque depends largely on the attraction of new immune cells¹⁹⁹. In order to enter the plaque from the blood stream, these cells need to extravasate through the endothelial cell layer, a process called diapedesis, in a way that is similar to other, non-atherosclerotic sites of inflammation^{200,201}. This is typically a three-step event where the cells initially roll over the vessel wall because of a low-affinity binding to the selectins that are present on the activated endothelial cells^{202,203}. Their enhanced affinity to endothelial adhesion molecules, mediated by chemokine-dependent conformational changes in their integrins, then results in firm adhesion and leukocyte arrest²⁰⁴. In a third step, the arrested leukocytes secrete proteases that degrade the extracellular matrix between the endothelial cells so they can transmigrate through the vessel wall towards the site of inflammation²⁰⁵.

Interference with this diapedesis cascade, for example by blocking antibodies against adhesion molecules or integrins, has been shown to reduce the nearby inflammatory burden²⁰⁶⁻²⁰⁸. Indeed, also in atherosclerosis, a reduced leukocyte attraction results in smaller lesions^{173,209-213} while an induction in recruitment promotes atherogenesis^{51,214}.

Quantification of new macrophages entering an atherosclerotic plaque has been challenging up to now due to a lack of good markers. In this thesis however, two novel markers are described that will facilitate the immunohistochemical detection of these cells.

Monocytes entering the atherosclerotic plaque differentiate into macrophages under the influence of the M-CSF growth factor present within the plaque. As a result, newly recruited macrophages still express monocytic markers like the one recognized by the ER-MP58 antibody²¹⁵⁻²¹⁷. This myeloid precursor marker has been proven useful in the detection of recently matured macrophages in several inflammatory diseases^{215,218,219} and is introduced in chapter 3 as a novel marker for newly recruited macrophages in the atherosclerotic plaque.

The M-CSF dependent expression of the inhibitor of apoptosis proteins (IAP) family member survivin was already described for macrophages entering the human

atherosclerotic lesion, while surrounding cells in the plaque and blood vessel were survivin-negative²²⁰. In chapter 4, it is now confirmed as a novel marker for newly recruited macrophages in murine atherosclerotic plaques as well.

This thesis

The macrophage and its recruitment to the atherosclerotic plaque is a returning theme throughout this thesis. In **Chapter 2**, we show that this recruitment is enhanced in a chemokine-dependent way by type I interferons, resulting in larger lesions upon interferon treatment and smaller lesions upon defective myeloid interferon signaling. Hereby we identify the type I interferons as a novel cytokine family with a key role in atherogenesis. Also in **Chapter 3**, enhanced macrophage recruitment explains the larger plaques in mice with a myeloid deficiency of I κ B α , an inhibitor of NF- κ B. In this chapter we also introduce ER-MP58 as a novel marker for newly recruited macrophages in the murine atherosclerotic plaque. In **Chapter 4**, we identify another protein that is specifically expressed in macrophages entering the murine atherosclerotic plaque, survivin. Subsequently, we target it with a lentiviral based immune therapy. We show that this immunization is indeed able to reduce the lesion size, opening new possibilities for future therapies. Finally, **Chapter 5** gives an overview of the current knowledge on the different macrophage subsets and hypothesizes how this heterogeneity might affect atherogenesis.

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CHAPTER 2

Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions

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Abstract

Inflammatory cytokines are well-recognized mediators of atherosclerosis. Depending on the pathological context, type I interferons (IFNs; IFN α and IFN β) exert either pro- or anti-inflammatory immune functions but their exact role in atherogenesis has not been clarified. Here we demonstrate that IFN β enhances macrophage-endothelial cell adhesion and promotes leukocyte attraction to atherosclerosis prone sites in mice in a chemokine-dependent manner. Moreover, IFN β treatment accelerates lesion formation in two different mouse models of atherosclerosis and increases macrophage accumulation in the plaques. Concomitantly, absence of endogenous type I IFN signaling in myeloid cells inhibits lesion development, protects against lesional accumulation of macrophages and prevents necrotic core formation. Finally, we show that type I IFN signaling is upregulated in ruptured human atherosclerotic plaques. Hereby, we identify type I IFNs as novel pro-atherosclerotic cytokines, which may serve as additional targets for prevention or treatment.

Introduction

The important contribution of inflammatory cytokines to atherosclerosis development is well recognized¹⁻³. The macrophage is both an important source as well as a major target of these inflammatory mediators. Specific cytokines influence macrophage effector functions and thereby affect plaque initiation, progression and cellular composition. Ultimately, macrophages hereby alter important plaque stability factors such as necrotic core formation and inflammatory cell content. Hence, the macrophage is a very attractive target for designing therapeutic interventions⁴, making it crucial to understand how cytokines regulate macrophage function in atherogenesis.

Cytokines of the type I interferon (IFN) family (IFN α and IFN β) are produced by immune cells in response to pathogenic challenges such as viruses, bacteria and tumor cells. They induce antiviral responses^{5,6}, have immunomodulating activities and are clinically used to treat viral diseases, multiple sclerosis and certain tumors^{5,7,8}. Recent data indicate that IFN β is also necessary for sustaining TNF driven inflammation⁹, promotes TNF-induced lethal shock¹⁰ and mediates MHC-I induction by TNF¹¹. In addition, constitutive low levels of type I IFN amplify IFN γ and interleukin-6 (IL-6) driven inflammatory responses^{12,13}. Thus, type I IFNs also modulate immune responses that are not directly induced by pathogens. In sharp contrast to the clinical application of IFN β as treatment for the pathologies mentioned above, type I IFNs have also been shown to promote disease in systemic lupus erythematosus (SLE), where inhibition strategies are being considered for therapy¹⁴. Interestingly, it was recently postulated that the increased risk of cardiovascular mortality in SLE patients¹⁵ is linked to their elevated levels of type I IFNs¹⁶. Thus, depending on the context, type I IFN may have either disease promoting or disease inhibiting properties.

The important immunomodulatory activities of type I IFNs prompted us to hypothesize that they also have a role in atherogenesis. We show that IFN β treatment induces chemokine dependent adhesion and migration of leukocytes and promotes atherosclerosis development *in vivo*. Consequently, we show that inhibition of type I IFN signaling in myeloid cells hampers cellular recruitment to lesions and thus atherosclerosis development. Finally, we found that type I IFN signaling is an integral feature of human atherosclerosis as well and is upregulated in plaque instability.

Results

IFN β treatment of macrophages induces chemotactic mediators.

To study the effect of type I IFN on macrophages we first performed *in vitro* studies with cultured bone marrow derived macrophages (BMM) and analyzed cytokine secretion, uptake pathways and factors mediating adhesion and migration. While TNF and IL-12 were not induced by IFN β , treatment did induce the production of the anti-inflammatory cytokine IL-10 (Figure 2.1A). Next, we analyzed uptake pathways by BMM that had been pretreated with IFN β . Neither endocytosis of oxidatively modified LDL (oxLDL) nor phagocytosis of fluorescently labeled latex beads was affected by treatment of cells with IFN β (Figure 2.1B and C). We further focused on molecules mediating attraction of cells to atherosclerotic lesions. FACS analysis of IFN β treated macrophages showed no differences for any of the major integrins necessary for rolling and arrest at inflammatory sites (Figure 2.1D). Interestingly, gene expression of the chemokine and chemokine receptors (Figure 2.1E and F) CCL5 (RANTES) and its receptor CCR5, as well as CCR2 were upregulated in macrophages activated with IFN β . However, further FACS analysis showed no significant upregulation of surface expression of both CCR2 and CCR5 (Figure 2.1G). In contrast, CCL5 secretion was strongly increased by activation of macrophages with IFN β (Figure 2.1H). Next, we studied the role of the main receptor for type I IFN, IFNAR1, and therefore macrophages from mice with a myeloid specific deletion of IFNAR1¹⁷ were used. Quantitative-PCR showed a deletion efficiency of IFNAR1 of 73.2% \pm 1.4, and CCL5 induction by IFN β was reduced to a similar extent in IFNAR1 deleted cells (IFNAR1^{del}) compared to control cells (IFNAR1^{wt}) (Figure 2.1I). Ablation of signal transducer and activator of transcription 1 (STAT1) signaling, as a key downstream transcription factor of type I IFN signaling, almost completely abolished induction of CCL5 by IFN β (Figure 2.1J). CCL5 ELISA showed similar reduction in secreted CCL5 for IFNAR1^{del} and STAT1^{-/-} cells (data not shown). Treatment of macrophages with another type I IFN, IFN α showed similar effects on CCR5 and CCL5 (Supplemental figure S2.1). Thus, in addition to inducing IL-10, type I IFN treatment of primary macrophages induces chemotactic factors, which may influence leukocyte attraction and atherosclerosis development.

IFN β treatment of macrophages enhances *in vitro* adhesion to endothelial cells and promotes leukocyte arrest at atherosclerosis prone sites *in vivo*.

In line with our data described above, we found that static adhesion of IFN β treated macrophages to endothelial cells was enhanced (Figure 2.2A). Endothelial adhesion of macrophages lacking IFNAR1 or STAT1 was not inducible by IFN β (Figure 2.2B and C). In line with a major role for CCL5-CCR5 signaling, we found that IFN β -induced adhesion

was not affected in CCR1 deficient macrophages, but completely blunted in macrophages lacking CCR5 (Figure 2.2D). Absence of CCR2 showed an intermediate phenotype, with a moderate effect of IFN β . *In vivo*, 4 days treatment of high fat fed apolipoprotein E deficient (*apoe*^{-/-}) mice with IFN β led to a strong increase of leukocyte

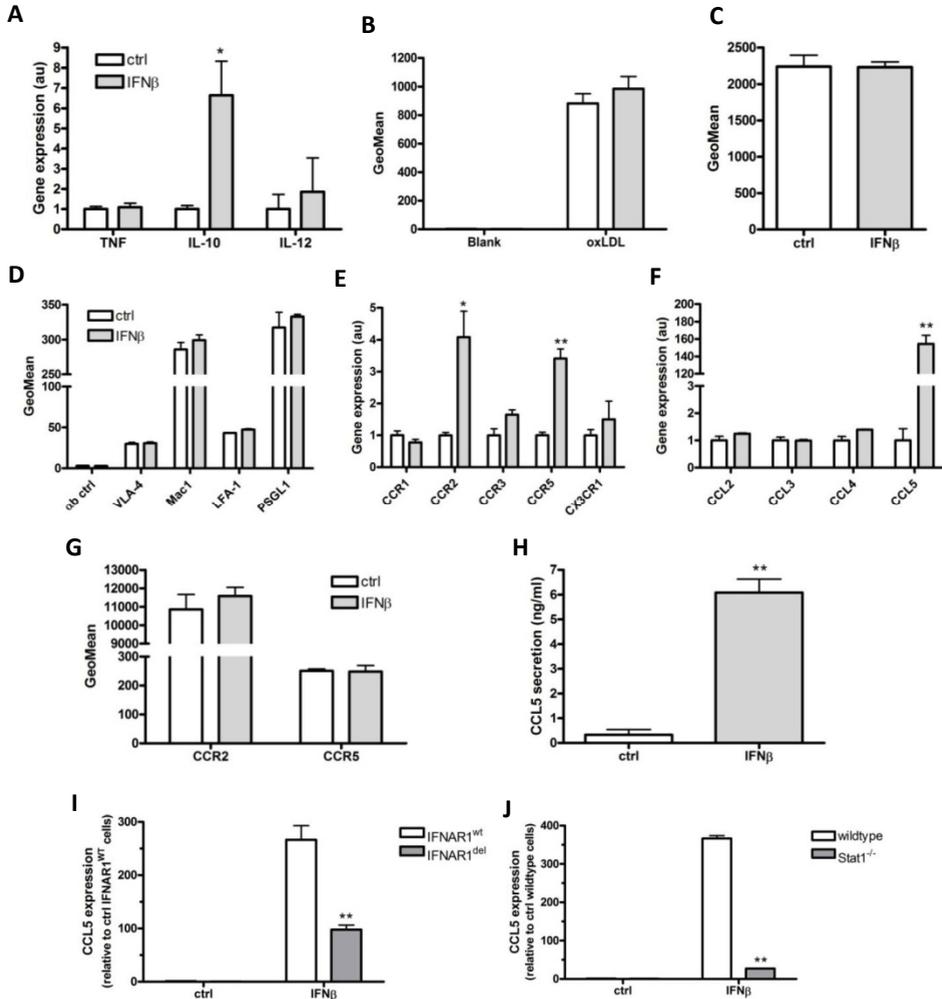


Figure 2.1: The effect of IFN β on cultured bone marrow derived macrophages. (A) Treatment of macrophages with IFN β induces IL-10 expression, without affecting TNF or IL-12. **(B)** Uptake of DiI-labelled oxLDL by control (ctrl) or IFN β treated macrophages. **(C)** Uptake of fluorescent latex beads by control or IFN β treated macrophages. **(D)** Surface expression of VLA-4, Mac1, LFA-1 and PSGL1 in control (ctrl) or IFN β treated macrophages. **(E)** Relative gene expression of chemokine receptors in control or IFN β treated macrophages. **(F)** Chemokine expression after IFN β treatment of macrophages. **(G)** FACS analysis of CCR2 and CCR5 after treatment with IFN β . **(H)** CCL5 secretion by macrophages after treatment with IFN β . **(I)** CCL5 expression in ctrl and IFN β stimulated IFNAR1^{wt} and IFNAR1^{del} macrophages. **(J)** CCL5 expression in ctrl or IFN β treated wildtype and STAT1^{-/-} macrophages. Graphs are representative for at least two independent experiments. Bars represent mean of triplicate wells \pm SEM (*P<0.05, **P<0.01).

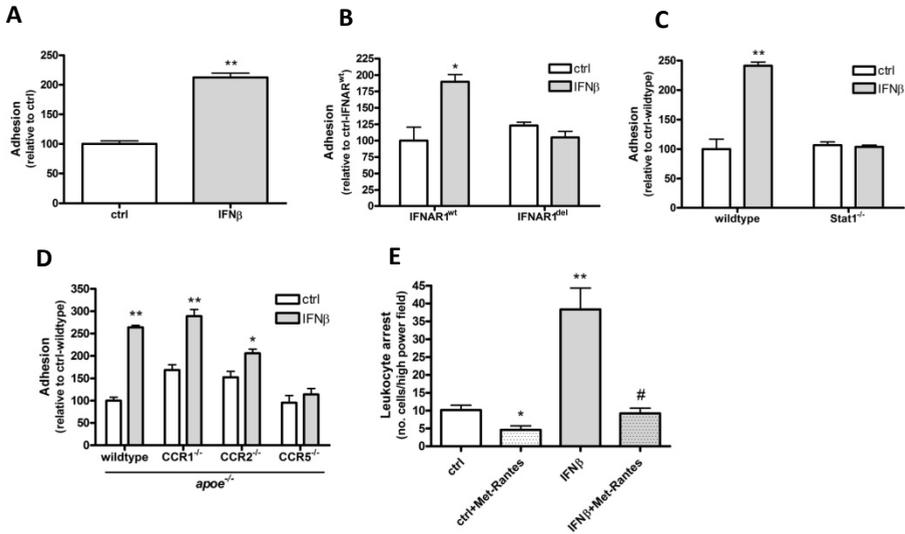


Figure 2.2: IFN affects static *in vitro* adhesion and *in vivo* leukocyte arrest. (A) Adhesion of untreated macrophages (ctrl) to endothelial cells compared to IFN β treated macrophages (** $P < 0.01$). (B) Adhesion of ctrl or IFN β treated IFNAR^{wt} and IFNAR^{del} macrophages (* $P < 0.05$). (C) Adhesion of ctrl or IFN β treated wildtype or STAT1^{-/-} macrophages (** $P < 0.01$). (D) Adhesion of macrophages in ctrl or IFN β treated CCR1^{-/-}, CCR2^{-/-} or CCR5^{-/-} macrophages, all on an apoE^{-/-} background (* $P < 0.05$, ** $P < 0.01$). All bars for *in vitro* adhesion represent mean of triplicate wells \pm SEM, stars indicate significant differences compared to ctrl treated cells of the same genotype. (E) Leukocyte arrest in the carotid artery of apoE^{-/-} mice (ctrl), after treatment with Met-Rantes, after 4-days IFN β treatment, or 4-days IFN β treatment combined with Met-RANTES treatment ($n = 8, 4, 7$ and 5 for the four groups respectively, error bars indicate SEM; * $P < 0.05$ or ** $P < 0.01$ compared to control (ctrl), # $P < 0.01$ compared to IFN β).

arrest in the carotid arteries of these animals (Figure 2.2E). Co-treatment of mice with the CCL5 antagonist Met-RANTES, just prior to analyzing leukocyte arrest, reduced leukocyte arrest in untreated mice and fully restored leukocyte adhesion to control levels in IFN β treated animals (Figure 2.2E). Thus, IFN β treatment *in vitro* and *in vivo* promotes leukocyte adhesion and attraction to the endothelium through CCL5-CCR5 dependent mechanisms.

IFN β treatment of mice accelerates atherogenesis in apoE^{-/-} and ldlr^{-/-} mice.

Next, we analyzed the effect of IFN β on atherogenesis in two well-established mouse models of atherosclerosis. Daily injections of IFN β in a collar-induced atherosclerosis model in apoE^{-/-} mice¹⁸ did not alter plasma cholesterol levels (27.89 \pm 2.10 and 26.98 \pm 2.04 mM; for the ctrl and 5 and IFN β treated, respectively) but increased plasma levels of IL-10 (3.90 \pm 2.1 and 19.21 \pm 5.8 pg/ml, $p < 0.05$; for the ctrl and IFN β treated,

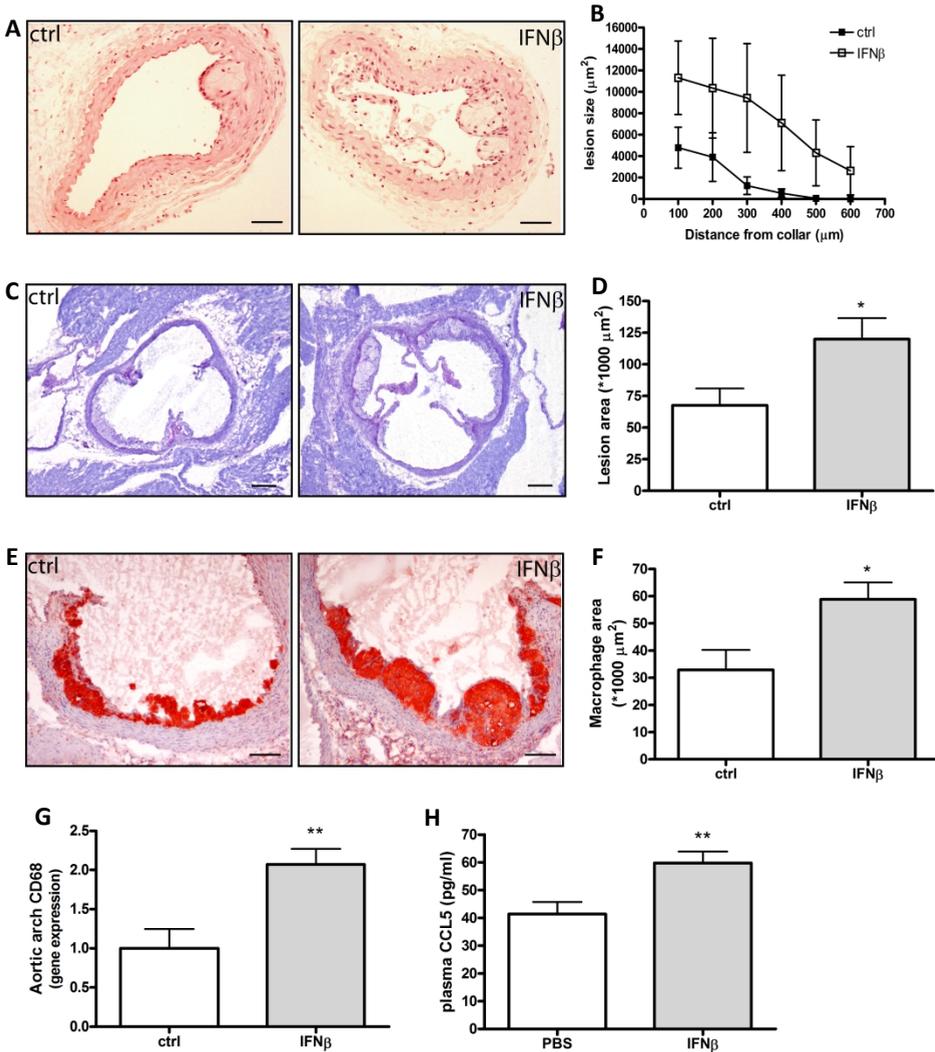


Figure 2.3: IFN β treatment accelerates atherosclerosis in *apoE*^{-/-} and *ldlr*^{-/-} mice. (A) Representative lesions of control (ctrl) or IFN β treated mice of collar induced atherosclerosis in *apoE*^{-/-} mice. Scale bar indicates 50 μ m. (B) Lesion area measured at six sequential locations proximal from the collar in *apoE*^{-/-} mice which were control or IFN β treated (** $P < 0.01$ by two-way ANOVA; $n = 9/12$). (C) Representative lesions in the aortic root of control (ctrl) or IFN β treated *ldlr*^{-/-} mice. Scale bar indicates 200 μ m. (D) Lesion area at the aortic root of control or IFN β treated *ldlr*^{-/-} mice (* $p < 0.05$; $n = 12/14$). (E) Representative MOMA-2 stained lesions from control and IFN β treated *ldlr*^{-/-} mice. Scale bar indicates 100 μ m. (F) Absolute macrophage area in lesions from control or IFN β treated *ldlr*^{-/-} mice (* $P < 0.05$; $n = 12/14$). (G) CD68 expression in aortic arches from control or IFN β treated *ldlr*^{-/-} mice (** $p < 0.01$; $n = 11/14$). (H) CCL5 levels in plasma from ctrl- or IFN β -treated *ldlr*^{-/-} mice (** $p < 0.01$; $n = 9/11$). Shown are mean \pm SEM.

respectively). Atherosclerotic lesion analysis (Figure 2.3A and B) showed a strong increase in lesion formation in mice that had been treated with IFN β . The same effect

was seen in low-density lipoprotein receptor deficient (*ldlr*^{-/-}) mice where IFN β treatment increased aortic root atherosclerotic lesion size by almost 2-fold (Figure 2.3C and D) without any effects on plasma cholesterol (26.30 \pm 1.78 and 26.86 \pm 1.66; for the ctrl and IFN β treated, respectively) or blood leukocyte levels (B220⁺ B-cells, CD3⁺ T-cells, Gr1^{hi}CD11b⁺ neutrophils and Gr1^{int/-}CD11b⁺ monocytes; data not shown). Staining for macrophage content showed an approximate two-fold increase in absolute macrophage area in IFN β -treated mice (Figure 2.3E and F). Gene expression analysis of aortic arches from these mice also showed increased expression of the macrophage marker CD68 (Figure 2.3G), further indicating increased macrophage accumulation in atherosclerotic lesions. More extensive immunohistochemical analysis of the aortic root lesions did not show any significant difference with respect to neutrophil or T-cell accumulation, TUNEL positive cells or necrotic core formation (data not shown). Of interest, we did find an induction of circulating CCL5 levels in IFN β -treated mice (Figure 2.3H). Thus, IFN β treatment enhances atherosclerosis development, despite the induction of IL-10.

Myeloid deletion of IFNAR1 reduces atherosclerosis development and prevents macrophage and neutrophil accumulation in lesions.

Subsequently, we investigated whether endogenous production of type I IFN also contributes to atherosclerosis development. We first analyzed whether typical type I IFN signature genes were expressed in lesions of atherosclerotic mice. Indeed, expression of OAS1, OAS2, MX2 and IRF9 could be readily detected in aortic arches from *ldlr*^{-/-} mice that had been fed a high fat diet for 6 or 9 weeks (supplemental figure S2.2), indicating that endogenous type I IFN signaling is present. We then performed a transplantation using bone marrow from IFNAR1^{wt} and IFNAR1^{del} mice, specifically lacking IFNAR1 in their myeloid lineage, to *ldlr*^{-/-} mice to yield atherosclerosis susceptible mice that were either wildtype (IFNAR1^{wt}) or deleted (IFNAR1^{del}) for IFNAR1 in their myeloid cells. Compared with IFNAR1^{wt} mice, mice lacking myeloid IFNAR1 showed a strong reduction in atherosclerotic lesion size (-34%) (Figure 2.4A and 4B) after 11 weeks of high fat diet. Lesions were mainly composed of macrophages (Figure 2.4C) and absolute macrophage area in the lesions was reduced in the IFNAR1^{del} mice (Figure 2.4D). Furthermore, the lesions were of intermediate phenotype, consisting mainly of macrophage derived foam cells with a fibrotic cap and some necrosis. IFNAR1^{del} mice showed reduced numbers of advanced lesions and increased moderate and early lesions (Figure 2.4E).

Plasma cholesterol levels did not differ between groups just before the start of the diet and after high fat feeding (Figure 2.4F). In addition, total leukocyte counts (Figure 2.4G) and blood levels of leukocyte subsets (Figure 2.4H) did not differ between IFNAR1^{wt} and IFNAR1^{del} mice. Plasma cytokine levels were reduced, with a strong reduction (-70%) of

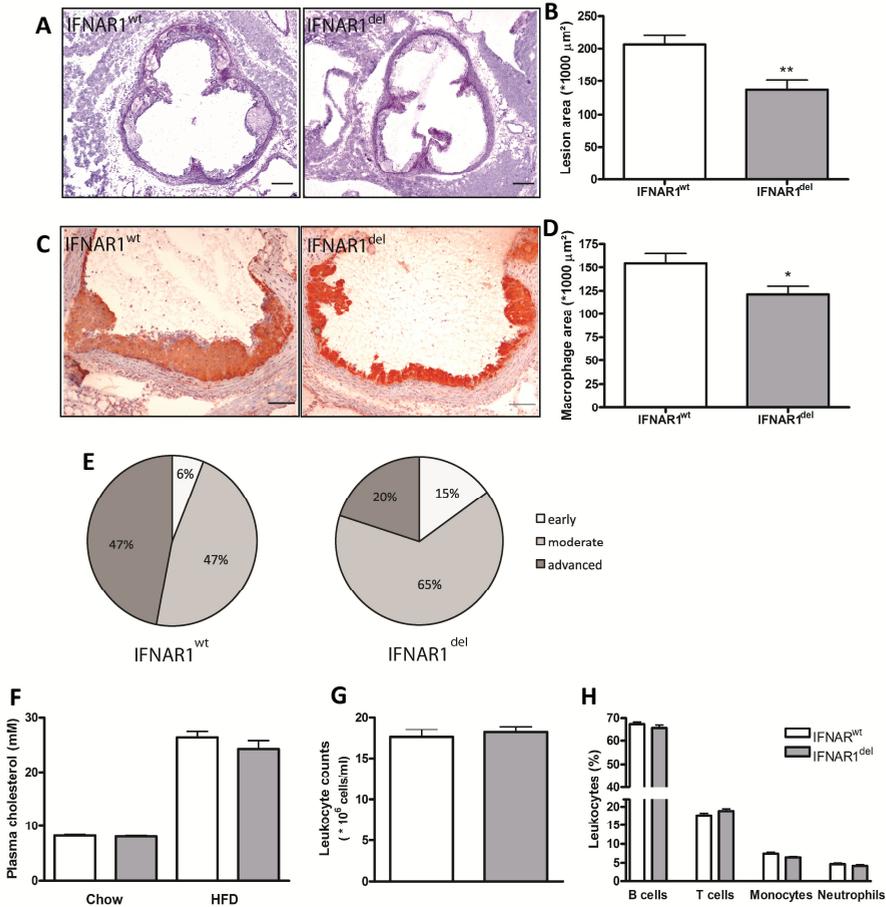


Figure 2.4: Absence of myeloid *IFNAR1* reduces atherosclerosis development. Bone marrow from conditional mice devoid of *IFNAR1* only on myeloid cells was transplanted to *Ildr^{-/-}* mice to yield atherosclerosis susceptible mice that were either wildtype (*IFNAR1^{wt}*) or deleted (*IFNAR1^{del}*) for *IFNAR1* in their myeloid cells. (A) Representative toluidin blue stained lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice. Scale bar indicates 200 μm . (B) Lesion area at the aortic root of *IFNAR1^{wt}* and *IFNAR1^{del}* mice (** $P < 0.01$; $n = 19/15$). (C) Representative MOMA-2 stained lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice. Scale bar indicates 100 μm . (D) Absolute macrophage area in lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice (* $P < 0.05$; $n = 17/14$). (E) Lesion severity in *IFNAR1^{wt}* and *IFNAR1^{del}* mice ($P < 0.05$ by Chi-square test; $n = 19/15$). (F) Plasma cholesterol levels before (chow) and after 8 weeks of high fat diet (HFD) in *IFNAR1^{wt}* and *IFNAR1^{del}* transplanted mice. (G, H) Total leukocyte counts and relative levels of leukocyte subsets. Shown are mean \pm SEM.

circulating IL-6 (supplemental figure S2.2) and a borderline ($P = 0.05$) reduction of the chemokine CCL2 (MCP-1) in *IFNAR1^{del}* mice.

Further examination of the lesions showed the neutrophil content to be decreased in atherosclerotic lesions of *IFNAR1^{del}* mice (Figure 2.5A and B), while T-cell content did

not differ (Figure 2.5C). Since type I IFNs have been implicated in the regulation of cell survival and proliferation⁵, cell proliferation and apoptosis were assessed by Ki-67 and TUNEL staining, but no differences were observed between the two groups (Figure 2.5D and E). We did however find a strong (>70%) reduction of necrosis in the lesions of *IFNAR1^{del}* mice (Figure 2.5F and G), which is often considered a plaque destabilizing consequence of impaired efferocytosis in the lesions¹⁹. Thus, endogenously produced type I IFNs activate myeloid cells, increase lesional accumulation of macrophages and neutrophils, promote necrotic core formation, and thereby promote atherosclerosis development.

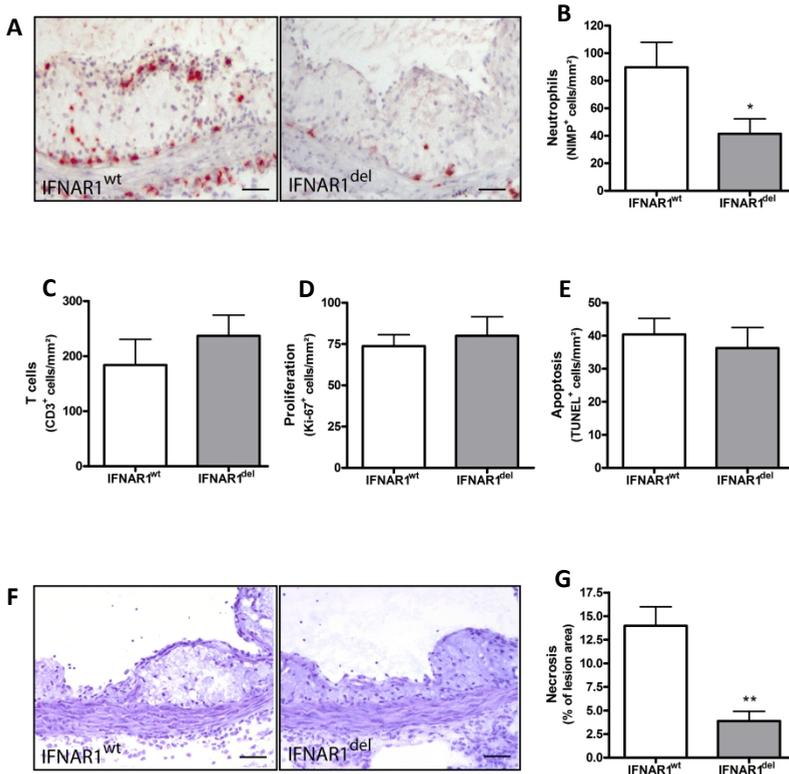


Figure 2.5: Absence of myeloid *IFNAR1* influences the phenotype of atherosclerotic lesions. (A) Representative NIMP stainings of lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice. Scale bar indicates 50 μ m. (B) Neutrophil influx in lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice (* $P < 0.05$). (C, D, E) T-cells, proliferation and apoptosis in lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice. (F) Representative examples of necrotic core in lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice. Scale bar indicates 50 μ m. (G) Necrosis in lesions from *IFNAR1^{wt}* and *IFNAR1^{del}* mice (** $P < 0.001$). For all analyses, $n = 19$ and 15 for *IFNAR1^{wt}* and *IFNAR1^{del}* mice respectively. Shown are mean \pm SEM.

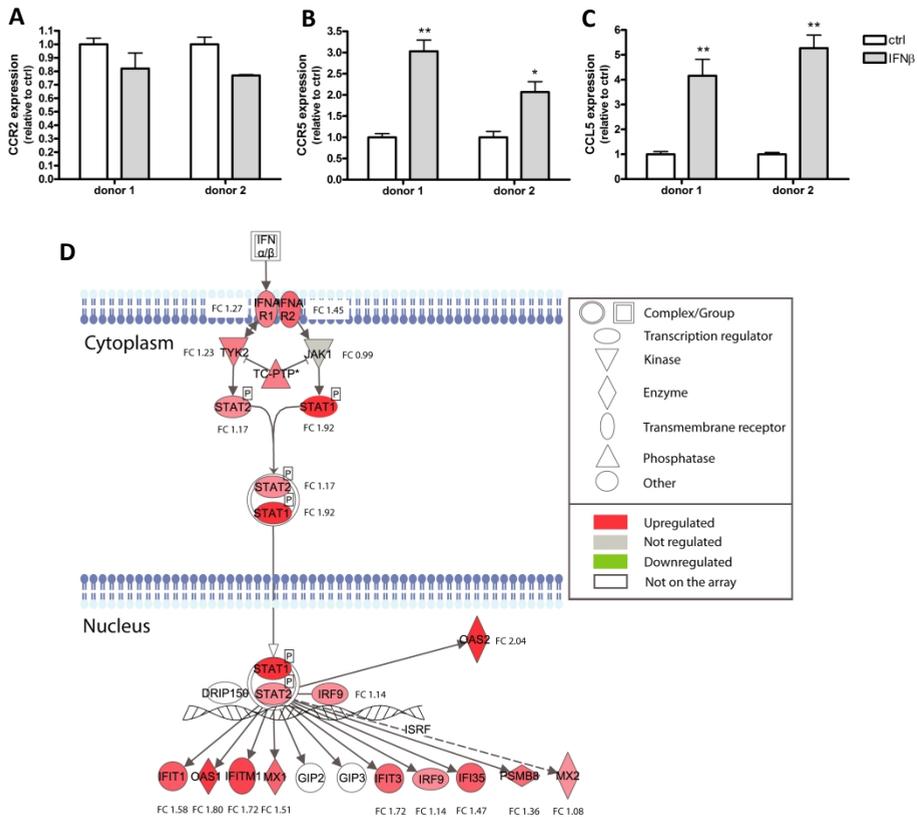


Figure 2.6: *IFNβ* induces chemotactic factors in human primary macrophages and type I IFN signaling is upregulated in unstable regions of human atherosclerotic lesions. **(A)** CCR2 expression in macrophages from two independent donors after *IFNβ* treatment. **(B)** CCR5 expression in macrophages from two independent donors after *IFNβ* treatment (* $P < 0.05$; ** $P < 0.01$). **(C)** CCL5 expression in macrophages from two independent donors after *IFNβ* treatment (** $P < 0.01$). Bars indicate mean of triplicate wells \pm SEM. **(D)** Ingenuity Pathway Analysis of the differentially expressed genes in stable sections compared to ruptured sections of carotid endarterectomy specimens. Red signals indicate upregulation and the pathway showed a strongly significant ($P = 2.36 \times 10^{-6}$, ratio 16/23 (0.696)) upregulation of type I IFN signalling. Indicated are the fold-changes (FC) of the respective genes.

Type I IFN induces chemotactic mediators in human macrophages and type I IFN signaling is associated with plaque instability in human atherosclerotic lesions.

To investigate whether type I IFNs also play a role in the pathogenesis of human atherosclerosis, we first analyzed the effect of *IFNβ* on primary human macrophages. Although *IFNβ* did not induce CCR2 (Figure 2.6A), it did increase expression of both CCR5 and CCL5 (Figure 2.6B and C), and induced secretion of CCL5 as analyzed by ELISA (Supplemental Figure S2.3), confirming our mouse data. Next, Ingenuity Pathway Analysis was performed on an expression database of human atherosclerotic lesions.

We compared data from stable carotid endarterectomy specimens and ruptured specimens, which had been classified according to the Virmani-classification²⁰. Interestingly the type I IFN pathway showed a highly significant upregulation in ruptured lesions when compared to stable lesions (Figure 2.6D). In addition to the upregulation of the type I IFN pathway, also several chemotactic factors including CCR5 and CCL5 were upregulated in ruptured atherosclerotic lesions (Supplemental Table ST2.1). These data show that type I IFN signaling correlates with plaque rupture in human atherosclerosis.

Discussion

In this paper we show that treatment with IFN β enhances atherogenesis in different models and that myeloid specific inhibition of type I IFN signaling reduces atherosclerosis development. We demonstrate that IFN β treatment induces chemotactic factors and thereby promotes leukocyte attraction to atherosclerosis prone sites. Accordingly, absence of endogenous myeloid type I IFN signaling reduced accumulation of cells from the myeloid lineage in the lesions. Thus, type I IFNs are pro-atherosclerotic cytokines that act by promoting chemokine dependent leukocyte recruitment to atherosclerotic lesions. Interestingly, upregulation of type I IFN signaling is also associated with plaque instability in human atherosclerotic lesions.

The family of type I IFNs consists of many members with IFN α and IFN β being the most abundant and best studied. It was previously shown that IFN α treatment promotes atherosclerosis in *ldlr*^{-/-} mice. However, this effect was accompanied by elevated plasma levels of triglycerides and cholesterol²¹, which complicates the interpretation of these data. Therefore, we chose to use IFN β in our treatment studies, although we found that IFN α had similar *in vitro* effects on chemotactic factors. Zhang et al. describe that IFN β administration attenuates lesion formation induced by carotid artery ligation in angiotensin-II infused *apoe*^{-/-} mice²², which is in contrast to our findings. Thus, angiotensin acceleration of atherosclerosis depends on other inflammatory factors than our models. We now show, using two different models of atherosclerosis, that IFN β treatment promotes atherosclerosis in high fat fed mice. In both a collar-accelerated model (in the *apoe*^{-/-} mice) and a solely hyperlipidemia-driven model (in the *ldlr*^{-/-} mice), atherogenesis was enhanced by IFN β , without influencing plasma lipid parameters.

We found that IFN β treatment of isolated primary macrophages induced IL-10. In addition, IFN β treatment of mice led to increased levels of circulating IL-10, similarly as is observed in MS patients and mouse models for MS²³. IL-10 is a classical anti-inflammatory cytokine and the beneficial effects of IFN β treatment in relapsing remitting MS patients is at least partly attributed to anti-inflammatory factors it induces²⁴. The induction of this anti-atherogenic²⁵ cytokine contrasts with the atherosclerosis promoting effect that we observe for IFN β . However, we did find IFN β

to specifically elicit chemotactic factors with a reported pro-atherogenic function. CCL2 (MCP-1) and CCL5 (RANTES) and their receptors (CCR2 and CCR5, respectively) are important in regulating attraction of cells to atherosclerotic lesions and thereby control atherosclerosis development²⁶⁻²⁸. We found that especially CCL5 was strongly upregulated after IFN β treatment of macrophages, coinciding with a modest induction of CCR5 and CCR2. CCL5 expression after IFN β was reduced in macrophages lacking either IFNAR1 or STAT1, indicating that CCL5 is either a direct target of IFNAR1-STAT1 signaling or regulated by interferon regulatory factors (IRFs) induced by STAT1 activation²⁹⁻³¹. We could also show that IFN β increased static adhesion of macrophages to endothelial cells, again in an IFNAR1 and STAT1 dependent manner, which was abolished by macrophage CCR5 deficiency, but not by deficiency for CCR1, the other major receptor for CCL5. CCR2 deficiency had a modest effect on IFN β induced macrophage adhesion. Furthermore, enhanced leukocyte arrest induced by IFN β in *apoe*^{-/-} mice could be completely blocked by co-treatment with the CCL5-receptor inhibitor, Met-RANTES. Therefore we think that despite the induction of IL-10 by IFN β , the atherosclerotic process is actually enhanced through the induction of especially the CCL5-CCR5 axis, thereby promoting attraction of leukocytes to lesions.

Type I interferons are massively produced by various cell types in response to viruses and other microbial stimuli, through engagement of toll-like receptors such as TLR3, TLR4 and TLR9. Major *in vivo* sources of type I IFN are plasmacytoid dendritic cells (pDCs). Interestingly, DCs and more specifically pDCs have been identified in human atherosclerotic lesions and have been associated with rupture prone areas of the lesions³²⁻³⁴. Using datasets from stable and ruptured human endarterectomy segments we now show that segments of ruptured plaque have an upregulation of the type I IFN signaling pathway, when compared to stable segments. Thus, ruptured sections of atherosclerotic lesions show induction of type I IFN signaling. This may consequently increase attraction of inflammatory cells, further contributing to matrix degradation and plaque destabilization.

Using conditional knockouts we demonstrate that myeloid cells are important targets for endogenously produced type I IFN in atherosclerosis. Through induction of chemotactic factors, type I IFN signaling promotes macrophage adhesion to endothelial cells. As such, deletion of IFNAR1 in myeloid cells ultimately leads to reduced accumulation of macrophages in atherosclerotic lesions and thereby reduces atherosclerosis development. We determined deletion efficiency to be approximately 70% in our bone marrow cultures, indicating some remaining type I IFN signaling. In addition, it was previously shown that circulating monocytes from *LysMCre-IFNAR1*^{floxed} mice show severely hampered but not fully absent type I IFN signaling¹⁷. Thus, the phenotype we observe is the likely result of reduced but not completely absent myeloid type I signaling. Whether this merely means an underestimation of the contribution of endogenous type I signaling in atherosclerosis or whether additional functions are therefore undetectable remains to be discovered.

In addition to affecting macrophages, myeloid IFNAR1 deficiency also reduces accumulation of neutrophils in the lesions, which have recently been found to contribute to atherosclerosis^{35,36}. Disturbed macrophage CCL5 production may impair lesional recruitment of neutrophils, which use CCR1 to respond to CCL5³⁷. Alternatively, IFNAR1 deficiency may have unexplored direct effects on neutrophils influencing migration to inflammatory sites. However, in our treatment approach with *Idlr*^{-/-} mice, neutrophil accumulation in the lesions was not affected (data not shown), indicating that IFN β treatment does not affect neutrophils in these studies. In addition, although we clearly identify that myeloid IFNAR1 signaling highly controls lesion development, we cannot exclude that other targets of type I IFN, such as endothelial cells or fibroblasts, will contribute to the effects that we observe upon treatment of the mice with IFN β .

We found that inhibition of endogenous myeloid type I IFN signaling reduces necrotic core formation and thus directly affects plaque stability. Reduced necrosis paralleled impaired progression of the lesions. However, upon analysis of a subset of lesions from both groups with similar size (84.5 ± 4.8 and $81.4 \pm 4.2 \times 1000 \mu\text{m}^2$ for the *IFNAR1*^{wt} and *IFNAR1*^{del} groups) and severity (moderate lesions), we found that necrotic core formation was still reduced in the *IFNAR1*^{del} group ($12.6 \pm 2.4\%$ and $4.0 \pm 1.3\%$ for the *IFNAR1*^{wt} and *IFNAR1*^{del} groups). Thus, inhibition of myeloid IFNAR1 function directly inhibits necrotic core formation independent of lesion size and severity and without any clear effects on endocytosis or phagocytosis. Depending on the experimental context, both pro- and anti-cell death functions have been attributed to type I IFN. The antitumor effects of type I IFN are at least partly attributed to the induction of cell death by type I IFN⁵, in which IFNs and interferon stimulated genes are both sensitizing tumor cells to immune cell mediated cytotoxicity and are augmenting lytic activity of immune cells. In line, several groups have reported that endogenous type I interferon signaling sensitizes cells to pathogen induced cell death^{38,39} or apoptosis induced by serum deprivation⁴⁰. In addition, treatment of MS patients with IFN β was shown to prime monocyte derived macrophages for apoptotic cell death⁴¹. We show that inhibition of myeloid type I IFN signaling reduces necrosis, but treatment of *Idlr*^{-/-} mice with IFN β did not enhance necrotic core formation. Thus, in atherogenesis the necrosis promoting effect of endogenous type I IFN is apparently already maximal and not further promoted by exogenously added IFN β .

Our findings are supported by previous studies demonstrating that type I IFNs are important inducers of cellular migration. The attraction of inflammatory cells is an important feature of type I IFN action in fighting microbial infections⁵. In addition, it was recently shown that type I IFNs are also essential mediators of TNF-induced lethal inflammatory shock by enhancing cell death and promoting white blood cell influx in tissues through induction of a set of chemokines¹⁰. Moreover, in search of IFN β related adverse side effects in MS patients several groups performed gene expression studies on peripheral blood mononuclear cells and demonstrated induction of chemotactic

factors in response to IFN β treatment^{42,43}. In line with this it was recently shown that a group of chemotactic genes, including CCL5 and CCL2, was among the most prominently upregulated ones in brain macrophages (microglia) treated with IFN β ¹⁷. We focused on the major chemokines and chemokine receptors with a demonstrated role in atherogenesis and found that especially the CCL5-CCR5 axis may be very important in mediating the pro-atherogenic function of IFN β .

In conclusion, we show that type I IFNs promote atherosclerosis development. Contrary to the antiviral effect of type I IFN acting on a multitude of cell types, we report that the specific interaction of endogenous type I IFN with myeloid cells enhances the recruitment of these cells to atherosclerotic lesions. This effect is in accordance with an observed type I IFN induced macrophage adhesion mediated by upregulation of specific chemokines and their receptors, mainly involving the CCL5-CCR5 axis. Furthermore, we present plaque instability as a direct consequence of myeloid IFN signaling. Collectively, these data raise the demand for further detailed analyses of cardiovascular risk in patients treated with type I IFN and may even imply that caution should be taken in using type I IFN as a therapeutic option. In contrast, targeting of type I IFN signaling may be an attractive target for prevention and treatment of atherosclerosis.

Experimental Procedures

Mice

C57BL/6 mice and *Id1r*^{-/-} mice on a C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME), *apoe*^{-/-} mice on a C57BL/6 background were obtained from Iffa Credo (Lyon, France). *IFNAR1*^{fl/fl}, *LysMCre-IFNAR1*^{fl/fl}, *apoe*^{-/-}, *apoe*^{-/-}*CCR1*^{-/-}, *apoe*^{-/-}*CCR2*^{-/-}, *apoe*^{-/-}*CCR5*^{-/-} and *STAT1*^{-/-} mice were all on a C57BL/6 background and have been described before^{17,44-46}. All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University or complied with German animal protection law.

Interferons

Recombinant murine IFN α or IFN β were obtained from Hycult Biotec (Uden, The Netherlands). Human IFN β was obtained from Peprotech (Rocky Hill, NJ, USA).

In vitro murine macrophage culture

Bone marrow cells were isolated from femurs and tibiae of wildtype mice (C57BL/6), *IFNAR1*^{fl/fl}, *LysMCre-IFNAR1*^{fl/fl}, *apoe*^{-/-}, *apoe*^{-/-}*CCR1*^{-/-}, *apoe*^{-/-}*CCR2*^{-/-}, *apoe*^{-/-}*CCR5*^{-/-} or *STAT1*^{-/-} mice. Cells were cultured in RPMI-1640 (Gibco Invitrogen, Breda, The Netherlands) with 10% heat inactivated fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), penicillin (100 U/ml), streptomycin (100 ug/ml), L-glutamine 2 mM (all Gibco Invitrogen, Breda, The Netherlands) (R10) supplemented with 15% L929-

conditioned medium (LCM) for 8-9 days to generate bone marrow derived macrophages (BMM), as described previously⁴⁷. Cells were treated for 24h with 100U/ml IFN α or IFN β .

In vitro human macrophage culture

Human mononuclear cells were obtained by apheresis of 2x the blood volume from healthy volunteers using a Cobe® Spectra (CaridianBCT Europe, Zaventem, Belgium). Monocytes were subsequently enriched by counter flow centrifugation using the Elutra Cell Separation System (CaridianBCT) and were cultured in R10 supplemented with 5ng/ml M-CSF (Peprotech) for 8 days to generate human macrophages. Cells were treated for 24h with 100U/ml human IFN β .

Gene expression

RNA was isolated from BMM or human macrophages with the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). 500 ng total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative PCR was performed using 10 ng cDNA, 300nM of each primer, IQ SYBR Green Supermix (BioRad) in a total volume of 20 μ l. Deletion efficiency in BMM of the LysMCre-IFNAR1^{fl/fl} mice was measured by Q-PCR on 25ng DNA as described before⁴⁷, using primers specifically detecting the floxed and not the deleted or wildtype IFNAR1 allele. All gene expression levels were corrected for cyclophilin A as house keeping gene and primer sequences are available upon request.

In vitro oxLDL and bead uptake

BMM untreated or treated for 24h with 100U/ml IFN β were incubated for 3h in Optimem-1 with fluorescently labeled latex beads or 25 μ g/ml Dil labeled oxLDL, generated through copper oxidation, as described previously⁴⁷. Uptake was assessed by flow cytometry after residual beads or oxLDL were washed away.

Flow cytometry

BMM (+/- 100U/ml IFN β for 24h) were stained with antibodies against VLA-4 (Cedarlane, Burlington, Ontario, Canada), Mac1, LFA-1, PSGL1 (all BD Pharmingen, Erembodegem, Belgium), CCR2 (Epitomics, Burlingame, CA, USA) or CCR5 (BD Pharmingen) and their mean fluorescence was measured by FACS analysis. For the CCR2 staining, cells were permeabilized using CytopermTM Plus Permeabilization Buffer and Perm/WashTM Buffer (both BD Pharmingen).

ELISA

Murine CCL5 secretion from BMM (+/- 100U/ml IFN β for 24h) was measured by ELISA using anti-mouse CCL5 (R&D Systems, Abingdon, UK) as coating antibody and biotinylated anti-mouse CCL5 (R&D Systems) as detection antibody with mouse CCL5

(Peprotech) as standard. Human CCL5 secretion in the supernatants from human macrophages (+/- 100U/ml IFN β for 24h) was measured by commercial ELISA (PBL Interferon Source, New Brunswick, NJ, USA). Absorbance was measured at 450nm using a microtiterplate reader (BioRad, Hercules, CA, USA).

In vitro adhesion assay

A confluent monolayer of bEND5 endothelial cells was grown in fluorescence 96 wells microplates (Greiner Bio-one, Frickenhausen, Germany). Triplicate wells were incubated for 30 min with 10⁵ BMM (+/- 100U/ml IFN β for 24h) that had been fluorescently labeled with a PKH dye according to the manufacturer's instructions (Sigma Aldrich, Zwijndrecht, The Netherlands). Subsequently, the wells were washed three times with R10 and adherent cells were measured by fluorometry in a Synergy HT microtiter plate reader (BioTek, Bad Friedrichshall, Germany) at an excitation of 485nm and an emission of 520nm.

In vivo leukocyte adhesion

Apoe^{-/-} mice were put on a high fat diet (0.15% cholesterol, 21% fat, Altromin) for 6 weeks and during the last 4 days daily injected subcutaneously with either saline or IFN β (5000U). Four mice receiving saline injections and five receiving IFN β injections were co-treated with an intravenous Met-RANTES (50 μ g/mouse) injection 30 min before the experiment. After sedation (intraperitoneal Ketamine/Xylazin) and intravenous rhodamin injection, the left carotid artery was exposed and 3 high power fields (hpf) near the carotid bifurcation were visualized by epifluorescence microscopy (Zeiss AxioTech, 20x water immersion objective) as described before⁴⁸. Short movies were recorded for each hpf, in which the cells attaching to the vessel wall were counted by eye.

Collar-induced atherosclerosis

Seventeen-week-old male *apoe*^{-/-} mice (n=21) were operated after 3 weeks of high fat diet (0.25% cholesterol, 16% fat) to introduce a 2mm long non-constrictive silastic tube around both carotid arteries, as described before¹⁸. During the 24 days post-operation, the high-fat diet was continued and the mice were treated by daily subcutaneous injection of either IFN β (5000U) or saline. Upon sacrifice the right carotid artery was isolated and embedded in paraffin. Sections of 5 μ m were made and after every 100 μ m, sections were stained with haematoxylin/eosin for lesion area analysis.

IFN β treatment in *ldlr*^{-/-} mice

Twelve-week-old male *ldlr*^{-/-} mice were fed a high fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 6 weeks in order to induce early atherogenesis. At this point, daily subcutaneous injections of either IFN β (5000U) or saline were started and continued for 3 weeks while the high fat diet was continued. Upon sacrifice the heart and aorta were taken out. The hearts were cut perpendicular to the heart axis just

below the atrial tips. Tissue was frozen in tissue-tec (Shandon, Veldhoven, The Netherlands) and cut into sections of 7 μ m as described before⁴⁷. Serial cross-sections from every 42 μ m were stained with toluidin blue. All lesion areas were quantified using Adobe Photoshop software. The aortas were snap-frozen and RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) including DNase treatment. Gene expression was assessed by Q-PCR, similarly as described above.

Bone marrow transplantation

One week before transplantation, female *Idlr*^{-/-} mice were housed in filter top cages and provided with acidified water containing neomycin (100mg/l; Gibco, Breda, The Netherlands) and polymyxin B sulphate (6x10⁴ U/l; Gibco). The animals received two times 6Gy total body irradiation on two consecutive days. On the second day, bone marrow was isolated from 6 LysMCre-IFNAR1^{fl/fl} mice (IFNAR1^{del}) and 6 IFNAR1^{fl/fl} littermates (IFNAR1^{wt}) and 10⁷ cells/mouse were injected intravenously to rescue the hematopoietic system of the irradiated mice. Four weeks after the transplantation, mice were fed a high fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 11 weeks. After sacrifice, the hearts from the bone marrow transplanted mice were taken out and lesion size in the aortic root was measured as described above. The lesions were also typed according to severity as early, moderate and advanced as described before⁴⁷.

Mouse blood parameters

At several time points during all *in vivo* atherosclerosis experiments, blood was drawn from the mice. Plasma lipid levels were monitored enzymatically (Sigma Aldrich, Zwijndrecht, The Netherlands) and plasma cytokine levels were measured by flow cytometry using a Cytometric Bead Array kit (BD-Pharmingen, San Diego). For the bone marrow transplanted mice, leukocytes were counted using a Coulter counter and blood cell distribution was quantified by flow cytometry after antibody staining with either Mac1-PE and Gr1-FITC for macrophages and granulocytes or 6B2-PE and KT3-FITC for B- and T-cells (BD-Pharmingen, Erembodegem, Belgium).

Immunohistochemical staining

Lesions from the aortic root were fixed in acetone and incubated with antibodies against macrophages (MOMA-2, a gift from G. Kraal), granulocytes (NIMP, directed against Ly6G, a gift from P. Heeringa), T-cells (KT3, directed against CD3, a gift from G. Kraal) and proliferating cells (Ki-67, Dako, Glostrup, Denmark). Apoptotic cells in the plaques were stained by the TUNEL staining (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. Necrotic areas were analyzed on toluidin blue stained sections and identified by the presence of pyknosis, karyorrhexis or complete absence of nuclei.

Human plaque transcriptomics

Microarray analysis was performed on RNA isolated from 44 (22 stable and 22 ruptured) human carotid plaque specimens using Illumina Human Sentrix-8 V2.0 BeadChip® technology to detect differential expression. For pathway analysis we used the Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) system.

Statistical analysis

The statistical analyses were performed using Graphpad Prism (Graphpad Software). Differences between 2 groups were evaluated using a *t*-test, unless stated otherwise. Values are represented as mean ± SEM. A *P* value of less than .05 was considered to be statistically significant. All mouse data passed a normality test.

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Chapter 3

Myeloid I κ B α deficiency promotes atherogenesis by enhancing leukocyte recruitment to the plaques

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Abstract

Activation of the transcription factor NF- κ B appears to be involved in different stages of atherogenesis. In this paper we investigate the role of NF- κ B inhibitor I κ B α in atherosclerosis. Myeloid-specific deletion of I κ B α results in larger and more advanced lesions in LDL-R-deficient mice without affecting the compositional phenotype of the plaques or systemic inflammatory markers in the plasma. We show that I κ B α -deleted macrophages display enhanced adhesion to an *in vitro* endothelial cell layer, coinciding with an increased expression of the chemokine CCL5. Also, *in vivo* we found that I κ B α ^{del} mice had more leukocytes adhering to the luminal side of the endothelial cell layers that cover the atherosclerotic plaques. Moreover, we introduce ER-MP58 in this paper as a new immunohistochemical tool for quantifying newly recruited myeloid cells in the atherosclerotic lesion. This staining confirms that in I κ B α ^{del} mice more leukocytes are attracted to the plaques. In conclusion, we show that I κ B α deletion in myeloid cells promotes atherogenesis, probably through an induced leukocyte recruitment to plaques.

Introduction

NF- κ B is a transcription factor that translates the inflammatory stimuli from the environment into gene expression patterns regulating cell differentiation, activation, proliferation and apoptosis as well as the production of a set of inflammatory mediators. It is activated in response to pathogen detection by Toll-like receptor signaling or, in the inflammatory milieu, through different cytokine receptors such as the TNF receptor. Also, non-pathogen related activation, called “sterile inflammation”, including stimuli such as free radicals, radiation and modified lipoproteins, can trigger NF- κ B^{1,2}.

Rather than one transcription factor, NF- κ B is in fact a family of homo- and heterodimers, with different possible combinations of the Rel-domain containing proteins NF- κ B1 (p50 and its precursor p105), NF- κ B2 (p52 and its precursor p100), RelA (p65), RelB and c-Rel. In the absence of an activating stimulus, the NF- κ B dimer is kept cytoplasmic because its nuclear localization signal is covered by an inhibitor belonging to the I κ B family³. The I κ B family consists of I κ B α , I κ B β , I κ B γ , I κ B ϵ and Bcl-3⁴. Following a signaling cascade initiated by an inflammatory stimulus, I κ B is phosphorylated by a complex consisting of I κ B kinase 1 (IKK1 or IKK α), IKK2 (or IKK β) and NEMO (or IKK γ). This phosphorylation leads to ubiquitination and subsequent proteasomal degradation of the I κ B, leaving NF- κ B free to translocate to the nucleus⁵. Being NF- κ B target genes themselves, the I κ B family members are part of a negative feedback loop, retracting NF- κ B from the nucleus back into the cytoplasm and thereby preventing excessive and irreversible NF- κ B activation⁶.

NF- κ B activation is an important response in different infectious as well as non-infectious pathologies. Also in the different stages of atherogenesis, from early endothelial activation to eventual plaque rupture, NF- κ B has been described as a key regulator^{7,8}. Atherosclerosis is a slowly progressing, chronic inflammatory disease of the large arteries representing the most common cause of death in western society⁹. This process is initiated when modified lipoproteins in the vessel wall activate the endothelial lining of the vessel, thereby attracting monocytes, which differentiate into macrophages upon migration through the endothelium. By taking up and storing the lipoproteins, these macrophages eventually become large foam cells and start secreting inflammatory mediators, cytokines and chemokines. The thereby created inflammatory environment attracts even more monocytes as well as other immune cells to the vessel wall, forming an atherosclerotic plaque¹⁰.

In the atherosclerotic plaque, a wide variety of NF- κ B inducers is present, ranging from modified lipoproteins to inflammatory mediators, free radicals and remnants of dead cells¹¹⁻¹³. Therefore, activated NF- κ B has been found in different cell types in the lesion, including macrophages, smooth muscle cells and endothelial cells^{8,14}. To investigate the importance of this activation, we have previously studied models with either a

macrophage-¹⁵ or endothelial cell-specific¹⁶ ablation in NF- κ B activation. While macrophage-specific deletion of IKK2 led to larger and more advanced lesions, endothelium-restricted NEMO deletion abrogated atherogenesis by impairing macrophage recruitment to the plaque.

In this paper we aimed at investigating the role of the NF- κ B inhibitor I κ B α in atherogenesis. Since full I κ B α knockout mice die neonatal of hypergranulopoiesis and severe dermatitis¹⁷⁻¹⁹, we used a conditional model with a myeloid specific deletion of I κ B α ²⁰. Bone marrow from these LysMCre-I κ B α ^{fl/fl} mice was transplanted into atherosclerosis-susceptible *Idlr*^{-/-} mice to study the effect of myeloid I κ B α deficiency on atherogenesis. We found that myeloid I κ B α deficiency promotes atherogenesis by causing increased attraction of myeloid cells to the developing plaques without affecting other phenotypical characteristics of the lesions. Quite surprisingly, macrophage I κ B α deficiency does not seem to affect the production of a number of NF- κ B target genes *in vivo* nor *in vitro* but appears to be involved in the adhesion and recruitment of these cells to the atherosclerotic plaque.

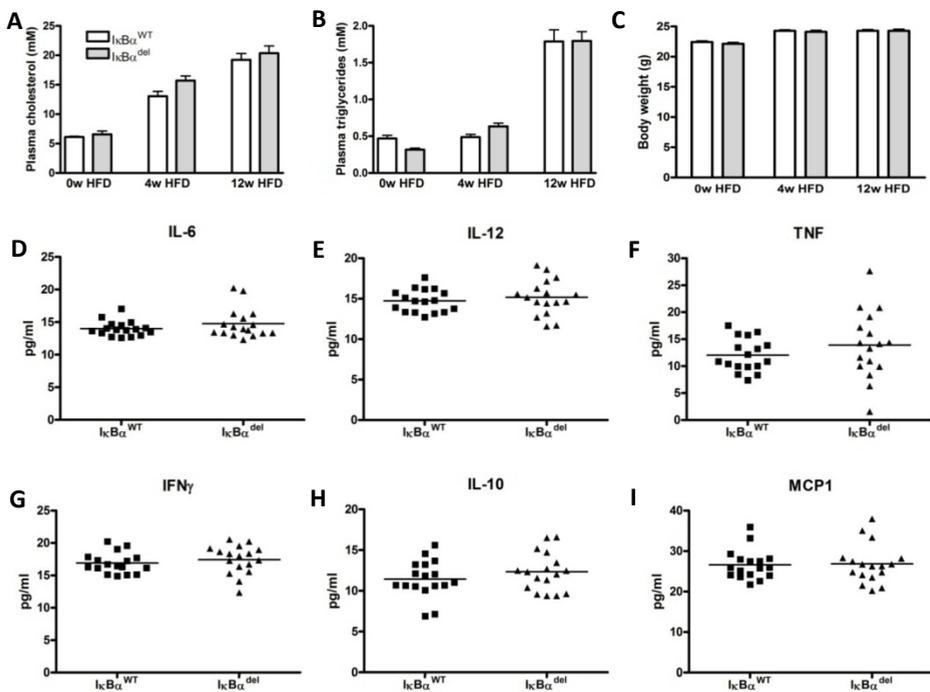


Figure 3.1: Plasma values and body weight of mice transplanted with either *I κ B α ^{fl/fl}* or *LysMCre-I κ B α ^{fl/fl}* bone marrow:

(A) Cholesterol levels, (B) triglyceride levels and (C) body weight measured before the start of the high fat diet (0w HFD), after 4 weeks of diet (4w HFD) and upon sacrifice of the mice (12w HFD). (D-I) Plasma cytokine concentrations at the end of the experiment, measured by a bead array.

Results

Myeloid IκBα deficiency promotes atherosclerosis without altering plaque phenotype, body weight, plasma lipids and cytokines

To study the effect of myeloid IκBα deficiency on atherosclerosis, bone marrow was isolated from $IκBα^{fl/fl}$ and $LysMCre-IκBα^{fl/fl}$ mice, the latter having a myeloid-specific deletion of the $IκBα$ gene²⁰. This bone marrow was transplanted into irradiated $Ildlr^{-/-}$ mice, resulting in atherosclerosis-susceptible mice that were either wildtype ($IκBα^{WT}$) or deleted ($IκBα^{del}$) for $IκBα$ in their myeloid cells. Four weeks after transplantation, these mice were put on a high fat diet for 12 weeks in order to induce atherosclerosis. The rise in plasma cholesterol, plasma triglycerides and body weight as a result of the diet was similar in both groups (Figures 3.1A, B and C). In addition, relative levels of circulating leukocyte populations (monocytes, granulocytes, T and B lymphocytes) after the transplantation did not differ between the groups (data not shown). After 12 weeks of diet, plasma levels of the NF-κB dependent pro-inflammatory cytokines IL-6, IL-12,

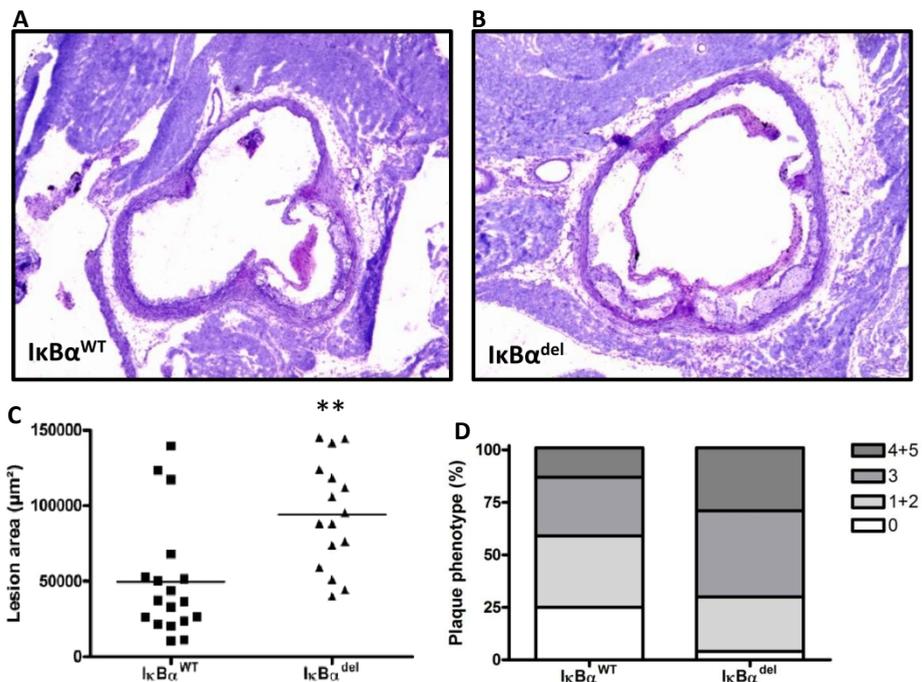


Figure 3.2: Myeloid $IκBα$ deficiency promotes atherosclerosis in $Ildlr^{-/-}$ mice:

Representative pictures of toluidine blue-stained sections in the aortic root of (A) $IκBα^{WT}$ or (B) myeloid-specific $IκBα^{del}$ mice, original magnification $\times 40$. (C) Lesion area in the aortic root of $IκBα^{WT}$ and $IκBα^{del}$ mice (** $p < 0.01$; $n = 18/16$). (D) Lesion severity in the aortic root of $IκBα^{WT}$ and $IκBα^{del}$ mice (Chi square test; *** $p < 0.0001$; $n = 54/48$) was typed as absent (0), early (1+2), or advanced (4+5), as described before¹⁵.

TNF- α and IFN γ , the anti-inflammatory cytokine IL-10 and the chemokine MCP-1 (CCL2) were not influenced by the absence of myeloid I κ B α (Figures 3.1D - I).

Upon sacrifice, atherosclerosis in the aortic root was analyzed. Interestingly, lesion area measurements using toluidine blue-stained sections (Figures 3.2A and B) showed a two-fold, significant increase in plaque formation in I κ B α ^{del} mice compared to I κ B α ^{WT} mice (49740 \pm 9142 μ m² vs. 94330 \pm 8803 μ m² for I κ B α ^{WT} and myeloid-specific I κ B α ^{del} respectively; $p=0.0014$) (Figure 3.2C). Moreover, classification of the lesions according to their severity showed that I κ B α ^{del} mice had relatively more advanced and less early atherosclerotic lesions (Chi square test; $p<0.0001$) (Figure 3.2D).

With additional analyses on sections of the aortic root, the phenotype of these plaques was further characterized. Plaque-stabilizing collagen was stained with Sirius Red but no significant differences were found in collagen content between the two groups (Figure 3.3A). Also analysis of T cell and neutrophil content revealed no significant differences (Figures 3.3B and C). Finally, both groups appeared to have the same number of proliferating and apoptotic cells in their plaques, as shown by Ki-67 and a TUNEL stainings (Figures 3.3D and E). Thus despite having larger and more advanced lesions, the I κ B α ^{del} lesions showed no other changes in their plaque characteristics.

Next to the lesions in the aortic root, atherosclerotic plaque formation was also assessed in the aortic arch. In RNA isolated from this tissue, a 49.6% increase in the expression of macrophage marker CD68 was found ($p=0.0384$, data not shown), indicating enhanced macrophage accumulation in the vessel wall of I κ B α ^{del}-transplanted mice.

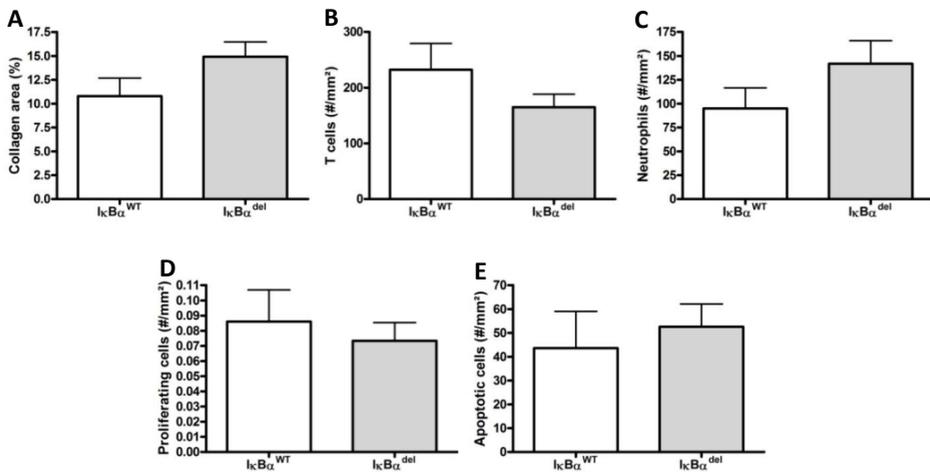


Figure 3.3: Myeloid I κ B α deficiency does not influence plaque phenotypic characteristics:

(A) Collagen content is not different between I κ B α ^{WT} and I κ B α ^{del} mice, as shown by a Sirius Red staining. Also the relative number of (B) T cells, (C) neutrophils, (D) proliferating and (E) apoptotic cells was similar in both groups.

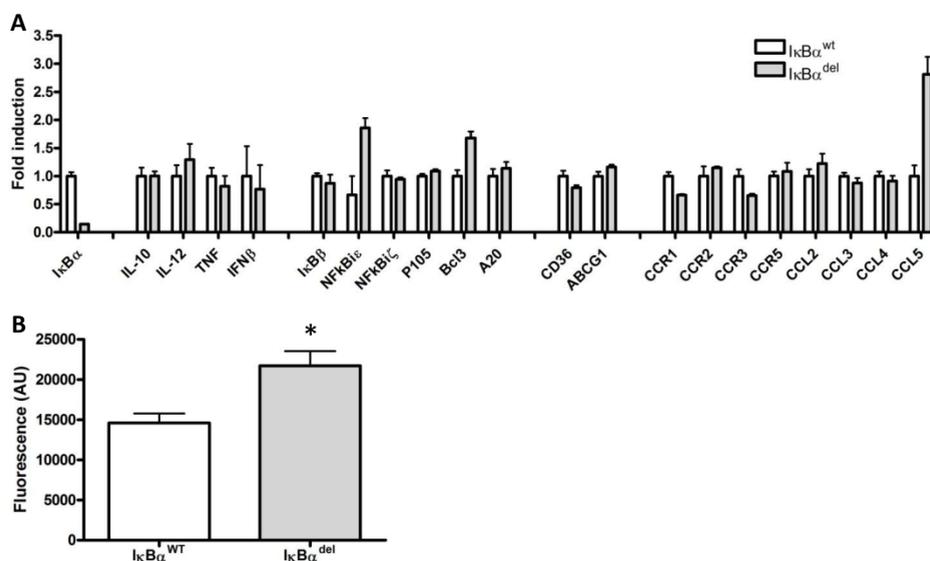


Figure 3.4: Deletion of I κ B α in bone marrow-derived macrophages has limited effect on the expression of NF- κ B-dependent and/or -regulating genes but enhances *in vitro* static macrophage adhesion:

(A) Gene expression in triplicates of LysMCre-I κ B α ^{fl/fl} macrophages was compared to I κ B α ^{fl/fl} macrophages by Q-PCR. (B) Adhesion of fluorescently labeled macrophages to an endothelial monolayer (bars represent triplicate wells \pm SEM; * $p < 0.05$). Data shown are representative for at least 3 experiments.

Myeloid I κ B α deficiency promotes *in vitro* macrophage adhesion

To investigate the mechanism behind the enhanced atherogenesis in the I κ B α ^{del} mice, *in vitro* experiments were done. Surprisingly, the reduced production of I κ B α shown in Figure 3.4A and Supplemental Figure S3.1A did not result in an increased activation of the p65 NF- κ B subunit (Supplemental Figure S3.1B-C), suggesting a potent compensatory mechanism operating in the absence of I κ B α . As a result, hardly any difference was found in the expression of some genes known to be NF- κ B targets, NF- κ B inhibitors or inflammatory mediators when assessed in unstimulated as well as LPS stimulated bone marrow-derived macrophages from LysMCre-I κ B α ^{fl/fl} mice and compared to wild type cells (Figure 3.4A and Supplemental Figure S3.1D). However, the expression of the chemokine CCL5 (or RANTES) was significantly elevated 2.8 fold in macrophages lacking I κ B α . CCL5 was previously shown to be involved in the adhesion of macrophages to endothelial cells. In line with these findings, static adhesion of I κ B α ^{del} macrophages to a monolayer of the bEND.5 endothelial cell line was significantly enhanced compared to wild-type macrophages (14610 \pm 1163 AU vs. 21720 \pm 1810 AU for I κ B α ^{WT} and I κ B α ^{del} respectively; $p = 0.0298$) (Figure 3.4B).

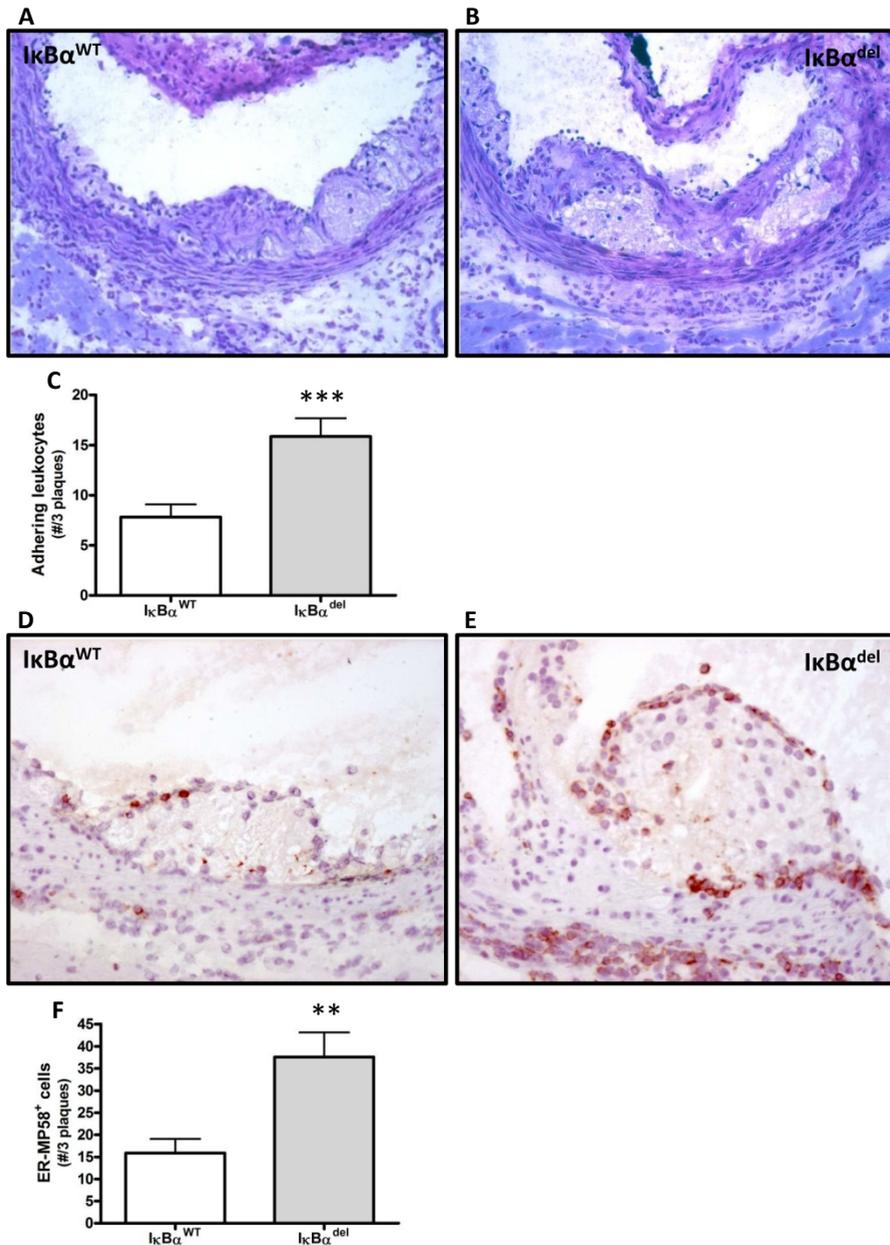


Figure 3.5: Increased leukocyte adhesion and newly recruited myeloid cells in $I\kappa B\alpha^{del}$ mice:

(C) In $I\kappa B\alpha^{del}$ mice, more leukocytes adhere to the endothelial cell layer delineating the lesion (***) $p < 0.0001$; $n = 17/17$). Representative pictures from (A) $I\kappa B\alpha^{WT}$ and (B) $I\kappa B\alpha^{del}$ lesions, original magnification $\times 200$. (F) Lesions from $I\kappa B\alpha^{del}$ mice contain more ER-MP58 positive cells, suggesting an increased attraction myeloid cells (** $p < 0.001$; $n = 19/17$). Representative pictures from (D) $I\kappa B\alpha^{WT}$ and (E) $I\kappa B\alpha^{del}$ lesions, original magnification $\times 200$.

Atherosclerotic lesions in mice lacking myeloid IκBα show increased leukocyte adhesion and migration.

To investigate whether the observed increased *in vitro* macrophage adhesion could also be observed in the *in vivo* atherosclerosis model, cells adhering to luminal side of the endothelial cells covering the atherosclerotic plaques were quantified in the toluidine blue-stained sections. Indeed, the IκBα^{del} mice displayed a significantly higher number of adhering cells compared to the IκBα^{WT} mice (7.8 ± 1.3 cells vs. 15.9 ± 1.8 cells for IκBα^{WT} and IκBα^{del} respectively; $p=0.0009$) (Figures 3.5A - C), suggesting enhanced recruitment of new cells to the plaque in mice lacking IκBα in their myeloid cells.

To further analyze this recruitment of monocytes to the lesions, we used a marker that is specifically expressed on circulating immature myeloid cells but that is lost upon differentiation to macrophages. This marker, detected by the antibody ER-MP58, has previously been used in other studies to analyze recruitment of cells from the circulation to tissues²¹⁻²³. Sections from the aortic root were stained with ER-MP58 and positive cells were quantified. As expected, these cells were predominantly observed on the luminal side of the plaque. However, also on the adventitial side, some myeloid recruitment could be observed. Interestingly, ER-MP58-positive cells were also seen in inflammatory regions in the vicinity of the plaques. Quantification showed that IκBα^{del} mice had significantly more newly recruited myeloid cells in their plaques compared to IκBα^{WT} mice (15.890 ± 3.164 cells vs. 37.590 ± 5.568 cells; $p=0.0014$) (Figures 3.5D - F). These data indicate that atherosclerotic lesions in the aorta of *ldlr*^{-/-} mice lacking myeloid IκBα may be larger because of enhanced recruitment and infiltration of myeloid cells, suggesting that myeloid IκBα is important in regulating the migratory phenotype of cells in atherogenesis. A possible role for CCL5 in this mechanism is illustrated by the observation that the expression of this chemokine was significantly elevated in the aforementioned aortic arch-derived RNA (40.7% increase, $p=0.0270$, data not shown).

Discussion

In this paper we show that myeloid-specific deletion of the inhibitor of NF-κB, IκBα, in *ldlr*^{-/-} mice results in larger atherosclerotic plaques without affecting the general plaque composition. Studying cell adhesion to the luminal side of the endothelial layer covering atherosclerotic plaques, we observed increased adhesion of leukocytes in the absence of myeloid IκBα. Through a marker specific for circulating myelomonocytic cells that is lost upon maturation to macrophages, ER-MP58, we found that lesions from mice lacking myeloid IκBα are characterized by more newly recruited leukocytes. Moreover, we found an increase in macrophage content in the vessel wall of the aorta.

These data suggest that I κ B α in myeloid cells may have a pivotal role in regulating the recruitment of cells to atherosclerotic lesions.

Atherosclerosis is known to be driven by inflammation and aggravated by the production of pro-inflammatory cytokines²⁴. Since cytokine expression is highly dependent on NF- κ B, it can be expected that the atherosclerotic process is proportional to the activation level of this transcription factor. Indeed, Gareus et al. showed that endothelial-specific inhibition of NF- κ B by NEMO deletion impaired macrophage recruitment to the plaque and hereby impaired atherogenesis¹⁶. Another paper, by Wolfrum et al., describes how haploinsufficiency for the NF- κ B activation inhibitor A20 results in enhanced atherogenesis while A20 overexpression reduced plaque formation²⁵. Our group also showed that the role of NF- κ B in macrophages is not as straightforward. Myeloid-specific blocking of the canonical activation of NF- κ B through deletion of IKK2 resulted in plaques that were not only larger but also more advanced and more necrotic, highlighting the fact that NF- κ B also acts as an anti-apoptotic transcription factor and is involved in regulating anti-inflammatory mechanisms¹⁵. In contrast, the present study demonstrates that deletion of myeloid I κ B α , aiming at myeloid-specific NF- κ B activation, also induces larger and more advanced plaques but without affecting the plaque composition.

Studying gene expression in LysMCre-I κ B α ^{fl/fl} bone marrow-derived macrophages and comparing it to wildtype cells, we found an increase in the expression of the chemokine RANTES (Released upon Activation, Normal T-cell Expressed and Secreted, or CCL5). This molecule belongs to a relatively limited set of chemokines and adhesion molecules that are described to be influencing atherogenesis through the recruitment of new cells to the plaque^{26,27}. In a recent publication, our group showed that IFN β -induced expression of CCL5 augments the static adhesion of macrophages to endothelial cells *in vitro* as well as the adhesion of leukocytes to the vessel wall at atherosclerosis-prone sites *in vivo*, thereby promoting the attraction of new cells to the plaques²⁸. Macrophages from LysMCre-I κ B α ^{fl/fl} mice indeed adhered more efficiently to an endothelial cell layer *in vitro*, while in the atherosclerosis model, more monocytes were found adhering to the endothelial layer covering the plaques in the I κ B α ^{del} mice compared to the I κ B α ^{WT} mice.

To confirm the hypothesis that the lesions in the I κ B α ^{del} mice were larger because of an increased attraction of leukocytes, we applied a staining detecting a marker specific for immature myeloid cells, ER-MP58. The target is an antigen with a yet unknown function, which is present on the bone marrow-derived, myeloid-committed progenitor cells. It continues to be expressed on neutrophils and monocytes but disappears progressively upon maturation of the M-CSF-responsive cells to macrophages^{21,29}. Thus, both Ly-6C-high and Ly-6C-low subsets of circulating monocytes are positive for ER-MP58 while tissue macrophages have lost the marker^{29,30}. Previously, detection of this marker has been used to distinguish infiltrating immature myeloid cells from the

resident macrophages already present within the site of inflammation, both in thioglycollate elicited macrophage recruitment to the peritoneum^{21,22} and in the restoration of the Kupffer cell population in the liver, following injection with liposome-entrapped dichloromethylene diphosphonate²³. In this paper, we show that ER-MP58 is also a valid marker for newly recruited myeloid cells to the atherosclerotic plaque, with a positivity that is limited to small, recently infiltrated cells in the vicinity of the luminal plaque surface and inflammatory regions.

Contrary to the upregulation of CCL5 expression in the LysMCre-I κ B α ^{fl/fl} macrophages, many typical NF- κ B-dependent genes were not influenced by the deletion of I κ B α . This suggests that in these cells, NF- κ B is not continuously activated, but inhibited by other feedback mechanisms which, like I κ B α , prevent NF- κ B translocation to the nucleus or terminate NF- κ B activation by exporting it back to the cytoplasm. Indeed, studying the nuclear translocation of several NF- κ B subunits, we found that the LysMCre-I κ B α ^{fl/fl} macrophages had the same degree of translocation as wildtype cells (data not shown) and no increase in p65 phosphorylation was observed in the I κ B α ^{del} macrophages (Supplemental Figure S3.1B-C). In addition, only mild to no upregulation of other I κ B family members was detected by gene expression analysis, indicating alternative mechanisms of regulation of NF- κ B dependent transcription. Recent studies indeed show that, besides I κ B inhibitors, also nuclear ubiquitin ligases can terminate chronic NF- κ B activation by its nuclear degradation, a mechanism that might compensate the deletion of I κ B α ³¹.

In conclusion, we found that myeloid-specific deletion of I κ B α resulted in an upregulation of the chemokine CCL5 and enhanced static *in vitro* adhesion of macrophages to an endothelial cell layer. Moreover, this correlated *in vivo* with increased leukocyte adhesion to the activated endothelial lining of the blood vessel, enhanced recruitment of ER-MP58⁺ immature myeloid cells to the atherosclerotic plaque and increased atherosclerotic lesion formation in the aortic root and arch. Hereby we show that the role of myeloid I κ B α in the regulation of inflammation is complex but that it is involved in the recruitment of macrophages to the atherosclerotic plaque.

Materials and methods

Mice

C57BL/6 mice and *Id1*^{-/-} mice on a C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME). I κ B α ^{fl/fl} mice on a C57BL/6 background were described before³². All animal experiments were approved by the DierExperimenten Commissie (DEC) of the Maastricht University (permit numbers 2005-090 and 2009-168).

Bone marrow transplantation

One week before transplantation, female *ldlr*^{-/-} mice were housed in filter top cages and provided with acidified water containing neomycin (100mg/l; Gibco, Breda, The Netherlands) and polymyxin B sulphate (6x10⁴ U/l; Gibco). The animals received 10Gy total body irradiation and on the following day, bone marrow was isolated from 6 LysMCre-IκBα^{fl/fl} mice (IκBα^{del}) and 6 IκBα^{fl/fl} littermates (IκBα^{wt}) and 10⁷ cells/mouse were injected intravenously to rescue the hematopoietic system of the irradiated mice. Four weeks after the transplantation, mice were fed a high fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 12 weeks.

Mouse blood parameters

At several time points during the *in vivo* atherosclerosis experiment, blood was drawn from the mice. Plasma lipid levels were monitored enzymatically (Sigma Aldrich, Zwijndrecht, the Netherlands) and plasma cytokine levels were measured by flow cytometry using a Cytometric Bead Array kit (BD-Pharmingen, San Diego, CA). Leukocytes were counted using a Coulter counter and blood cell distribution was quantified by flow cytometry after antibody staining with either Mac1-PE and Gr1-FITC for macrophages and granulocytes or 6B2-PE and KT3-FITC for B- and T-cells (BD-Pharmingen, Erembodegem, Belgium).

Atherosclerosis analysis

Upon sacrifice, the hearts from the bone marrow transplanted mice were taken out and cut perpendicular to the heart axis just below the atrial tips. Tissue was frozen in tissue-tec (Shandon, Veldhoven, The Netherlands) and cut into sections of 7 μm as described before¹⁵. Serial cross-sections from every 42 μm were stained with toluidine blue. All lesion areas were quantified using Adobe Photoshop software. The lesions were also typed according to severity as early, moderate and advanced, as described before¹⁵.

Immunohistochemical staining

Lesions from the aortic root were fixed in acetone and incubated with antibodies against neutrophils (1A8, BD-Pharmingen), T cells (KT3, directed against CD3, a gift from G. Kraal), proliferating cells (Ki-67, Dako, Glostrup, Denmark) and newly recruited macrophages (ER-MP58, P. Leenen), followed by detection with a biotin labeled rabbit anti-rat antibody and staining with the ABC kit (Vector Labs, Burlingame, CA). Apoptotic cells in the plaques were stained by the TUNEL staining (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Collagen areas were analyzed on Sirius red stained sections. Adhering leukocytes were quantified on toluidine blue-stained sections.

***In vitro* murine bone marrow macrophage culture**

Bone marrow cells were isolated from femurs and tibiae of either wild-type (I κ B α ^{fl/fl}) or deleted (LysMCre-I κ B α ^{fl/fl}) mice. Cells were cultured in RPMI-1640 (GIBCO Invitrogen, Breda, The Netherlands) with 10% heat-inactivated fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), penicillin (100 U/ml), streptomycin (100 ug/ml), and L-glutamine 2mM (all GIBCO Invitrogen, Breda, The Netherlands) supplemented with 15% L929-conditioned medium (LCM) for 8–9 days to generate bone marrow-derived macrophages (BMM), as described previously¹⁵.

Western blotting

Protein was isolated from BMM with an SDS lysis buffer, supplemented with complete protease inhibitor cocktail (Roche Diagnostics) and PhosSTOP phosphatase inhibitor cocktail (Roche Diagnostics). After Western blotting, blots were incubated with P-p65 antibody (1:500, Cell Signaling, Danvers, MA) in PBS with 0.05% Tween and 5% BSA (Sigma Aldrich).

Gene expression

RNA was isolated from BMM with the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) or from snap-frozen aortic arches with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). 500 ng total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative PCR (Q-PCR) was performed using 10 ng cDNA, 300 nM of each primer, and SensiMix (Quantace-Bioline, London, UK) in a total volume of 20 μ l. All gene expression levels were corrected for cyclophilin A as housekeeping gene. Primer sequences are available upon request.

***In vitro* adhesion assay**

A confluent monolayer of bEND5 endothelial cells was grown in fluorescence 96-well microplates (Greiner Bio-one, Frickenhausen, Germany). Triplicate wells were incubated for 30 min with 10⁵ BMM that had been fluorescently labeled with a PKH67 dye according to the manufacturer's instructions (Sigma Aldrich, Zwijndrecht, The Netherlands). Subsequently, the wells were washed three times with the aforementioned macrophage medium, and adherent cells were measured by fluorometry in a Synergy HT microtiter plate reader (BioTek, Bad Friedrichshall, Germany) at an excitation of 485 nm and an emission of 520 nm.

Statistical analysis

The statistical analyses were performed using Graphpad Prism (Graphpad Software). Differences between 2 groups were evaluated using a t-test, unless stated otherwise.

Values are represented as mean \pm SEM. A *P* value of less than .05 was considered to be statistically significant. All mouse data passed a normality test.

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Chapter 4

A survivin-targeted lentiviral vaccination strategy reduces murine atherogenesis

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Submitted

Abstract

Survivin is an inhibitor of apoptosis and is highly expressed in virtually all tumors but has low to undetectable levels in healthy cells. Therefore, survivin has been a target in cancer therapies, including immunotherapy against survivin-positive cells. Recently, survivin expression was observed in macrophages that infiltrate early human atherosclerotic lesions. In this paper, we confirmed the presence of survivin-positive cells in murine atherosclerotic plaques. Subsequently, we targeted atherosclerosis by inducing a cytotoxic T cell response against survivin-positive plaque macrophages. We immunized atherosclerosis-susceptible mice using a lentivirus-based vaccination strategy, which indeed reduced atherosclerotic lesion size and promoted the migration of CD3⁺ T cells and CD8⁺ cytotoxic T cells into the plaque. These experiments are the first to show the therapeutic potential of immunization against plaque-infiltrating macrophages and thereby offer a novel perspective for immunotherapy-based strategies in combating atherosclerosis.

Introduction

Survivin (also known as baculoviral inhibitor of apoptosis repeat-containing 5, BIRC5) is a member of the Inhibitor of Apoptosis (IAP) family of proteins¹ and is predominantly studied as a tumor-associated protein. While its expression in healthy cells is usually low to undetectable² and restricted to the G2/M phase of mitosis with a rapid downregulation in the G1 phase, virtually all tumors exhibit an excessive and cell cycle-independent expression, making survivin the fourth most upregulated gene in tumor transcriptomes³.

Survivin regulates two essential cellular processes that both contribute to tumor growth. As an IAP family member, survivin inhibits apoptosis. Indeed, its overexpression attenuates cell death, while inhibiting its function results in spontaneous cell death and a higher sensitivity to apoptotic stimuli⁴. It is, however, not a direct caspase inhibitor⁵, and the mechanism behind its effect on apoptosis is still unclear. In addition to blocking apoptosis, survivin also regulates mitosis through its effects on multiple spindle microtubule functions and mitotic checkpoints, promoting proliferation by facilitating accurate sister chromatid segregation and stabilization of microtubules in late mitosis^{6,7}. Because cell cycle progression and resistance to cell death are two factors that are essential to tumor formation, survivin upregulation may be a key step in the initiation of tumorigenesis⁸. These properties make survivin an attractive universal target for anti-tumor drugs and therapies⁴. Several strategies, including small-molecule antagonists, dominant-negative mutants, small interfering RNA (siRNA), ribozymes, anti-sense oligonucleotides, immunotherapy and survivin promoter-induced TRAIL expression, have been tested in experimental models, and some strategies have advanced to human phase I or II clinical trials, all without significant cross-reactivity-related side effects⁹⁻¹².

Interestingly, Blanc-Brude et al. described survivin to also be detectable in human atherosclerotic plaques¹³. Atherosclerosis is a chronic inflammatory disease of the vascular wall that gradually causes a variety of acute, life-threatening outcomes, such as myocardial infarction and stroke. It is initiated when low-density lipoproteins (LDL) accumulate in the subendothelial space and are chemically modified. This mobilizes circulating monocytes to the vessel wall to clear the modified LDL. The accumulation of these cells forms an early atherosclerotic plaque or fatty streak. Subsequently, the plaque evolves to a more advanced state by the attraction of more blood cells, the growth of a fibrous cap and the appearance of a necrotic core, a region in the plaque where the debris of inefficiently cleared apoptotic and necrotic cells gathers. The susceptibility of the plaque to rupture and cause acute symptoms is determined by the balance between stabilizing (e.g., cap thickness) and destabilizing (e.g., necrotic core size) factors.

In the aforementioned study, macrophage colony-stimulating factor (M-CSF) is able to upregulate survivin expression in macrophages *in vitro*¹³. M-CSF is abundantly present in atherosclerotic plaques where it promotes macrophage attraction and survival¹⁴. Prolonged *in vitro* exposure to oxidized LDL (oxLDL), however, abolishes survivin expression¹³. Indeed, immunohistochemical analysis of human atherosclerotic plaques revealed that CD68⁺ macrophages are positive for survivin at the luminal side of early fatty streaks, while in more advanced plaques, survivin-positive cells were rarely found¹³. This suggests that newly infiltrated macrophages in early atherosclerotic plaques express survivin under the influence of M-CSF, possibly as a protective mechanism against apoptosis provoked by radical oxygen species and lipid degradation products within the lesion¹⁵. Once the macrophages become foam cells, however, they lose their survivin expression. Thereby they become vulnerable to apoptosis, contributing to necrotic core formation.

In this paper, we identify survivin as a new specific immune target in atherosclerosis by showing that survivin-expressing cells are detectable in murine plaques. Analogous to cancer models, in which cellular immunity against survivin-expressing cells selectively kills tumor cells without significant side effects^{16,17}, we applied a lentiviral vaccination strategy to induce cellular immunity against newly infiltrating macrophages in the plaques. We show that such survivin-based immunotherapy effectively reduced atherosclerotic plaque size and was accompanied by an enhanced recruitment of CD3⁺ T cells and CD8⁺ cytotoxic T cells to the lesions.

RESULTS

Survivin is detected in murine atherosclerotic plaques and macrophages

Cryosections of atherosclerotic lesions from LDLR^{-/-} mice fed a high-fat diet for 12 weeks were immunohistochemically stained for survivin. In the plaques, survivin-positive cells were clearly detectable (Figure 1 A-B). Morphologically, these cells were identified as macrophages, and they were located predominantly on the luminal side of the plaque, similar to the survivin-positive cells found in human fatty streaks¹³. No staining was observed in the media or in the adjacent aorta, indicating that survivin expression was specific to the plaque-infiltrating macrophages, in addition to its usually assumed restriction to tumor cells, apoptosis-related fetal tissues¹⁸, sites of vascular injury¹⁹ and a selection of self-renewing cells².

Analysis of microarray data generated by our group²⁰ showed that treating murine bone marrow-derived macrophages *in vitro* with oxLDL, but not with acetylated LDL (acLDL) or unmodified LDL, reduced *survivin* expression by 48% ($p=0.0068$; Figure 1 C).

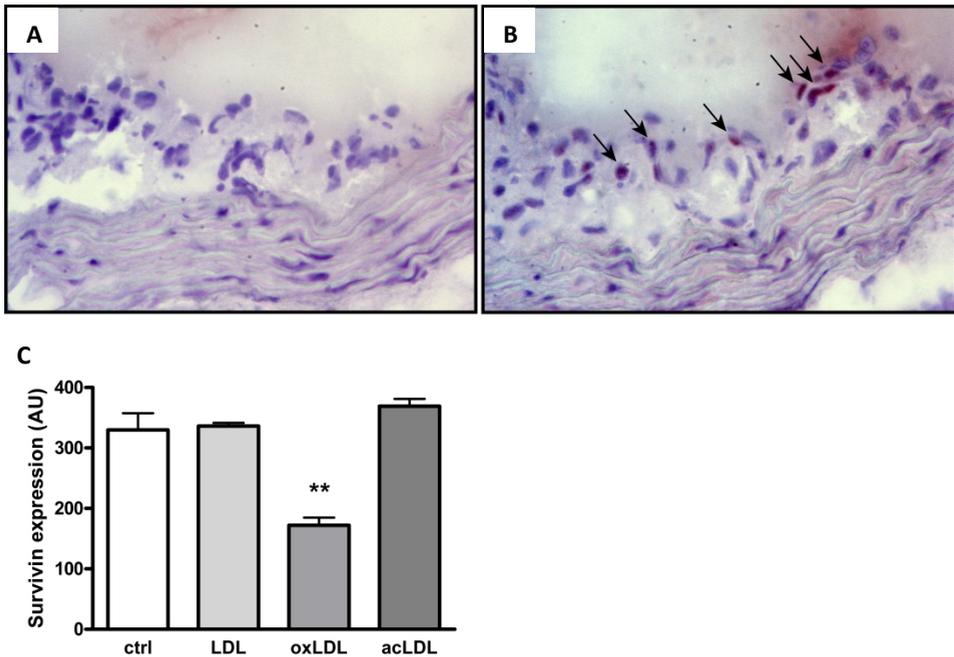


Figure 4.1: Survivin expression in murine atherosclerotic plaques and macrophages:

A-B: Murine atherosclerotic plaques were immunohistochemically stained without (A) or with (B) a survivin-detecting antibody (original magnification $\times 200$). The survivin-expressing cells are indicated by arrows. **C:** Survivin gene expression in murine bone marrow-derived macrophages incubated for 24h with non-modified LDL, oxidized LDL or acetylated LDL.

Validation of a survivin-specific cytotoxic T cell response upon lentiviral vaccination

Inhibition of survivin expression and elimination of survivin-positive cells have been successfully applied as therapies in murine tumor models and cancer patients¹⁰. To evaluate whether survivin-positive cells in atherosclerotic plaques can be targeted in a similar way, we developed a lentiviral vector-based vaccination strategy aimed to skew the immune system toward autoreactivity against survivin-presenting cells. The murine *survivin* gene was cloned into a lentiviral vector containing a CMV promoter. Moreover, this vector encodes the non-functional truncated nerve growth factor receptor (tNGFR) as a marker gene²¹. Viruses generated from this vector ('SURV') or from the empty control vector ('tNGFR') were subsequently used for vaccination.

To verify the ability of these viruses to induce a murine survivin-specific immune reaction, BALB/c mice were injected in the footpad with one of the viral vectors. Four days after immunization, splenocytes from the mice were isolated and re-stimulated *in vitro* with the survivin-expressing BALB/c-derived B cell lymphoma cell line A20. Subsequently, the induction of cytotoxic T cells was evaluated in a ⁵¹Cr-release assay. In this experiment, the splenocytes were co-cultured with radioactively labeled A20 cells

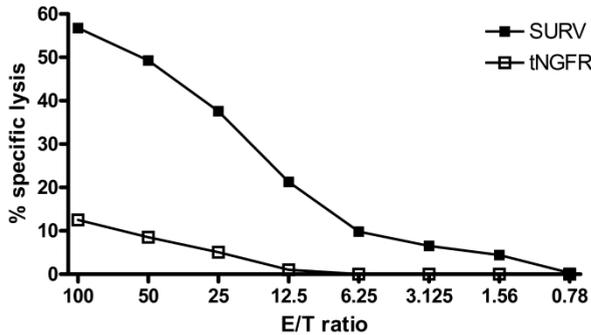


Figure 4.2: In vitro cytotoxicity assay:

Splenocytes from mice that were vaccinated with either control (tNGFR) or survivin (SURV) lentiviruses were re-stimulated with survivin-expressing cells, after which they were co-cultured with ^{51}Cr -labeled A20 cells at different E/T ratios, and lysis was analyzed.

at different effector/target (E/T) ratios, and specific cell lysis was analyzed. The results show that vaccination with a survivin-encoding lentivirus significantly induced cytolytic activity against A20 cells (two-way ANOVA, $p=0.0141$; Figure 2).

LDLR^{-/-} vaccination and general pathology

To assess the effect of a survivin-targeted vaccination on atherosclerosis development, 9-week-old LDLR^{-/-} mice were injected with either 'SURV' (n=16) or 'tNGFR' (n=15) lentiviruses. They received additional boost injections after 1 and 4 weeks. One week after the first injection, the mice were placed on a high-fat diet to induce atherogenesis, which was maintained for 11 weeks (Supplemental Figure S4.1). During this period, both groups of mice gained equally in total body weight (Supplemental Figure S4.2A), and both groups had similarly raised plasma cholesterol and triglyceride levels (Supplemental Figure S4.2B-C). No mice died or showed any sign of discomfort during the experiment. Upon sacrifice, no macroscopic signs of pathology were visible in either of the vaccinated groups, and the microscopic examination of the small intestine, kidneys, liver and skin did not show any indication of tissue damage or immune cell aggregation. The plasma values and pathology of the lentivirus-injected groups did not differ from that of a group of PBS-injected control mice (n=15).

Atherosclerotic plaque analysis

After 11 weeks on a high-fat diet, the mice were sacrificed, and cryosections of the aortic root were obtained to analyze the effect of vaccination on atherosclerosis. Compared with the 'tNGFR' group, 'SURV'-vaccinated mice had plaques that were 31% smaller ($124,900 \pm 6,472 \mu\text{m}^2$ vs. $85,950 \pm 10,250 \mu\text{m}^2$, respectively, $p=0.0080$; Figure 3 A-B). We observed no effect from the lentiviral treatment itself; lesions of the 'tNGFR'-vaccinated mice were equal in size compared with PBS-injected mice (data not shown).

Both groups of vaccinated mice had equal percentages of plaque-stabilizing collagen in their lesions ($7.002 \pm 1.050\%$ for 'tNGFR' vs. $6.340 \pm 1.050\%$ for 'SURV', $p=0.6647$; Supplemental Figure S4.2D), while the necrotic core area was remarkably yet insignificantly increased upon 'SURV' treatment ($3.222 \pm 0.6864\%$ for 'tNGFR' vs. $5.705 \pm 1.181\%$ for 'SURV', $p=0.0834$; Supplemental Figure S4.2E).

Because the lentiviral vaccination was designed to induce a cytotoxic response against survivin-expressing cells, the number of T cells within the plaque was assessed using CD3 staining. We observed an increase in $CD3^+$ T cells in the plaques of 'SURV'-vaccinated mice (113.2 ± 21.65 T cells/ mm^2 for 'tNGFR' vs. 611.7 ± 92.19 T cells/ mm^2 for 'SURV', $p=0.0001$; Figure 3 C-D). Focusing further on cytotoxic T cells, we also found a higher lesion content of $CD8^+$ cells in 'SURV'-vaccinated mice (37.64 ± 8.472 $CD8^+$ cells/ mm^2 for 'tNGFR' vs. 84.68 ± 18.06 $CD8^+$ cells/ mm^2 for 'SURV', $p=0.0306$; Figure 3 E-F), suggesting the recruitment of survivin-targeted cytotoxic T cells.

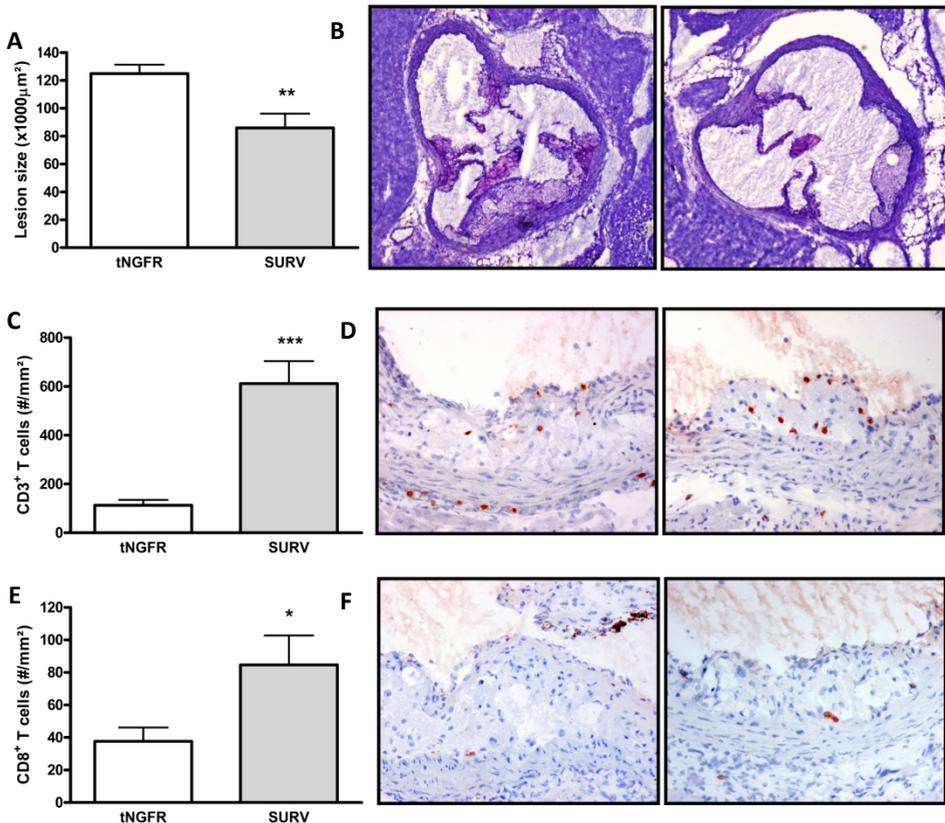


Figure 4.3: Atherosclerotic plaque size and phenotype:

A-B: Sections of the aortic root of mice treated with either control (tNGFR) ($n=15$) or survivin (SURV) ($n=16$) lentivirus were stained with toluidine blue to quantify the lesion size. **C-D:** The numbers of $CD3^+$ T cells in the plaques of tNGFR or SURV treated mice with representative pictures. **E-F:** The numbers of $CD8^+$ cytotoxic T cells in plaques of tNGFR or SURV treated mice with representative pictures.

DISCUSSION

In chronic diseases where leukocyte recruitment to the site of inflammation has a detrimental effect on outcome²², knowledge of specific markers for the infiltrating cells can be very useful for both analysis and treatment purposes. While no survivin expression is present in healthy blood vessels^{18,19}, the presence of survivin-positive cells has been demonstrated in human atherosclerotic lesions¹³. In this paper, we evaluated murine lesions for the presence of cells that express survivin and subsequently targeted them with a lentiviral vaccination strategy.

Blanc-Brude et al. described how M-CSF induces the expression of survivin in macrophages entering the human plaque. However, these survivin-positive cells were absent in the adventitial region as well as in more advanced plaques, which was attributed to a downregulation of the expression evoked by long-term exposure to oxidized lipids within the plaque¹³. We confirmed the presence of survivin-positive cells in a confined region within murine plaques and identified them morphologically as newly recruited macrophages. Furthermore, we demonstrated *in vitro* that murine bone-marrow-derived macrophages cultured in the presence of M-CSF express survivin and lose this expression upon treatment with oxLDL, but not with acLDL or unmodified LDL. This indicates that the survivin downregulation in foam cells is specifically related to the oxidative modification of lipoproteins rather than the intracellular accumulation of cholesteryl esters. Taken together, these data indicate that survivin is a specific marker for newly recruited macrophages in murine atherosclerotic plaques that is lost upon foam cell formation.

The specificity of this marker allows targeting of these cells through the induction of a cytotoxic immunity. A lentiviral vaccination strategy was designed in analogy to tumor cell-targeting vaccines, where it was shown previously that direct administration of similar lentiviral vectors to mice results in the induction of transgene-specific T cell responses^{23,24}. In order to evaluate the potency of the lentiviral injections to induce cytotoxicity against survivin-expressing cells, we measured the level of cell death in a survivin-positive cell line co-cultured with splenocytes from vaccinated mice. The observed induction in cytotoxicity is comparable to results from earlier immunization studies using the same constructs with a different insert²⁴.

Until now, therapeutic options in atherosclerosis have been mainly restricted to plasma lipid lowering, either through dietary changes or pharmaceutical intervention. Recently, experimental immune modulation gained attention and has begun to be used in the clinic. To achieve a more targeted effect than systemic immune suppression, however, an immune response against atherosclerosis-specific antigens is needed. Active or passive immunization with oxLDL or with specific epitopes derived from oxidized phospholipids or apoB-100 has been shown to reduce plaque size^{25,26}. Other immune therapies that successfully attenuated experimental atherosclerosis include promoting

regulatory T cells through oral HSP60 tolerance induction²⁷, skewing the helper T cells towards a regulatory T cell or Th2 response²⁸ and inducing cellular immunity against CD99²⁹ or vascular endothelial growth factor receptor 2 (VEGFR2)³⁰.

Analysis of the atherosclerosis in LDLR^{-/-} mice revealed that vaccination with the 'SURV' lentiviruses resulted in significantly smaller lesions containing more CD3⁺ T cells and CD8⁺ cytotoxic T cells, without affecting other tissues or the mice' health. Moreover, their lesions displayed a trend towards an increase in necrotic core area, which may be a reflection of the lysis of survivin-expressing cells within the plaques and may suggest that 'SURV' vaccination provokes plaque instability over time, an important issue that should be further investigated in the future.

In conclusion, the lentiviral vaccination strategy applied in this murine atherosclerosis model reduced the lesion size and induced the presence of intra-plaque T cells and cytotoxic T cells. This confirms the feasibility of this novel approach, which specifically targets cells in the atherosclerotic plaque with a survivin-specific immune response without significant side effects. This approach is analogous to existing cancer therapies that target survivin-expressing tumor cells. Future studies should further investigate the long-term effects of lentiviral vaccination on plaque size and composition and also study whether therapeutic and prophylactic vaccinations have similar outcomes.

METHODS

Mice

BALB/c mice and *Idlr*^{-/-} mice on a C57BL/6 background were obtained from Harlan (Horst, The Netherlands) and Jackson Laboratory (Bar Harbor, ME), respectively. All animal experiments were approved by the Leiden University and the Vrije Universiteit Brussel.

Survivin expression

Macrophage *survivin* expression was assessed by Affymetrix microarray, as described before²⁰.

Lentivirus production

The murine *survivin* gene (a kind gift from Dr. E.M. Conway, Katholieke Universiteit Leuven, Belgium), was cloned into the pSINT vector, which was previously described²¹. This transfer plasmid ('SURV') or the empty vector ('tNGFR') was co-transfected into the human embryonal kidney (HEK) 293T cell line together with the envelope (pMD.g) and packaging (pCMVΔR8.9) plasmids (a kind gift from Dr. Trono, University of Geneva, Switzerland), using the FuGENE[®] HD transfection reagent (Roche, Mannheim, Germany). Viruses were harvested from the supernatant on three consecutive days and

concentrated by ultracentrifugation, as previously described²¹. Virus titers were determined using the NucleoSpin RNA Virus kit (Macherey-Nagel, Düren, Germany) and the Lenti-X qRT-PCR Titration kit (Clontech, Mountain View, CA).

Mouse immunization and cytotoxicity assay

BALB/c mice were immunized by injection of 10^7 transducing units (TU) of the 'SURV' or 'tNGFR' lentivirus in the footpad. The induction of survivin-specific T cells was analyzed by isolation of spleen cells and re-stimulation for 5 days by co-culture with mitomycin C-treated A20 cells at a responder/stimulator ratio of 1:2. Subsequently, splenocytes were co-cultured for 4 hours with ⁵¹Cr-labeled A20 cells at different effector/target (E/T) ratios. Cell lysis was measured by ⁵¹Cr-release in the supernatant with a β counter as previously described^{21,24}.

Atherosclerosis analysis

Ldlr^{-/-} mice were immunized by injection of 10^7 TU of 'SURV' (n=16) or 'tNGFR' lentivirus (n=15) in the footpad. The effect of the virus injection was assessed by injecting a parallel group with PBS (n=15). The mice were fed a high fat diet (15% cocoa butter, 0.25% cholesterol) for 11 weeks and subsequently sacrificed for analysis as described previously²². Survivin staining was performed using a rabbit polyclonal antibody (Novus Biologicals, Littleton, CO).

Statistical analysis

Statistical analyses were performed using Graphpad Prism (Graphpad Software). Differences between 2 groups were evaluated using a t-test, unless stated otherwise. Values are represented as mean \pm SEM. A *P* value of less than .05 was considered to be statistically significant. All mouse data passed a normality test.

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Chapter 5

Macrophage heterogeneity: relevance and functional implications in atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease involving many cell types with a well-accepted key role for macrophages. A wide array of different properties and functional characteristics are attributed to macrophages present in the atherosclerotic plaque. As an increasing body of evidence strengthens the consensus that macrophages comprise a heterogeneous population, several co-existing subtypes with diverse, even opposing specialties have already been described in fields like parasitology, tumour biology and metabolic disorders. However, macrophage heterogeneity within atherosclerotic lesions has not been studied in detail yet. In this review we will introduce the characteristics of macrophage subsets in other pathologies and address the presence and possible roles of distinct macrophage subtypes in the rapidly evolving field of atherosclerosis. Finally, we make an effort to relate these subtypes to disease progression and explore a number of opportunities for novel diagnostic and therapeutic approaches.

Introduction

Atherosclerosis is a multifactorial disease that afflicts the medium and large sized arteries of the body. Formerly perceived as a mere lipid storage disease, the treatment of atherosclerosis in patients has focused mainly on the lowering of plasma cholesterol. Although effective lipid lowering can be achieved pharmaceutically, cardiovascular disease (e.g. myocardial infarction, stroke) still constitutes the main cause of mortality in modern societies. Since these acute clinical events are largely attributable to complications of atherosclerotic lesions, novel strategies for prevention and management of atherosclerosis are in high demand. From this perspective, the fundamental role of inflammation as a mediator of all stages of atherogenesis is being appreciated to an ever increasing extent. Not only does the chronic inflammatory response associated with atherosclerosis act alongside well-known environmental and genetic risk factors to induce lesion initiation, it also promotes progression and ultimately leads to plaque rupture by reducing lesion stability^{1,2}. Consequently, extensive scientific efforts have been made over the past decade to illuminate the inflammatory processes that underlie atherosclerosis development at the molecular and cellular level.

Atherosclerosis, as an inflammatory condition, involves many cell types and mediators of the immune system. In this regard, monocyte-derived macrophages are imperative as these immune cells partake in innate and adaptive (Th1 and Th2-mediated) immune responses, which are both intricately engaged in atherogenesis². Macrophages do not only form one of the main cellular constituents of the lesions (e.g. as foam cells), they also serve as a major source of inflammatory mediators. Thereby they determine the inflammatory equilibrium (i.e. the balance between pro- and anti-inflammatory factors) during atherogenesis, greatly impact the activation, migration and survival of other cells in the plaque and ultimately affect plaque stability. As such, macrophages are generally accepted as key players in the pathophysiology of atherosclerosis that profoundly shape atherosclerotic plaque development. The morphology of macrophages in atherosclerotic lesions can vary dramatically, from a large quiescent lipid-laden foam cell to a small active inflammatory cell. Recent work in the field of innate immunity has led to the identification of macrophage subsets based on their immune polarization. These subsets portray divergent characteristics and identification of specific markers and their functional role *in vivo* has recently gained strong interest in different fields of inflammatory disease. Knowledge on the *in vivo* contribution of different macrophage populations to the development of atherosclerosis however is scarce. Nevertheless, based on their distinct physiological roles, macrophage subsets are expected to highly determine plaque composition, stability and consequently clinical outcome. Taking approaches with experimental animal models as lead, in this review we will highlight the typical features of macrophage subsets and discuss their relevance and

contribution in a number of pathologies. Moreover, we speculate on the functional characteristics of macrophage subsets in atherosclerosis and address opportunities that may arise in using macrophage subsets for therapeutic targeting and the diagnosis of atherosclerosis.

Macrophage heterogeneity

Cells of the mononuclear phagocyte lineage (i.e. monocytes, macrophages) are characterized by a substantial degree of heterogeneity^{3,4}. Especially macrophages are known to express functionally different phenotypes in response to tissue-derived signals and the immunological micro-environment, thus skewing them towards their many tasks in homeostasis, host defence and pathology⁴⁻⁹. Furthermore, macrophages display notable plasticity, as they are capable of rapidly switching between activation states in response to a specific incentive⁹⁻¹¹. Accordingly, under inflammatory conditions, macrophage populations may initially partake in promoting inflammation and later contribute to its resolution¹²⁻¹⁴. Over the last decade, a conceptual framework has been devised and gradually expanded to account for the polarized functional properties of different macrophage populations. In reflection of the Th1 and Th2 nomenclature in lymphocytes, polarized macrophage subsets are mostly referred to as M1 and M2 macrophages^{5,6,15}.

Classically activated or M1 macrophages are elicited through stimulation with the Th1-cytokine interferon- γ (IFN- γ) alone, through a concomitant stimulus of IFN- γ with microbial products, such as lipopolysaccharide (LPS) or by activation with the Th1 cytokine tumour necrosis factor (TNF). Although these cells show extensive morphological diversity based on their tissue localization, M1 cells share the ability to secrete large quantities of pro-inflammatory cytokines, such as TNF, interleukin (IL)-1 β , IL-6, IL-18 and IL-12^{5,13}. Additionally, they produce high levels of anti-microbial effector molecules (reactive oxygen and nitrogen species; ROS, RNS). These qualities allow M1 macrophages to act as potent inducers and effectors of polarized type I immune responses, consequently associating them with resistance to pathogens and tumours as well as tissue destruction¹⁶⁻¹⁹. Recently, Martinez et al. have suggested a further subdivision of the M1 subset in M1a and M1b macrophages to distinguish between classical and innate activation types¹³. Whereas the latter phenotype is said to be induced through ligation of for instance Toll-like receptors (TLR) by so-called Pathogen-Associated Molecular Patterns (e.g. LPS) and somewhat resembles the classical activation profile, the different phagocytic properties and inability to produce functional amounts of IL-12, sets it apart from classically activated M1a macrophages^{20,21}. However, in our view, macrophages derived through these activation pathways still need to be characterized more thoroughly.

M1	M2a	M2b	M2c
IFN- γ + LPS or TNF- α	IL-4, IL-13	IC + IL-1 β or TLR-ligands	IL-10 (TGF- β , glucocorticoids)
IL-10 ^{low}	IL-23 ^{low}	IL-23 ^{low}	IL-23 ^{low}
TNF	TGF- β	TNF	TGF- β
IL-1 β		IL-1	IL-1Ra
IL-6		IL-6	
IL-18			
NO, ROS (iNOS)	polyamines (Arg-1)		
CCL2, CCL3, CCL4, CCL5	CCL17, CCL18, CCL22, CCL24	CCL1	CCL18
MHC-II	MR	CD86	MR
CD86	decoy IL1-RII		CD163
CD80	SR		SR
IL-1RI			
TLR2/ TLR4			

Table 5.1: Simplified overview of the phenotypical characteristics of M1 and M2 macrophages.

Abbreviations: IFN- γ , interferon- γ ; LPS, lipopolysaccharide; TNF- α , tumour necrosis factor- α ; IL, interleukin; IC, immune complexes; TGF- β , transforming growth factor- β ; GC, glucocorticoids; NO, nitric oxide; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; Arg-1, arginase-1; IL-1Ra, IL-1 receptor antagonist; CCL, chemokine C-C motif ligand; MHC-II, major histocompatibility complex class II; CD, cluster of differentiation; IL-1RI, IL-1 receptor I; MR, mannose receptor; SR, scavenger receptor.

In recent years, the term M2 has served as a generic name indicating the various forms of alternatively activated or anti-inflammatory macrophages^{4,22}. As opposed to classical activation by IFN- γ , alternative activation of macrophages is induced by several different stimuli and a subdivision of M2 cells has been made accordingly. M2a cells are generated by stimulation with the Th2-cytokines IL-4 and IL-13²³; M2b cells correspond to concomitant stimulation with immune complexes and either LPS or IL-1 β (initially termed type II activation)^{24,25}, whereas M2c macrophages are triggered by IL-10 (and to some extent by transforming growth factor- β (TGF- β) or glucocorticoids). Functionally, M2 macrophages are integrated in type II immune responses, accounting for the attenuation of excessive Th1-mediated inflammation, tissue remodeling, allergy, tumour progression and host defence against extracellular parasites^{5,7,15,22}. Although the diversity between M2 macrophages is apparent^{14,26}, usage of this term is justified by the fact that various forms of M2 macrophages do share a number of phenotypical similarities⁴. Generally, M2 cells produce low amounts of pro-inflammatory factors (e.g. IL-12), in contrast to an increased production of anti-inflammatory mediators such as IL-10 and the pro-fibrotic factor TGF- β . However, in this regard the M2b subset stands out by the fact that it is characterized by a higher inflammatory cytokine production (e.g. TNF, IL-1 and IL-6) and does not show induction of arginase-1 (Arg-1) or TGF- β . On the other hand, these cells still clearly portray a high IL-10 and low IL-12 profile, which warrants their M2 classification¹⁹. M2 macrophages additionally display high levels of scavenger receptor activity and express a chemokine repertoire that is dissimilar from

that of M1 macrophages^{4,27}. Finally, in M2a macrophages as opposed to M1 cells, the arginine metabolism is generally shifted to the production of proline and polyamines through activity of the profibrotic enzyme Arg-1. This shift inversely correlates with inducible nitric oxide synthase (iNOS)-mediated nitric oxide (NO) production²⁸. A simplified summary of the characteristics of each respective phenotype is presented in Table 5.1.

Macrophage heterogeneity in health and disease

Although the contribution of different macrophage subsets to atherosclerosis is very scarcely studied^{29,30}, the concept of macrophage heterogeneity has already been well explored in other pathologies with macrophage-related origins (i.e. infectious disease, cancer and metabolic disorders). Logically, knowledge from lessons learnt in other fields might be useful when applied to atherosclerosis research, provided that the underlying mechanism by which macrophage dysfunction causes morbidity is taken into account³¹. Often, when conditions manifest with a disturbed inflammatory balance, immune cells (e.g. macrophages) are either unable to react to environmental changes or by contrast exaggerate their response to such events. In these situations, one macrophage subset may be favoured over another. It is very likely that such an imbalance also plays a role in the pathogenesis of atherosclerosis, making it very relevant to study the dynamics in subset distribution throughout the course of this disease. Thereby, we might better appreciate how each subset contributes to the many specialized macrophage functions in the atherosclerotic plaque.

Macrophage heterogeneity in infectious disease

Regulation of the delicate balance between M1 and M2 type polarization has been shown to be critical in host defence against many types of pathogens. Based on the analysis of host transcriptomes in several studies, Jenner et al. identified a set of genes that together comprise the 'common host response' of innate immune cells in reaction to a range of bacteria, viruses and fungi³². Especially monocyte-derived macrophages seem to develop a common pattern of gene expression when challenged by bacteria³³. Interestingly, Benoit et al. reported more recently that this shared response to bacterial infection is mainly associated with increased expression of genes that are involved in polarization towards a functional M1 program. More specifically, these include the genes that encode for TNF, IL-6, IL-12, iNOS and monocyte chemoattractant protein-1 (MCP-1), also known as chemokine ligand 2 (CCL2)³⁴. Up-regulation of these genes partially accounts for the enhanced microbial killing activity that is regarded as a typical characteristic of M1 macrophages. Thus, by augmenting the immediate resistance to bacteria, M1 polarization promotes immunity and serves a protective role in host defence during acute infectious disease. However, an extended period of M1 skewing

may lead to a deleterious outcome for the host if not kept in check. This is illustrated by the correlation between the amount of circulating M1-type cytokines and increased sepsis severity in a human population³⁵. In their review, Benoit et al. further provide a detailed account of the approaches that several bacterial pathogens have developed to hamper the M1 polarization of macrophages in an attempt to endure in their surroundings. Different strategies include inhibition of the oxidative microbicidal capacity of macrophages (NO release), inhibition of the expression and secretion of M1-type cytokines (primarily IL-12 and TNF), the release of virulence factors that interfere with M1-associated cellular signaling pathways and finally, direct promotion of M2 polarization³⁴. Skewing of macrophages towards an M2 phenotype is associated with the continual presence of bacteria in tissues and the conversion of some infectious diseases to a chronic state. For instance, replication of *Coxiella burnetii*, the causative agent of Q fever, was shown to be related to macrophages expressing a M2 signature that featured up-regulation of Arg-1, YM-1 and the mannose receptor (MR) and down-regulation of iNOS and M1-type cytokines. Activation by IFN- γ however, inhibits replication of this pathogen and reprograms macrophages towards M1³⁶⁻³⁸. Additionally, Whipple's disease, which is caused by *Tropheryma whippelii*, has also been linked to M2 polarization. Whereas this pathogen is eradicated in an M1-driven immunological micro-environment, it actively replicates in a M2 setting³⁹. Notably, these are only a few examples of the growing list of pathogens that take advantage of changes in macrophage activation profiles.

Over the last few years, studies on macrophage function in parasite infections have also yielded great knowledge on the existence and function of different macrophage subsets in disease states⁴⁰. In general, infection of a host organism with both intracellular and extracellular parasites triggers a specific inflammatory response. As a first line of defence preceding adaptive immunity, macrophages mediate both anti-parasitic immunity and parasite clearance by phagocytosis and immunosuppression. A pro-inflammatory response by M1 macrophages, targeted at parasite elimination, is crucial for limiting parasite growth during the acute phase of the infection. After this initial immune reaction, a type II inflammatory response ensues aiming to resolve the inflammatory process. Any disturbance of the equilibrium between these two phases will consequently worsen the outcome of the infection. Whereas lack of the initial M1 response would result in uncontrolled parasite colonisation, an insufficient M2 response would lead to more efficient parasite killing that is however accompanied by excessive systemic inflammation. The subsequent anaemia and liver damage by ROS might eventually result in host death. The infection with *Trypanosoma congolense*, the African parasite responsible for sleeping sickness, exemplifies the influence this fragile balance of macrophage polarization has on parasite infection and even how this parasite can exploit these changes in the inflammatory response. Upon infection, this extracellular parasite will primarily settle in the liver, spleen and brain. It is covered by variant-specific surface glycoproteins (VSG) that are very immunogenic and cause a T-

cell dependent B-cell response. However, by frequently transforming their molecular structure, these VSGs avoid targeting by the adaptive immune response and make vaccination against this parasite impossible⁴¹. Trypanosomes can be phagocytosed upon binding of antibodies to VSG, but this binding simultaneously releases these molecules to the circulation as soluble VSG (sVSG). Initially, sVSG modulate macrophage function towards systemic macrophage activation with M1 characteristics, which is sustained by T-cell-derived IFN- γ and characterized by production of the trypanotoxic agents NO and TNF. Following this early response, a transition towards a type II response takes place that mainly involves M2a macrophages and is characterized by high plasma levels of IL-10, IL-4 and IL-13. The end result is tolerance towards the parasite with long host survival and limited tissue damage⁴²⁻⁴⁴. However, C57BL/6 mice lacking either TNF⁴⁵ or IL-10⁴⁶ signaling are both susceptible to trypanosomes and die upon infection, by excessive parasite growth or by an IFN- γ mediated shock syndrome respectively⁴⁷. Concluding, fighting bacterial and parasitic infections necessitates an adequate regulation of the balance between M1 and M2 macrophages to ensure proper microbial killing. Increasing our knowledge on this part could eventually lead to more efficient ways to treat infections. Although selectively promoting or inhibiting M1 or M2 phenotypes at the right moment is likely to enhance the effectiveness of the inflammatory response, one should be aware of the risks involved with intervening in this delicate balance. Overstimulation of the M1 phenotype for a prolonged period will result in excessive tissue damage. Preventing an appropriate M2 macrophage response from counteracting M1-mediated inflammation might pose a similar hazard. Promotion of the M2 phenotype on the other hand will probably facilitate the survival and growth of pathogens. Additionally, the way in which the M1/M2 balance shifts during the course of different infections may prove another pitfall. Temporal variability in the macrophage response to distinct micro-organisms would hamper large-scale application of macrophage-based approaches. Furthermore, this would necessitate detailed knowledge of the macrophage kinetics in certain types of infection if successful timing of such a therapeutic approach were to be achieved.

Tumour associated macrophages

Another model illustrating the relevance of the macrophage M1/M2 paradigm in pathologic situations concerns the macrophages that are found within tumours⁴⁸. A tumour micro-environment not only exists of malignant cells but also includes stroma and cells belonging to the immune system. The latter are part of a system of immune surveillance that aims to eliminate tumour cells through innate and adaptive responses⁴⁹. Tumour cells evade this targeting through their genetic variability, active immune suppression and secretion of tolerogenic effectors. Because of their contribution to both the targeting and the protection of the tumour cells, tumour associated macrophages (TAMs) are a great example of the possible implications of macrophage heterogeneity in vivo^{8,50,51}. Moreover, their ambivalent functional

characteristics clearly show the caveats to be dealt with when contemplating macrophage directed therapies⁵².

Macrophages that are attracted to tumour sites by factors like M-CSF and CCL2^{53,54}, are initially cytotoxic through secretion of ROS and slow down tumour cell proliferation. This M1 phenotype induces an inflammatory environment that attracts and activates cells of the adaptive immune system^{55,56}. However, with sustained activation, these cells increase the risk of DNA damage and tissue structure abnormalities, thus promoting carcinogenesis⁵⁷. However, tumour cells that are lysed by these M1 macrophages release factors including TGF- β and sphingosine-1-phosphate, which reprogram the macrophages towards an alternatively activated phenotype⁵⁸. Subsequently, these M2 macrophages will temper the inflammatory response and create a more tolerogenic environment by secreting IL-10 and TGF- β ⁵⁸. Moreover, these cells mediate cell proliferation and tissue remodelling through arginase induction and tumour invasiveness through secretion of chitinase-like proteins, further contributing to tumour development. Another important feature of the alternatively activated TAMs is their role in the formation and remodeling of new blood vessels in the tumour area by releasing both pro-angiogenic factors such as vascular endothelial growth factor (VEGF), TNF, IL-8 and basic fibroblast growth factor (bFGF) as well as angiogenesis modulating enzymes such as matrixmetalloproteinase (MMP)-2, MMP-7, MMP-9, MMP-12 and cyclooxygenase (COX)-2⁵⁹. As the rapid tumour growth demands increasing perfusion to prevent hypoxia and subsequent necrosis, angiogenesis is essential for disease progression⁶⁰.

The formation of tumour promoting TAMs has been linked directly to the nuclear factor- κ B (NF- κ B) signal transduction pathway, which activates transcription factors that play an essential role in regulating inflammation and immunity⁶¹. Especially the NF- κ B subunit p50 has been identified as an important regulator of macrophage polarization in tumours. The observation that TAMs are defective in their production of the NF- κ B target gene IL-12 but rather show higher levels of IL-10 indicated that a regulation at the level of NF- κ B could be expected⁶². It was shown that p50 can form inhibitory homodimers that are able to bind the NF- κ B consensus binding sites, thus repressing transcription of a subset of NF- κ B-dependent inflammatory genes⁶³. Indeed, TAMs show tolerance toward both LPS and other pro-inflammatory signals as indicated by a defective IL-12, IL-6 and TNF expression and relatively enhanced IL-10 expression⁶⁴. Moreover, TAMs were characterized by massive nuclear overexpression of p50. Interestingly, the anti-inflammatory phenotype of TAMs was reversed in macrophages lacking p50 and both p50-deficient mice and wildtype mice that received p50-deficient bone marrow displayed a delayed tumour progression with increased survival⁶⁴. Hence, activation of the NF- κ B subunit p50 appears to induce an M2 phenotype in TAMs. Continuing on the role of NF- κ B signaling in regulating TAM function, it was more recently shown that the main kinase mediating NF- κ B activation, inhibitor of NF- κ B kinase 2 (IKK2 or IKK β), inhibits M1 differentiation of macrophages by affecting signal

transducer and activator of transcription-1 (STAT1) signaling. Consequently, macrophage-specific deletion of IKK2 led to a more pronounced M1 phenotype, characterized by high IL-12, iNOS and MHC-II expression and low levels of IL-10^{65,66}. This phenotypic shift resulted in macrophages that were better capable of actively eliminating tumour cells, both directly by enhanced production of oxygen radicals and indirectly through IL-12-dependent natural killer (NK)-cell anti-tumour activity⁶⁶. Thus, NF- κ B activation is intricately involved in the induction of anti-inflammatory tumour repressive TAMs at different levels.

Since high TAM density in tumours is correlated with a poor prognosis^{59,67}, blocking the attraction of macrophages to the tumour might seem a promising therapeutic target. Indeed, in experimental models this approach showed to inhibit further tumour growth⁶⁸⁻⁷¹. However, such a strategy would also rule out the potential of attracting more M1 polarized macrophages to engage in tumour cell lysis and promote an anti-tumour immune reaction. Therefore, induction of macrophage polarization towards an M1 phenotype would be a more desirable treatment option than merely interfering with macrophage attraction to tumours. For now, the major challenge will be to find techniques that locally and specifically skew macrophages to the desired phenotype. Until such methods are developed, it remains unclear if any phenotype promoting or targeting treatment will suffice as a stand-alone therapy or will merely serve as an adjuvant therapeutic option.

Macrophage heterogeneity in obesity and insulin resistance

In recent years, mounting evidence suggests that low-grade inflammation contributes to the development of obesity and progression to insulin resistance⁷². More specifically, recent work has recognized adipose tissue macrophages (ATM) as the main source of pro-inflammatory mediators in adipose tissue and has correlated their function with the degree of insulin resistance⁷³. Interestingly, in lean mice, resident ATMs exhibit characteristics of M2a macrophages with expression of Arg-1 and IL-10⁷⁴. From a functional perspective, this IL-10 production was shown to protect adipocytes from TNF-induced insulin resistance⁷⁴, advocating these M2 macrophages as being protective in obesity. Rather than altering macrophage activation state, the induction of obesity through a high fat diet superimposes a new population of macrophages to adipose tissue that possess an M1-polarized phenotype with expression of pro-inflammatory markers such as iNOS and TNF⁷⁵. With progressive obesity, these M1 macrophages accumulate in adipose tissue through recruitment by MCP-1 dependent mechanisms. Absence of MCP-1 or its receptor CCR2 abolishes migration of macrophages into adipose tissue, thereby preventing adipose tissue inflammation and improving insulin resistance^{76,77}.

The M2 phenotype of ATMs in lean mice was subsequently shown to be maintained by the nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ). Induction of

alternatively activated macrophages by IL-4 was reduced in the absence of PPAR γ and was accompanied by reduced Arg-1 activity, decreased suppression of IL-6 and inhibition of fatty acid oxidation in macrophages. Furthermore, macrophage-specific deletion of PPAR γ in vivo reduced the M2 phenotype of ATMs, impaired adipocyte function by reducing mitochondrial oxidation and ultimately resulted in enhanced obesity and insulin resistance⁷⁸. In line with these findings, Hevener et al. showed that macrophage-specific PPAR γ deficiency propagates impaired insulin sensitivity in muscle, liver and adipose tissue and worsens insulin resistance upon high fat feeding of mice⁷⁹. Thus, PPAR γ is necessary for sustaining the M2 phenotype in adipose tissue of lean individuals and thereby protects against obesity and insulin resistance.

Remarkably, contrary to ATMs, disruption of PPAR γ does not affect Kupffer cells, the liver's resident macrophages. In these cells, it is the transcriptional activation of another nuclear receptor known as PPAR δ by mono-unsaturated fatty acids that induces an alternative activation state. Analogous to PPAR γ deficiency, abrogation of PPAR δ impairs the alternative activation of Kupffer cells, thereby causing hepatic dysfunction, diet-induced insulin resistance and obesity⁸⁰. Similar observations were described by Kang et al.⁸¹, demonstrating that myeloid-specific PPAR δ deficiency leads to insulin resistance and severe steatohepatitis. Hence, macrophage polarization in adipose tissue and liver is chiefly regulated by PPAR γ and PPAR δ respectively and provides an important incentive in the pathogenesis of metabolic diseases. Whereas the anti-inflammatory phenotype displayed by resident ATMs in healthy conditions appears to be protective, the obesity-induced recruitment of pro-inflammatory M1 macrophages alters the macrophage polarization balance in adipose tissue and aggravates insulin resistance.

The macrophage as central player in atherosclerosis initiation and progression

From the above it is evident that macrophage polarization is a main determinant in the aetiology of many different diseases. In terms of atherosclerosis, this is no different. The major initiating events in atherosclerosis development encompass endothelial dysfunction and modification of low-density lipoprotein (LDL)⁸². Aberrant blood flow renders specific areas of the vessel wall more susceptible to atherogenesis, since the altered haemodynamics increase permeability of the endothelial barrier to macromolecules such as LDL¹. These molecules subsequently accumulate in the subendothelial matrix, where they undergo modification through oxidative or enzymatic processes. Retention of these modified lipoproteins exerts a pro-inflammatory stimulus on the micro-environment, thereby promoting expression of a range of adhesion molecules (e.g. vascular cell adhesion molecule 1; VCAM-1), chemotactic proteins (e.g. MCP-1) and growth factors (e.g. macrophage-colony stimulating factor; M-CSF) by the overlying endothelium⁸³. This in turn mediates

leukocyte recruitment and monocyte migration into the intima at lesion prone sites¹. Upon M-CSF-induced differentiation, monocyte-derived macrophages will start to internalize substantial amounts of modified LDL (mLDL) via scavenger receptors (SR)^{1,83,84}. Although this process of foam cell formation may at first serve a protective role in removing pro-inflammatory modified lipids and apoptotic cell debris from the vessel wall, the increasing build-up of these cells ultimately leads to the development of fatty streaks. The progression of a clinically insignificant fatty streak to a more complex lesion is hallmarked by the migration of smooth muscle cells (SMC) from the media to the subendothelial space of the vessel wall^{1,83}. In conjunction with a continuous influx of immune cells, these SMCs can proliferate within the lesion, contribute to foam cell formation but primarily secrete extracellular matrix components. Concurrently, apoptotic foam cells that are improperly scavenged will contribute their lipid-filled contents to the growing necrotic core of the plaque⁸². Ultimately, an advanced lesion is formed, in which the lipid-rich necrotic core is shielded from the lumen by a fibrous cap of SMCs and extracellular matrix. In disease progression to clinical manifestation, rupture of this fibrous cap is generally thought to elicit thrombosis, as exposure of plaque lipids and tissue factor to blood components will consequently initiate the coagulation cascade^{1,85}. The physical integrity of the fibrous cap therefore is vital for plaque stability. Interestingly, the balance between trophic and degenerative factors that determine fibrous cap thickness can be profoundly affected by both cellular and humoral factors of immunity. While IFN- γ , for instance, inhibits extracellular matrix production by SMCs, plaque macrophages enable the degradation of extracellular matrix proteins by the production of MMPs^{82,86}. As such, the macrophage is acknowledged as a key player in atherosclerosis in the regulation of plaque development and stability (see Figure 5.1).

Macrophage heterogeneity in atherosclerosis

As detailed above, the functional characterization of macrophage heterogeneity in numerous different macrophage-related pathologies has been ongoing for several years now. Based on their inflammatory characteristics, M1 macrophages can be expected to promote atherosclerosis development, while M2 macrophages in general may be considered to be protective in this regard. However, to this point, the functional contribution of individual macrophage subsets to atherosclerosis has barely been studied, despite the fact that macrophage heterogeneity in human atherosclerotic lesions was already demonstrated early on. Using differentiation markers and stainings for lipids and lysosomal phosphatase, van der Wal and co-workers reported that more centrally localized macrophages in lesions have more differentiated and matured characteristics, than their counterparts in the superficial layers of these plaques⁸⁷. This

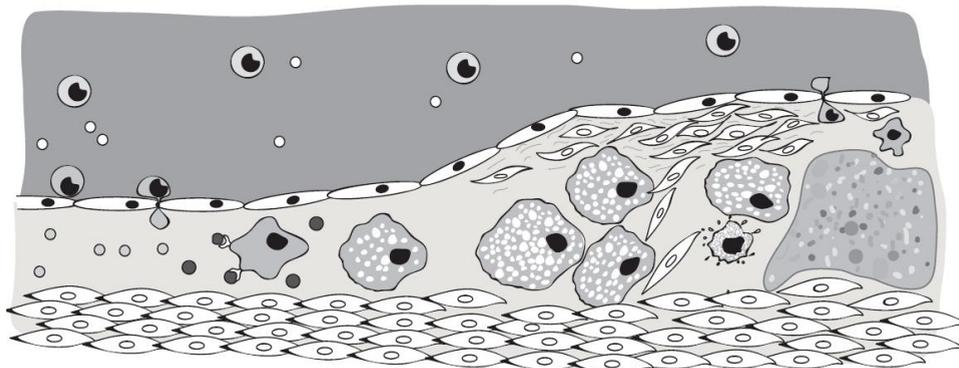


Figure 5.1: Macrophages are paramount to plaque development:

A schematic outline of the lumen and vessel wall is shown in which, from left to right, the development of an atherosclerotic plaque is depicted. Early phases of the disease involve the subendothelial retention and accumulation of LDL. These particles are subsequently modified and induce endothelial activation and chemokine secretion. In response, attracted monocytes migrate into the vessel wall where they differentiate into macrophages. These macrophages commence continual scavenging of these modified lipoproteins, which transforms them into foam cells that characteristically contain intracellular lipid droplets. Persisting foam cell accumulation eventually results in the formation of a fatty streak. Macrophages in atherosclerotic lesions also secrete lipid modifying enzymes, which promote further LDL oxidation, and chemokines and cytokines that control the inflammatory balance and regulate recruitment, activation and proliferation of cells. Upon further progression of atherogenesis, T-cells are attracted and activated which propagate the chronic inflammatory state of the lesion. When foam cells die through apoptosis, improper scavenging of these cells leads to secondary necrosis and the release of cellular debris (i.e. scavenged lipids) to the growing lipid-rich necrotic core of the lesion. As smooth muscle cells proliferate and migrate, they form a fibrotic cap that encloses the lesion and its necrotic core. In time, inflammatory factors compromise the integrity of this fibrous cap, resulting in thrombus formation when rupture of the fibrous cap exposes thrombogenic plaque contents to the blood. This process of rupture is promoted by the local recruitment of macrophages, producing matrix-degrading enzymes. Abbreviations: LDL, low density lipoproteins.

implied functional differences between macrophage populations within the same lesions. Similarly, using a set of macrophage specific monoclonal antibodies, it was shown that macrophages in murine atherosclerotic lesions show a large degree of heterogeneity as well⁸⁸. More recently, Waldo et al. confirmed the heterogeneity of human macrophages in atherosclerotic lesions yet again by using CD14 positivity to discriminate between subsets²⁹. Thus, heterogeneity of macrophages has been well established in human and mouse atherosclerotic lesions. However, the amount of data that provide a direct functional link between the M1 and M2 subsets and atherosclerotic lesions is limited.

Recently, Bouhlef et al.³⁰ were the first to describe the expression of M1 and M2 markers in atherosclerotic lesions. Concretely, they demonstrated M1-associated marker genes such as MCP-1, IL-6 and TNF and MR, CD163, IL-10 and CCL18, markers of M2 macrophages, to be expressed in human atherosclerotic lesions. Moreover, they

established a direct correlation between the expression of M2 markers and that of PPAR γ , indicating a role for PPAR γ in regulating M2 differentiation in atherosclerosis, similar to what was reported previously in murine diet-induced obesity (see above)⁷⁸. Notably, through cell cultures they were able to confirm that PPAR γ activation polarizes human macrophages towards an M2 phenotype. Interestingly, patients treated with PPAR γ agonists showed a more pronounced M2 phenotype in their circulating mononuclear cells, whereas M2 marker expression in atherosclerotic lesions did not change. Although certain questions regarding the therapeutic benefit of this approach remain unanswered, this paper offers an interesting functional perspective by identifying the expression and localization of certain subset markers in atherosclerosis.

More recently, Boyle et al. reported intraplaque haemorrhage, a main contributor to lesional development and instability, to induce a novel protective macrophage phenotype in atherosclerotic plaques of human coronary arteries⁸⁹. Primarily defined by high levels of the scavenger receptor CD163, these macrophages use this surface molecule to bind and clear haemoglobin-haptoglobin complexes from the vessel wall. As a result, CD163^{high} macrophages secrete a number of anti-oxidant and anti-inflammatory mediators (e.g. IL-10 and haeme oxygenase-1) in an attempt to counter the atherogenic effects of plaque haemorrhage⁸⁹. However, in spite of its beneficial qualities, this phenotype is by no means fully atheroprotective as is illustrated by the use of post-mortem specimens of culprit lesions in this study. This can be partly explained by the fact that even lesions with substantial haemorrhage predominantly featured pro-inflammatory macrophages, whereas plaques devoid of haemorrhage lacked CD163^{high} macrophages altogether. As such, the relatively small size of the CD163^{high} subpopulation seems to limit its impact on the surrounding tissue. Taken together, these data imply that CD163 might in fact be a suitable marker for high-risk atherosclerotic plaques. This notion is confirmed by Papaspyridonos et al.⁹⁰, who found the expression of CD163 to be upregulated almost sevenfold in unstable plaque regions as compared to stable areas. Interestingly, the presence of neutralizing IL-10 antibodies prevented the development of CD163^{high} macrophages in vitro, hereby showing a role for IL-10 in inducing this phenotype. Moreover, glucocorticosteroids have also been found to elicit CD163, be it to a lesser extent than haemoglobin-haptoglobin complexes or IL-10, which further enhances the analogy to the M2c subtype⁹¹. Unfortunately, to our knowledge, this is the only report to date that directly associates an M1/M2 subset marker with the functional phenotype (i.e. stability) of an atherosclerotic lesion.

Macrophage subset effector function and atherosclerosis

From a theoretical perspective and knowing the many specialized functions macrophages exert in all stages of atherogenesis, it can be expected that specific features of the M1 and M2 polarized macrophage subsets described before in this review contribute differently to plaque development. First of all, a range of cytokines

that has been associated to macrophage polarization (see table I) has been the subject of extensive atherosclerosis research (as reviewed by Tedgui and Mallat, Kleemann et al.)^{92,93}. In general, cytokines that are specifically produced by M1 macrophages have a proatherogenic effect. Using knockout, transgenic, treatment or inhibitory approaches, it is firmly established that cytokines such as TNF, IL-1 and IL-12 promote disease progression in different animal models of atherosclerosis. In contrast, M2 associated cytokines are either anti-atherogenic (e.g. IL-10), or combine anti-atherogenic characteristics with profibrotic properties (e.g. TGF- β). These cytokine data are in line with the concept that M1 macrophages promote atherosclerosis, whilst M2 macrophages inhibit plaque growth and mediate plaque stability.

For chemokine patterns associated with macrophage polarization⁴, the story is more complex. M1 inducers (e.g. IFN- γ and TLR-ligands) usually act through signal transduction pathways such as NF- κ B and STAT-1 to evoke transcription of inflammatory chemokines (CCL2, 3, 4 and 5 amongst others)^{94,95}. Moreover, both CCL5 (RANTES) and CCL2 have been studied to some extent in atherosclerosis⁹⁶. While CCL5 blockade reduces atherosclerosis⁹⁷, inhibition of its two receptors (i.e. CCR1 and CCR5) has opposing effects. CCR5 acts pro-atherogenic as genetic deletion of this receptor reduces atherogenesis and inhibits Th1 responses⁹⁸⁻¹⁰⁰, while in contrast CCR1-deficient mice show accelerated atherosclerosis, with IFN- γ being an important mediator^{101,102}. CCL2 and its receptor CCR2 seem to have a more clear-cut role in promoting atherosclerosis¹⁰³⁻¹⁰⁵ by regulating the specific recruitment of inflammatory monocytes to atherosclerotic lesions¹⁰⁶. Interestingly, M2 stimuli (e.g. IL-4, IL-13 and IL-10) interfere with the general expression of M1-associated chemokines through inhibition of the same pathways that mediate M1 responses. Moreover, these signals elicit expression of a set of chemokines that is linked to type II immune responses⁴. As such, M2a macrophages display expression of CCL17, 18, 22 and 24 for instance^{107,108}, whereas both the M2a and M2c subsets express CCL18¹⁰⁹. Many other chemokines are currently still in need of better association with particular macrophage subsets or have yet to be investigated for their role in atherosclerosis development in general.

In accordance with the atherogenic function of M1 macrophages and the putative atheroprotective effect of M2 macrophages, the ways in which these subtypes utilize the common substrate arginine also influence lesion fate. As mentioned before, M2 skewing induces Arg-1 expression that enables the conversion of arginine to collagen precursors such as ornithine and subsequently proline and polyamines. In macrophages, this diverts arginine metabolism away from M1-associated iNOS-derived NO synthesis. Hereby, M2 macrophages may contribute to enhancing the physical integrity of the fibrous cap, thus promoting plaque stability, and reduce atherogenesis. This was recently substantiated by genetic analysis of two rabbit strains with high and low susceptibility for atherosclerosis, showing that the rabbits which had reduced atherosclerosis development were characterized by elevated levels of Arg-1 activity, regulated through a polymorphism in the 3'-untranslated region¹¹⁰.

However, certain characteristics of M2 polarized macrophages, do not conform to the straightforward distinction between pro- and anti-inflammatory properties that has been put forth by the M1/M2 paradigm. Firstly, M2 macrophages are highly phagocytic and express increased levels of scavenging receptors such as scavenger receptor class A (SR-A) and CD36. Both these receptors have originally been described as major mediators of foam cell formation and promoters of atherosclerosis in general. However, recent studies have questioned the function of these scavenger receptors as being mere facilitators of lipid uptake and even advocated them as mediators of inflammation in atherogenesis^{111,112}. Accordingly, M2 polarization may very well promote foam cell formation and thereby lesion growth, but may also add to a lesion's inflammatory burden through changes in scavenger receptor activity. Secondly, as described above, through tumour biology research it is well known that M2 macrophages promote angiogenesis¹¹³. Especially the so-called angiogenic switch, which concerns the induction of tumour vasculature that is conditional for tumour growth and disease progression, is considered to be at least partly mediated by TAMs^{113,114}. Hypothetically, M2 macrophages in the vessel wall may similarly promote microvessel growth into atherosclerotic lesions. However, as these newly formed vessels often lack proper structural integrity, they can give rise to intraplaque haemorrhage that in turn promotes lesion development and compromises plaque stability¹¹⁵.

Hence, despite the fact that the cytokine and chemokine repertoires raise high hopes for M2 macrophages as therapeutic targets in atherosclerosis, not all functional characteristics exerted by these cells are by definition beneficial. Even so, it is important to recognize that any undesirable effects need not necessarily concern all M2 subtypes, since these factors might just as well be attributable to a single M2 phenotype. Selecting an appropriate subset that promotes the desired properties will therefore be crucial for the development of diagnostic and treatment options. In this view, characterizing the temporal and spatial balance between macrophage subsets and its underlying mechanisms provides an interesting challenge that might open doors towards new methods of atherosclerosis diagnosis and therapy, namely by targeting, favouring or inhibiting specific cell types and functions. That said, one of the major obstacles that will have to be overcome when studying macrophage polarization in atherosclerosis is the current lack of a good readout system that uses markers to specifically distinguish the different phenotypes. Despite the existence of many accounts listing typical genes, proteins and metabolic products associated with the different subpopulations, these markers are usually more abundant in, but rarely specific for, a particular subset. To be able to recognize the different subsets within one tissue, a more clear-cut readout system would be advantageous. Moreover, most of the M1 and M2 associated markers have not been tested for their expression and intralésional localization in either human or experimental atherosclerosis models. Hence, to better understand the functions exerted by the different subsets, their

characteristics should be examined further on a molecular and phenotypic level. In addition, since atherosclerotic plaques contain a unique macrophage stimulus in mLDL, it will be of great interest to study if this at all alters the response of macrophages to the typical M1 and M2 inducing signals. Still, lipid-laden foam cells could possibly be seen as yet another phenotype with characteristics distinct from the current M1 and M2 phenotypes. Finally, since different subsets are present in the plaque and probably co-exist for extended periods within one tissue, it would also be valuable to study the interplay between macrophages with different phenotypes. An important feature to investigate here is the ability of macrophages to redifferentiate into another phenotype in response to stimuli from their environment¹⁰.

Macrophage polarizing cytokines in atherosclerosis

To actually study the role of the different macrophage subsets in atherogenesis, specific gene targeting in different murine models of atherosclerosis remains the method of choice. Up until now, a broad range of studies deleting or over-expressing cytokines with key functions in macrophage polarization have been performed in both ApoE^{-/-} and LDLR^{-/-} mice. However these investigations never focused directly on how a specific intervention affects macrophage phenotype in the plaque, but rather on atherosclerosis development in general⁹³.

In the setting of atherosclerosis, IL-10 is one of the best studied polarizing cytokines. As described earlier, IL-10 is involved in M2c polarization, but has also been shown in several studies to exert atheroprotective qualities, especially in early atherogenesis. Several knockout models for this cytokine describe a significant increase in lesion size and inflammation, which is accompanied by reduced plaque stability and an increased influx of T-cells^{116,117}. In line with these findings, different transgenic models overexpressing IL-10 demonstrate a reduced lesion size¹¹⁸⁻¹²⁰. However, since IL-10 also favours Th2 responses and has anti-inflammatory effects on most other cell types present in the atherosclerotic plaque, we are currently unable to appreciate exactly how much of its anti-atherogenic effects is mediated by macrophage phenotype skewing. For this reason, additional studies addressing IL-10 signaling specifically in macrophages should be performed that clearly dissect the inability of these macrophages to respond to or produce and secrete IL-10 from that of other immune cells. Moreover, such studies may provide novel insights into the expression of markers for macrophage subsets in atherosclerosis and their dependence on polarizing signals.

Another polarizing cytokine, IL-4, rather induces an M2a phenotype in macrophages. Although these are potentially anti-atherogenic, IL-4 is also known to be involved in the up-regulation of VCAM-1^{121,122} and MCP-1^{123,124} in endothelial cells, both important positive mediators of atherogenesis. This possibly blurs the effect of studies investigating the effects of IL-4 on atherosclerosis in mouse models and may be the reason why ApoE^{-/-}IL-4^{-/-} double KO mice or LDLR^{-/-} mice transplanted with IL-4^{-/-} bone

marrow initially rather showed an atherosclerosis promoting effect in a time and site specific manner^{125,126}. However, an initial study on IL-4 deficiency in C57BL/6 mice showed no effect¹²⁷ and more recently King et al. performed an extensive study with both ApoE^{-/-} and LDLR^{-/-} mice with IL-4 knockout or treatment approaches and also found no effect on atherosclerotic lesion formation¹²⁸. These results raise questions concerning the contribution of M2a macrophages to atherogenesis, but may also be caused by the aforementioned opposing effects on different cellular populations in the plaques. Finally, contradicting evidence on IL-4 may even represent differences between mouse and man.

Comparable to the difficulties that arise in distinguishing the M2 and Th2 promoting capacities of IL-10, the effect of macrophage polarization by IFN- γ on atherogenesis is also difficult to reveal, since this pro-inflammatory cytokine affects both M1 macrophages and Th1 cells. Most experimental studies performed clearly show a reduction in lesion size upon absence of this cytokine or its receptor¹²⁹⁻¹³¹ and report accelerated atherosclerosis upon intraperitoneal recombinant IFN- γ administration¹³². These data confirm the atherogenic effect of this M1 polarizing cytokine. However, as is the case with IL-10, it is still unclear which cell types mediate the observed effects and whether genetic modification or treatment with IFN- γ actually altered macrophage populations and functionality in the plaques of these mice.

Last but not least, the pro-inflammatory TNF is probably one of the most difficult macrophage polarizing cytokines to study, due to its broad range of activities in atherosclerosis. Besides promoting an M1 phenotype, it also induces cell attraction through upregulation of intercellular adhesion molecule 1 (ICAM-1), VCAM-1 and MCP-1 in endothelial cells and influences foam cell formation through regulation of scavenger receptor mediated LDL uptake¹³³. This abundance in TNF's possible interactions within several key stages of atherogenesis complicates the comparison of different experimental models, making it very difficult at this moment in time to distil a concrete theory on the effect of TNF-induced M1 macrophages on the atherosclerotic plaque. In general however, absence of TNF has been found to decrease atherosclerotic lesion size and progression¹³³⁻¹³⁶.

In conclusion, the data above clearly shows association of polarizing cytokines with atherosclerosis development, as depicted in Figure 5.2 as well. However, studies on the direct effect of M1/M2 skewing on atherosclerosis in experimental models are lacking and more specifically, identification of subsets and their functional roles *in vivo* are scarce. To investigate this further, it may be important to realize that an approach of phenotypic switch should be preferred over the selective depletion of a specific subset. Targeting specific cell types with a cell death inducing method may lead to an accumulation of cell debris in the plaque, which causes plaque instability, especially in the more advanced lesions¹³⁷. Another obstacle to keep in mind when further studying this matter is that both macrophage heterogeneity¹³⁸ and atherogenesis^{139,140} are known to be differently regulated between species. Therefore, the major challenge in

the end will still be to translate novel findings from murine atherosclerosis models to the human situation.

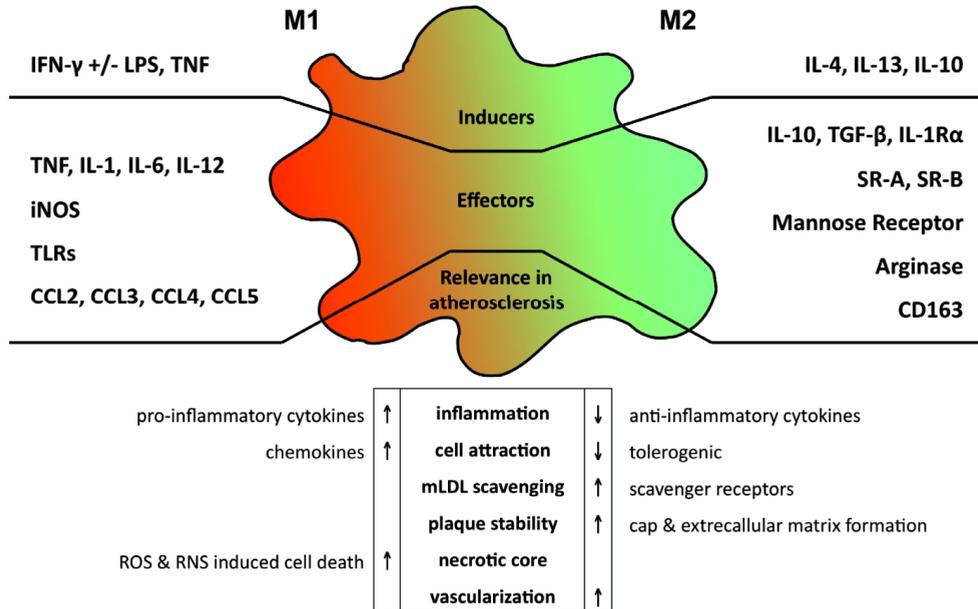


Figure 5.2: Macrophage polarization in atherosclerosis:

Summary of macrophage properties that are altered by phenotype skewing and are known to play a role in the atherosclerotic plaque. The top part of the figure shows a list of cytokines and other effectors that are typically associated with macrophage heterogeneity and have already been described to be relevant in terms of atherosclerosis development. Underneath (in bold) is a list of macrophage functions that are known to be involved in atherogenesis. The arrows indicate how macrophage polarization affects these processes. They are accompanied by the mechanism or effectors that are responsible for this altered function.

Macrophage polarization: beneficial effects of therapeutics and future opportunities for imaging?

Numerous studies provide an indication for therapeutic agents, of which the function can at least partly be ascribed to their immuno-modulatory properties. Hypothetically, distinct macrophage subsets might be involved in mediating the (pleiotropic) actions of some of these drugs. In turn, these agents might drive macrophage polarization to a certain extent as well and may thereby present ways to manipulate macrophage reprogramming. Although a multitude of therapeutics may be worth considering in this respect (e.g. type I interferons, corticosteroids), we opted to highlight just a selection of compounds that are readily prescribed in current cardiovascular medicine. Nevertheless, it would most certainly be interesting to pursue other drugs for their

possible influence on macrophage phenotypes and even explore potential synergies between different immune-modulators. In addition, imaging of macrophages as a diagnostic tool has recently gained great interest. Since macrophage subsets are likely to be differentially involved in regulating plaque instability, future approaches based on the identification of certain detrimental macrophage subsets (or associated characteristics) may be of prognostic value in atherosclerosis. We focus on a number of studies reporting macrophage-based strategies for atherosclerosis imaging and speculate on potential methods for discriminating between macrophage subpopulations.

Therapeutic interventions modulating macrophage polarization?

Statins (3-hydroxy-3-methylglutaryl coenzyme A or HMG-coA reductase inhibitors), provide a proficient approach to achieve lowering of serum cholesterol in patients and have accordingly become one of the most commonly used drugs in the primary and secondary prevention of cardiovascular disease¹⁴¹. Over the last decade, several in vitro and in vivo studies have established that statins conduct at least part of their beneficial effects through a manner that is independent of their lipid-lowering properties^{142,143}. As a result, statins have gained strong interest in different fields of inflammatory disease, as several of their pleiotropic effects were demonstrated to be attributable to the modulation of inflammatory processes. First of all, these drugs have been demonstrated to inhibit inducible MHC-II expression by IFN- γ in monocyte-macrophages and endothelial cells¹⁴⁴. Moreover, in 2002, Youssef et al. showed that the HMG-coA reductase inhibitor atorvastatin significantly alters the inflammatory balance in a murine model of auto-immune encephalomyelitis¹⁴⁵. Whilst inhibiting secretion of the pro-atherogenic Th1-cytokines IL-12, IFN- γ and TNF, atorvastatin promoted a shift towards a Th2-type immune response through the release of cytokines such as IL-4, IL-5, IL-10 and TGF- β . Moreover, this study provides further confirmation that statins affect both T-cell and antigen-presenting cell (APC) compartments. Similarly, a decrease in Th1 bias was reported in human study populations^{146,147}, providing additional in vivo evidence for the immunoregulatory potential of statins. With regard to macrophage polarization, these findings may be of particular interest, since changing the immune response through treatment with statins might shift the phenotype of macrophage populations. Apart from being acknowledged as anti-atherogenic factors, Th2 effector molecules are evidently engrossed in the skewing of macrophages towards M2 phenotypes¹³. For this reason, statin-mediated alterations in inflammatory processes might well offer an interesting mechanism for shaping macrophage polarization. Admittedly, as a therapeutic class, statins are comprised of many individual agents that may have varying degrees of immunomodulatory potential and thus may differently influence macrophage phenotype switching. However, to our knowledge, work on this subject is yet to be reported. Finally, rather than being limited to the development of atherosclerosis, differential

involvement of macrophage subsets may potentially be incorporated in other inflammatory disorders as well. As such, statins have been shown to exert anti-inflammatory effects in rheumatoid arthritis¹⁴⁸ and to inhibit the amount and size of brain lesions in multiple sclerosis (MS)¹⁴⁹.

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are PPAR γ agonists that are used to increase insulin sensitivity in type 2 diabetes patients. In addition to their metabolic action, these compounds have also been shown to exhibit profound anti-atherosclerotic effects in several animal models¹⁵⁰⁻¹⁵². These atheroprotective functions (e.g. reduced M1-cytokines and MMP-9 production^{153,154}) are largely attributed to their immuno-modulatory properties, since PPAR γ can directly interfere with inflammatory signal transduction cascades such as NF- κ B, STAT-1 and activating protein-1¹⁵⁵. In line with this anti-inflammatory action, rosiglitazone treatment has been shown to reduce plasma levels of C-reactive protein in type 2 diabetes patients¹⁵⁶. As discussed above, it was shown that PPAR γ agonists can directly drive macrophage polarization towards an M2 programme in both human and mouse cells^{30,78}. Thus, an important part of the anti-atherosclerotic and perhaps even the anti-diabetic action of PPAR γ activation may be attributed to polarization of macrophages to an M2 phenotype. In a recent randomized placebo-controlled trial in non-diabetic patients, rosiglitazone was indeed shown to reduce lesional inflammation and increased plaque collagen content and stability¹⁵⁷. Interestingly, all of these effects are associated features of M2 macrophages. However, examination of macrophage gene expression identified certain M1 and M2-associated genes to be unresponsive to TZDs^{79,158}, implying that activation of PPAR γ through agonists or ligands is influential, but not solely responsible for M2 polarization. More investigation into this area is needed to clarify the contribution of M1 and M2 macrophages and the role of PPARs in both atherosclerosis and diabetes.

The macrophage as a diagnostic target

As a large body of evidence has designated a lesion's inflammatory burden, rather than the degree of stenosis, as the major determining factor for clinical outcome in atherosclerosis^{1,82}, newly developed imaging strategies accordingly aim to assess the key inflammatory components that decide a plaque's fate. Seeing that polarized macrophage subsets are hypothesized to highly affect plaque stability, supposedly with contrasting outcomes, imaging strategies that target specific macrophage populations or the polarization balance in general might prove valuable tools in the prevention of acute ischemic events.

Above all, magnetic resonance imaging (MRI) is considered to be of great potential in terms of atherosclerosis imaging¹⁵⁹. Due to its excellent soft-tissue contrast, this non-invasive imaging modality is recognized as being supremely adept for serially assessing atherosclerotic vessels¹⁶⁰. Through application of MRI in experimental animal models

(i.e. rabbits and mice) and human research populations, adequate in and ex vivo imaging protocols for the assessment of atherosclerotic plaque composition have been defined¹⁶¹⁻¹⁶³. By discriminating plaque constituents based on differences in magnetic relaxation properties, this technique is able to detect cellular elements, lipid core, fibrosis, and thrombus formation in atherosclerotic lesions with high sensitivity and specificity¹⁶⁴. Interestingly, the use of contrast enhancement provides additional opportunities for depicting inflammatory mediators in atherosclerosis (e.g. macrophages). One such an approach utilizes ultrasmall superparamagnetic particles of iron oxide (USPIO) as a contrast agent for MRI. Once internalized by macrophages, these nanoparticles cause a focal loss of signal intensity on MRI and thus enable non-invasive localization of macrophages in vivo¹⁶⁵. At present, this technique has been used to assess atherosclerotic plaques in hyperlipidemic rabbits^{166,167}, ApoE^{-/-} mice¹⁶⁸ and was demonstrated to be a feasible strategy for macrophage detection in a human population¹⁶⁹. Interestingly, the latter study reported accumulation of USPIO especially in macrophages in ruptured or rupture-prone atherosclerotic lesions. Hence, by appraising plaque macrophage content, USPIO-enhanced MRI might help distinguish between stable and vulnerable lesions.

In a similar fashion, several studies evaluated alternative imaging modalities for their ability to portray macrophages in atherosclerosis. Recently, Hyafil et al. demonstrated that the iodinated contrast agent N1177 is readily taken up by macrophages and subsequently induces an increased density of macrophage-rich tissues on computed tomography (CT) imaging¹⁷⁰. Upon histological examination this effect co-localized with macrophage infiltration. In addition, Fayad and colleagues reported a CT-based approach to be suitable for plaque characterization¹⁷¹. Finally, positron emission tomography (PET) imaging techniques can visualize macrophages by detecting uptake of ¹⁸F fluorodeoxyglucose (FDG). Macrophage-rich atherosclerotic lesions show augmented uptake of FDG due to the higher metabolic activity of activated macrophages. Interestingly, symptomatic lesions exhibit more FDG accumulation in comparison to their asymptomatic counterparts¹⁷², further implicating macrophages in plaque complication.

Our growing insights into the molecular mechanisms that drive atherosclerosis emphasize the need for imaging techniques that are able to depict functional and biological processes (e.g. inflammation) as opposed to anatomical abnormalities (e.g. stenosis). Taken together, the accounts above use a myriad of ways to visualize macrophages in atherosclerotic lesions and thereby show these cells to be both promising and versatile targets for future imaging strategies. Although these methods need to be optimized before routine application in clinical practice can be realized, they may ultimately extend to the depiction of macrophage subsets in inflammatory disease. Striving to further unravel the involvement of distinct macrophage subsets in atherogenesis will surely propagate the development of novel imaging strategies that grant us information regarding the macrophage polarization balance in selected parts

of the vasculature. Attaining such data prior to the patient developing symptoms might revolutionize the ways by which we assess atherosclerotic arteries, stratify individual risk and might even allow us to intervene accordingly. For instance, selectively visualizing a lesion's M1 macrophage content could point out plaques containing a relatively high inflammatory load that as such have a higher likelihood to experience rapid progression or even rupture. The role of different M2 macrophage subsets in atherosclerosis however is still somewhat controversial. As we mentioned before, specific features that are possibly attributable to a M2 subset (i.e. angiogenesis) may actually enhance atherogenesis, leaving that particular phenotype less desirable in atherosclerosis. Conversely, we certainly expect other M2 phenotypes to be beneficial for plaque stability. For this reason, reflecting on how imaging of M2 macrophages would likely be implemented in the diagnostic work-up of atherosclerosis is difficult. To illustrate this, we would like to refer to an earlier section of this review, in which we elaborately discuss that an M2 population of CD163^{high} macrophages in atherosclerotic lesions could identify plaques that are predisposed to rupture through intraplaque haemorrhage. This particular example creates a remarkable controversy that shows the complexity of the M1/M2 paradigm, since an atheroprotective M2 subset may possibly also serve as a diagnostic tool for recognizing certain detrimental plaque characteristics. Therefore, depending on the M2 subset in question, a visual appraisal of lesional M2 content might provide additional, but diverging clues as to a plaque's eventual outcome.

However, at this time we can only speculate on possible diagnostic techniques that will allow for a dynamic application of macrophage heterogeneity in atherosclerosis. Ideally, continued efforts to characterize macrophage subsets will identify better (e.g. subset- but not species-specific) markers that could then be specifically targeted for imaging. In addition, differential affinity of polarized macrophage subtypes for the internalization of certain molecules and compounds might offer another window of opportunity for subset visualization.

Conclusions

Over the past few years, we gained considerable insight into the inflammatory processes that lie at the core of atherosclerosis development. Macrophages are progressively acknowledged as major determinants of atherosclerosis, joining both innate and adaptive immune responses in its pathogenesis. Currently, several polarized M1 and M2 macrophage subsets have been identified and both populations were demonstrated to be present in atherosclerotic lesions. Although the differential expression of effector phenotype implies macrophage function in atherosclerosis to be highly ambiguous (i.e. M1 and M2 macrophages as atherogenic and atheroprotective effectors respectively), evidence supporting their functional implications *in vivo* is

largely lacking. In this respect, several arising challenges complicate the implementation of macrophage polarization in our understanding of the atherosclerotic process. Firstly, additional markers are required that will allow us to better distinguish the current macrophage subsets and possibly help identify new ones. Preferably, these markers will be adept for characterizing both murine and human macrophage populations, as inter-species variability is becoming increasingly apparent. Any such markers might subsequently facilitate the exploration of means by which the inflammatory balance in atherogenesis can be affected (e.g. by pharmaceutical intervention). Additionally, we need direct functional studies in mice that lack a specific macrophage subset to clarify the *in vivo* relevance of that particular subtype and possibly increase our insight into the underlying mechanisms.

Ultimately, the current M1/M2 paradigm offers a comprehensive take on the concept of macrophage heterogeneity. However, due to its simplified nature, it is highly unlikely that this digotomy represents the full scope of functional macrophage phenotypes. Instead, the current M1 and M2 states are better regarded as extremes of a continuum that remains to be explored. Hence, if we are to generate a classification that is a more accurate portrayal of *in vivo* atherosclerotic conditions, we should advance our knowledge on this phenomenon. Only by negotiating the aforementioned difficulties will we be able to develop strategies that allow us to skew macrophage polarization in such a manner that it serves our diagnostic and therapeutic goals, be it in the context of atherosclerosis or other macrophage-mediated disorders such as obesity and cancer.

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Chapter 6

General discussion

Through its versatility, the macrophage is a key player in both innate and adaptive immune responses. Its broad range of functions is illustrated at its best in infectious diseases, where they participate in pathogen killing and engulfment, attract other leukocytes to the site of infection, and ultimately help in directing the response of these newly recruited cells¹. But also the outcome of non-infectious diseases like tumorigenesis² and insulin resistance³ depends on the inflammatory regulation and phagocytic capacity of the macrophage.

In atherosclerosis, the multi-functionality of the macrophages is reflected in their different actions throughout the course of the disease⁴. After they initiate atherogenesis by taking up modified lipids in the vessel wall to a point where they are unable to leave the subendothelial space, they create a local inflammatory environment by producing and secreting chemokines, cytokines and growth factors. Hereby, they promote the attraction, activation and proliferation of new macrophages and other cells to the growing lesion. Their subsequent contribution to different processes can be either plaque stabilizing or destabilizing, a heterogeneity that is described in chapter 5.

Atherosclerosis and its acute clinical complications are the number one cause for mortality in Western society. Although not a newly emerging disease, the combination of a sedentary life style, high-caloric diet pattern and several environmental factors has increased our morbidity and mortality to alarmingly high levels. Through our knowledge of risk factors contributing to this disease, a number of preventive, lifestyle changing actions can be advised in order to reduce the risk. However, our advancing understanding of the molecular and cellular mechanisms underlying the pathogenesis has yet resulted in a disappointingly limited array of pharmaceutical or therapeutical options. In addition to tackling the hyperlipidemia that is caused through lifestyle or genetic predisposition⁵ and surgically correcting the life-threatening clinical manifestations⁶, therapeutics that can alter the course of the disease would be more than welcome.

Experiments in animal models have illustrated several strategies to interfere with molecular pathways and cellular interactions involved in atherogenesis that may present useful future treatment strategies but are still not readily applicable in the clinic. Because it is the most abundant cell type during plaque growth, the target of these therapies is often the macrophage and the several ways it contributes to the pathogenesis, from recruitment over foam cell formation to cell death and necrotic core formation. In this thesis, we further investigated the role of macrophages in atherogenesis, focusing on their recruitment to the plaque and inflammatory signaling pathways.

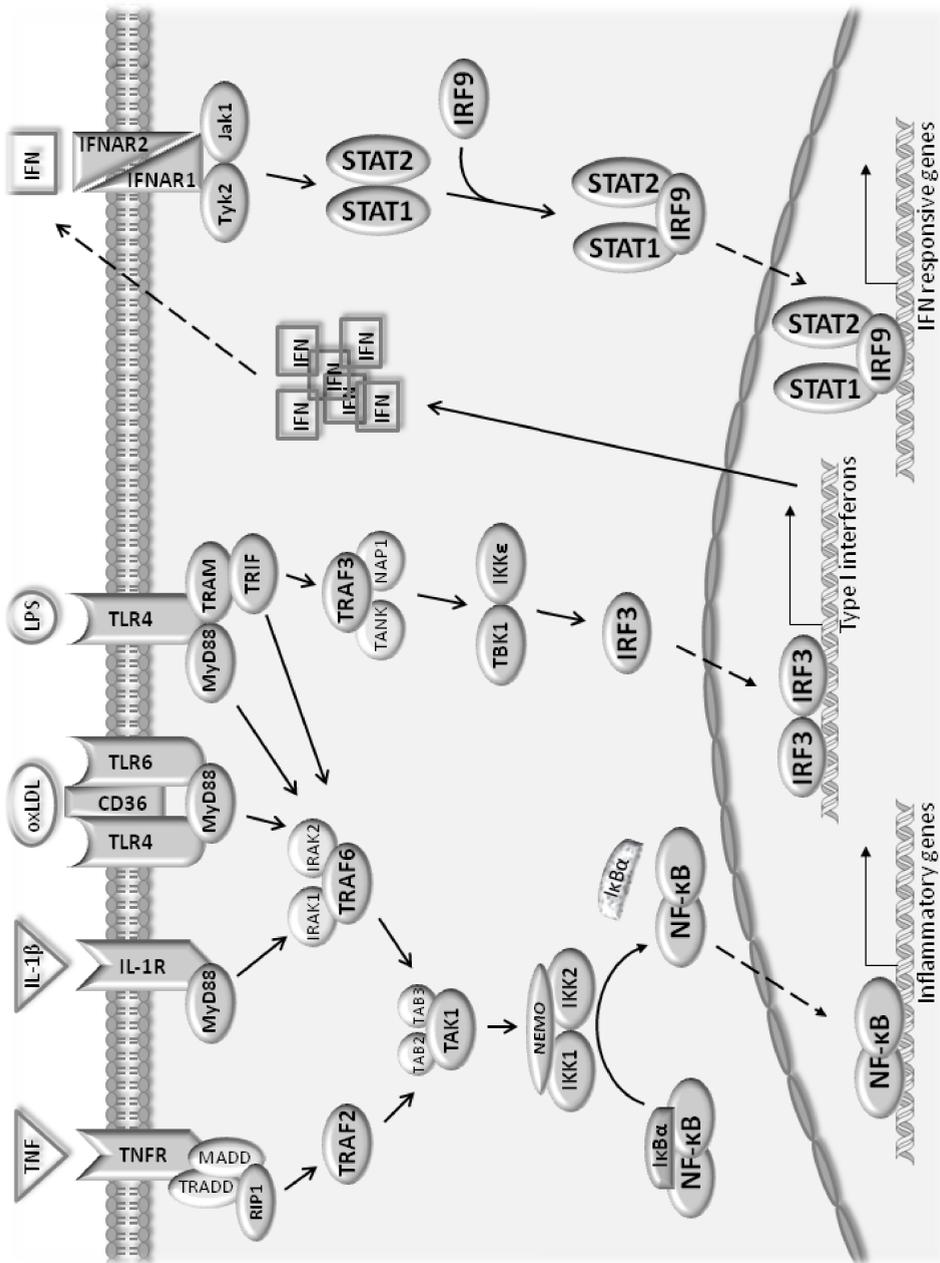


Figure 6.1: The NF-κB and IRF3 signaling pathways that lead to the expression of NF-κB dependent genes and type I interferons, followed by type I interferon signaling.

Type I interferons promote atherosclerosis

In **Chapter 2**, the effect of type I interferon (IFN α and IFN β) treatment as well as the role of endogenous interferons on different mouse models of atherosclerosis was investigated.

Interferons were described more than 50 years ago as a cytokine family that is produced and secreted in response to virus infection and induces an antiviral state in the surrounding cells⁷. Exposure to specific viral or bacterial components activates Toll-like receptor signaling which then leads to interferon production, parallel to the activation of the inflammation-regulating transcription factor NF- κ B (Figure 6.1). Upon secretion, autocrine or paracrine binding of interferons to their heterodimeric receptor (IFNAR1 and IFNAR2) will induce a Jak/STAT pathway that ensures the expression of a set of interferon-stimulated genes (ISGs)⁸. This diverse group of more than 300 genes regulates the cell's defense against microbial threats, with multiple redundant actions including active elimination of pathogens, raising the cell's resistance to infection and slowing down the protein production in order to halt the *de novo* assembly of parasites⁹⁻¹¹. Additional to these antiviral properties however, the ISGs were also found to be regulating other cell functions that can play a role in case of infection. They can facilitate cell death, presumably to kill infected cells before they can contribute to the microbial expansion, and they have immune-regulatory functions, simultaneously restricting a potentially exorbitant innate immune response to the infection and encouraging an adaptive immune response.

Already since the early '80s, the therapeutic potency of these different type I interferon features is exploited in the clinic for the treatment of several diseases⁸, with side effects that are mainly limited to flu-like symptoms and injection site irritation¹². While patients infected with certain viruses, e.g. hepatitis C virus, benefit from the antiviral effects of interferons¹³, some cancer patients have increased tumor cell necrosis upon interferon injections^{14,15}. A more prolonged treatment is applied in patients suffering from relapsing-remitting multiple sclerosis (MS), a T cell-driven, chronic inflammatory disease that is characterized by episodic attacks of progressive neurological deterioration. Daily to weekly injections with interferons extends the period between the acute attacks through a yet largely unclear mechanism¹⁶. Both in these patients as well as in the experimental autoimmune encephalitis (EAE) mouse model for MS, treatment with interferons raised the plasma levels of IL-10¹⁷, suggesting that this anti-inflammatory cytokine is playing a role in the therapeutic mechanism.

The induction of plasma IL-10 levels appeared to be an interesting feature of these type I interferon injections, since this interleukin not only reduces inflammation but has also been described as one of the strongest anti-atherogenic cytokines¹⁸. It suggested that an existing therapy may be applied in the treatment of atherosclerosis.

While the role of other cytokines of the interleukin, TNF and type II interferon family was already well documented, with pro-inflammatory cytokines most often promoting atherogenesis and anti-inflammatory cytokines attenuating plaque growth¹⁹, the few studies on the effect of type I interferons showed conflicting results. In the earliest published study, Wilson et al. show how treatment of cholesterol-fed rabbits with type I interferons resulted in a further unexplained reduction in lesion size accompanied by a decreased plaque lipid content, while plasma lipids were unaffected²⁰. However, the interferons used in this experiment were isolated from cell cultures without detailed identification or assessment of the purity of the isolate. Therefore, other factors influencing atherogenesis may have been administered along with the interferons, although in a previous study, treatment with two different interferon-inducing agents caused a similar effect on the rabbit plaques²¹. In a second study, a low dose of IFN α was given to hyperlipidemic LDLR^{-/-} mice for five weeks. Here, the treatment accelerated atherogenesis, which is explained by the higher plasma levels of both cholesterol and triglycerides in treated compared to control mice²². A third and final experiment used a more aggressive, hypercholesterolemia-independent atherosclerosis model, surgically ligating the carotid artery in ApoE^{-/-} mice and locally perfusing angiotensin II (AngII) through a mini-pump. Here, IFN β treatment attenuated atherogenesis as well as the AngII-induced but not the ligation-induced vascular remodeling. In their control ApoE^{-/-} mice without AngII and on a normolipidemic diet, IFN β treatment did not affect plaque formation²³.

In our experiments²⁴, daily injections with IFN β , in doses comparable to treatment studies in the EAE model, promoted atherogenesis in two different murine models, despite raised plasma IL-10 levels and without influencing plasma lipids. The mechanism involved a CCL5-dependent enhanced recruitment of leukocytes to the vessel wall at atherosclerosis-prone sites and a resulting sub-endothelial macrophage accumulation, as shown in both *in vitro* and *in vivo* experiments. Moreover, deleting the *IFNAR1* gene specifically in lysozyme M-expressing cells and thereby inhibiting IFN signaling in myeloid cells resulted in smaller lesions, indicating that also endogenous IFN contributes to atherogenesis.

Whereas many cell types can produce type I interferons in response to an array of specific stimuli, the endogenous IFNs in the plaque are believed to be predominantly produced by plasmacytoid dendritic cells (pDC). This subset represents about one third of all dendritic cells and has, compared to myeloid dendritic cells (mDC), a low presence of the CD11c membrane marker for DC's, a high expression of IL-3 receptor CD123 and a less dendritic phenotype. They also express less extracellular but more intracellular Toll-like receptors and are therefore more specialized in the detection of viruses as well as nucleotides derived from dying cells. These viral- and autoantigens induce a massive production and secretion of type I interferons²⁵.

Since the presence of IFN-producing pDCs is demonstrated in human plaques²⁵ and *in vitro* treatment of human macrophages with IFN β resulted in an upregulation of

chemokines and chemokine receptors²⁴, similar to mice, it should be investigated if our findings in murine models can be translated to human atherogenesis. These novel findings may be of importance in the search for new diagnostic and therapeutic approaches. For obvious reasons of susceptibility to viral or bacterial infections²⁶ however, it will not be possible to inhibit interferon production, interfere with its signaling or deplete pDCs as long as no methods are developed yet to target these therapies specifically to the atherosclerotic plaque. Also the ability to upregulate the chemokine CCL5 may be essential in the reaction to other pathologies and can therefore not be inhibited systemically. One has to keep in mind as well that human lesions differ significantly from the murine models, being far more complex and especially developing over several years instead of a few weeks²⁷. It is therefore still uncertain if the contribution of cell recruitment to human atherogenesis is equally important as in murine plaques.

A more direct consequence of our results is the awareness that type I interferon treatment may not be as harmless as previously reckoned. Perhaps short-time treatment, as applied in hepatitis or tumor patients, might not affect a slowly progressing disease that much, but care has to be taken when treating for extended periods. Therefore, a thorough assessment of the cardiovascular risks in IFN-treated MS patients and a comparison with patients receiving other treatment options should be made as soon as possible.

Moreover, our findings may shed new light on the mechanisms that cause the 50-fold increased risk of developing atherosclerotic cardiovascular disease in patients suffering from systemic lupus erythematosus (SLE)²⁸. This systemic autoimmune disease is characterized by elevated plasma levels of type I interferons²⁹, which is thought to play a central role in its pathogenesis³⁰ and is therefore targeted in experimental and clinical attempts to treat SLE³¹. Although the correlation between SLE and atherosclerosis in mice and patients was known before, it was until now explained by the combined contribution of different atherosclerosis-promoting factors, including traditional risk factors as well as a chronic inflammatory environment, elevated levels of auto-antibodies, endothelial dysfunction, decreased vascular repair and increased levels of oxidized lipids^{28,32,33}. We, and simultaneously with us also other groups, now postulate that the high plasma levels of type I interferons may be an additional and important risk factor^{24,34,35}.

Similarly to SLE, other autoimmune inflammatory diseases including psoriasis, psoriatic arthritis and rheumatoid arthritis combine an essential role for type I interferons in their pathogenesis with an increased cardiovascular risk³⁶⁻⁴⁰. Also in these diseases, interferon signaling may contribute to this risk, in combination with traditional risk factors and other immune mediators like IL-1⁴¹ and TNF⁴².

A yet unpublished observation in our experiment in which ApoE^{-/-} mice were treated with IFN β may potentially shed a light on the importance of macrophage recruitment during the course of atherogenesis. In our experimental approach, we initiated the daily IFN injections simultaneously with the surgical positioning of the collars around the carotid arteries, implicating that the elevation of plasma IFN β levels in these mice coincided with the initiation of carotid atherogenesis. At that moment however, these ApoE^{-/-} mice were already fed a high-fat diet for more than three weeks and therefore, plaques had already started to form in other, more hyperlipidemia-dependent locations like the aortic root, the innominate artery and the aortic arch. While IFN injections promoted atherogenesis proximal of the collar, as shown in Chapter 2, plaques in the aortic arch and innominate artery were unaffected by this treatment (**Figure 6.2**). Since the plaque size in these locations was not assessed in our other models, there is no certainty that atherogenesis in these locations is similarly dependent on CCL5-mediated leukocyte recruitment as plaques in the aortic root. A more plausible and instinctive explanation however would be that this effect of IFN treatment on plaque growth is most outspoken in newly-formed plaques. Since early lesions are rapidly expanding through the recruitment of new cells, they may be more affected by CCL5 upregulation than the larger, more advanced lesions, in which plaque stability parameters become more relevant. More evidence is needed to support this theory, for example by assessing the number of newly-recruited macrophages in plaques of different stages and sizes with the ER-MP58 marker that was described in Chapter 3. The outcome will be of high importance when designing future experiments and therapies that target the cell recruitment to the lesions.

It is therefore also possible that the results of the IFN treatment we described in Chapter 2 are highly dependent on the experimental setup we applied. In our models, daily IFN injections were initiated at the expected start of the high-fat diet-induced plaque formation (in the LDLR^{-/-} model) or immediately after positioning the collars (in the ApoE^{-/-} model) and therefore coincided with the earliest atherogenesis. In future experiments, it has to be verified if a similar atherosclerosis-promoting effect can still be observed when the treatment starts later in the disease progress. In the more advanced plaques, IFN treatment may affect the plaque stability, for example through TRAIL-mediated promotion of apoptosis⁴³ or inhibition of smooth muscle cell proliferation⁴⁴, rather than promote further plaque growth.

Other future experiments should focus on the effect of type I interferons in other cell types within the atherosclerotic lesion. Repeating the studies of high-fat diet-induced atherosclerosis in LDLR^{-/-} mice that have a cell type-specific deletion of IFNAR1 in smooth muscle cells (smooth muscle actin promoter⁴⁵) or endothelial cells (Tie-2 promoter⁴⁶) may reveal additional mechanisms in which IFNs contribute to plaque growth or (in)stability.

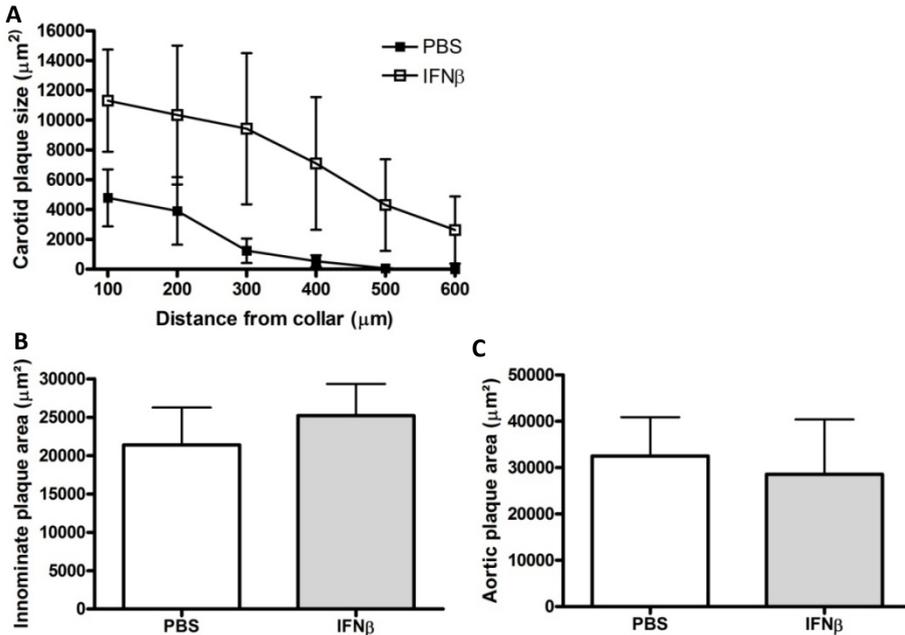


Figure 6.2: IFN β treatment in ApoE^{-/-} mice with a collar induced atherosclerosis in the carotid artery (A) but did not affect plaque size in the innominate artery (B) nor in the transcervical aorta (C).

Myeloid I κ B α deficiency promotes atherosclerosis

It has been shown extensively before that inflammation has a major effect on atherosclerosis^{4,47}. This is not only illustrated by the local presence of different inflammatory cell types⁴⁸ but also the proven influence of inflammatory cytokines on the development of the plaque¹⁹. The transcription factor NF- κ B plays a key role in many of the cellular responses that drive inflammation, including the production of many of these cytokines⁴⁹. This homo- or heterodimer is kept inactive through binding of the inhibitor of NF- κ B (I κ B). Phosphorylation of this inhibitor by the I κ B kinase (IKK) complex and subsequently ubiquitination and degradation releases NF- κ B and enables it to translocate to the nucleus, resulting in a specific gene expression pattern. This IKK complex can be activated through many different converging signaling pathways that respond to a wide range of stimuli, including microbial molecules, cytokines⁵⁰ and, notably in the atherosclerotic plaque, oxidized LDL particles⁵¹ (Figure 6.1).

Because of its role in the initiation of inflammation and because atherosclerosis is an inflammatory disease, NF- κ B activation was thought to contribute to the pathogenesis of this disease. In experiments targeting upstream signaling molecules that were previously published by our group and other groups however, it was shown that the role of NF- κ B is far more complex and yet to be thoroughly investigated⁵²⁻⁵⁴.

In **Chapter 3**, we aimed at further clarifying the effect of NF- κ B activation in macrophages on murine atherogenesis by genetically and myeloid-specifically deleting its main inhibitor of the I κ B family, I κ B α . Contrary to the full I κ B α ^{-/-} mice, these conditional LysMCre-I κ B α ^{fl/fl} mice have no dysregulation of myelopoiesis⁵⁵. Instinctively, they should result in a continuous NF- κ B activation and a sustained NF- κ B-dependent gene expression pattern in macrophages and neutrophils. However, both *in vitro* and *in vivo* experiments revealed disappointingly little increase in macrophage NF- κ B translocation to the nucleus or upregulation of some typically NF- κ B-dependent genes. Without clear experimental evidence for compensatory mechanisms, functional redundancy⁵⁶ or interference with other transcription factors⁵⁷ in this complicated and tightly regulated pathway, we still don't know yet why I κ B α deletion cannot be used as a model for NF- κ B hyper-activation. Knowing that the deletion of a directly binding inhibitor is still insufficient to deregulate this pathway, it can be questioned if it will even be possible to design genetic models of sustained NF- κ B activation. Perhaps the closest useful approach would involve mice with a myeloid-specific deletion of the de-ubiquitinating enzyme A20⁵⁸, but one has to bear in mind that these mice also display a continuous IRF3 activation and thus produce elevated levels of the atherosclerosis-promoting type I interferons.

Remarkably, even without demonstrable effect on NF- κ B activity, the chemokine CCL5 was upregulated in I κ B α ^{del} macrophages, promoting their adhesion to endothelial cells in a similar way as we described for IFN β in Chapter 2. Although considered an NF- κ B-dependent gene⁵⁹, we and others demonstrated the importance of IFNs in macrophage CCL5 expression^{24,60}. This raises the question whether the observed upregulation is a consequence of the NF- κ B or IFN signaling. As we have not detected an induced production of IFNs in the I κ B α ^{del} cells, future experiments may investigate whether these cells perhaps have an increased sensitivity to IFNs. In order to completely understand the effect of I κ B α deletion on the gene expression pattern, a full microarray should be performed on these macrophages, instead of the confined list of some typical NF- κ B-dependent or atherosclerosis-influencing genes we assessed.

The introduction of ER-MP58 as a marker for newly-recruited macrophages in the plaque may offer new possibilities for further research. As mentioned above, quantification of the ER-MP58⁺ macrophages throughout the course of plaque development will bring valuable insights in the role of cell recruitment in different stages of the disease. It can also serve as a tool for assessing the effect of future therapeutic strategies and dietary changes in murine atherogenesis models.

A lentiviral vaccination targeting survivin potently reduces plaque size

In **Chapter 4**, a novel vaccination strategy for murine atherosclerosis was demonstrated. After confirming the presence of the mostly tumor-specific protein

survivin in newly-recruited macrophages of murine plaques, similarly to the human lesions⁶¹, the evocation of a targeted immunity by injections with survivin-expressing lentiviral vectors successfully reduced diet-induced atherogenesis.

Despite the wide range of survivin-targeting therapeutic strategies that have been developed in tumor models and are already applied in the clinic⁶², most of these approaches are aimed at a one-time elimination of survivin-positive cells and are therefore not applicable in the treatment of a chronic disease. Moreover, as discussed above, the recruitment of new macrophages to the lesion may contribute more to early pathogenesis and gradually reduce in importance when the plaques become more advanced. Survivin-targeting interventions would therefore be at their most effective against newly-forming plaques and thus should be prophylactic rather than therapeutic. This makes immune therapy the desired option for treating atherosclerosis, over the short-lived small-molecule antagonists, siRNA, anti-sense oligonucleotides and other survivin-targeting methods.

But although this vaccination strategy has been applied before in mice as well as in the clinic to combat survivin-expressing tumors with limited side effects and now appears to successfully target murine atherosclerosis as well, this therapy is not yet ready for application in atherosclerosis patients. First of all, one may question the ethics of vaccinating against a disease that affects a whole population but is, when well managed, invisible and non-terminal. On the other hand, vaccinating only the people that are proven to be at risk may come too late for optimal efficacy. Moreover, survivin-targeting vaccinations already were shown to be strongly varying in efficiency between different types of tumor cells, ranging from phase I trials that achieved a decrease in tumor markers in less than half of the treated patients⁶³ to patients that have temporary or even complete remission after vaccination⁶⁴. Thence, the efficiency against plaque macrophages remains uncertain.

Also, the long-term effects of this vaccination should be assessed thoroughly first. Other than in cancer, where short-term and aggressive immunity against survivin should suffice to eliminate as many tumor cells as possible, usually in addition to other tumor-targeting therapies, inhibiting atherogenesis requires a life-long, latent immunity. Before this can be attempted, the role of survivin in non-tumoric, healthy cells should be investigated more exhaustively. However survivin is thought to be predominantly tumor cell specific and expressed only shortly and in low levels in healthy tissues^{65,66}, there may still be yet unknown cell type-specific and situation-specific requirements for survivin-expression⁶⁷, similar to the expression that was just recently discovered in plaque-infiltrating macrophages⁶¹. Certitude of the specificity is required before long-term immunity can be induced safely.

Before the above mentioned requirements can be met, a survivin-targeting immune therapy for atherosclerosis will not be ready for clinical application. Meanwhile, these results should rather be regarded as an illustration of the possibility to specifically

target cells in the atherosclerotic plaque, not only for vaccinations but potentially for plaque-specific delivery of other future therapeutics as well. Furthermore, immune histochemistry for survivin could be used in a similar way as described above for ER-MP58 to monitor macrophage recruitment to the plaques.

Future Perspectives

Other than a change in lifestyle and cholesterol-lowering drugs, no pharmaceuticals or therapies are yet available for intervening with the disease progression of atherosclerosis. Similarly to the difficulties in finding cancer treatments, the main problem may be that the cells we would wish to target are not differing enough from the healthy cells, while their functions we hope to block are still necessary in other, physiological processes. Therefore, it will be very important to focus further research on finding the properties that make the cells in the atherosclerotic plaque significantly different from healthy cells, enabling targeted therapies.

The abundance and the central role of macrophages in the plaque make this cell type the desired target for different therapeutic strategies⁶⁸. From the very first phases of atherogenesis up until plaque rupture, macrophages drive the disease progression and influence the attraction and function of the surrounding cell types. Unlike the earliest proposed models of macrophage heterogeneity, with pro-inflammatory M1's and anti-inflammatory M2's, we now know that there is a wide range of macrophage phenotypes in between these two extremes and that each of these phenotypes is induced by their specific environment during differentiation, priming and activation⁶⁹. Most likely, even the plaque macrophages are a collection of different phenotypes, depending on the cells' exposures to growth factors, cytokines, lipids etc. Therefore, macrophage markers related to the uptake and storage of lipids may be more attractive as a target than the classic M1 and M2 markers. Only a few genes are already described to be more or less specifically upregulated in (subsets of the) plaque macrophages, including the genes for the fatty acid binding protein aP2⁷⁰, the reverse cholesterol transporter ABCA1^{71,72} and the nuclear receptor PPAR γ ^{73,74}. Also the survivin gene is, as discussed earlier, upregulated in newly recruited plaque macrophages. Knowledge of these specific genes and pathways may allow therapeutic interventions but may also be useful for targeting vaccinations, like we and others demonstrated before^{75,76}, and as a target in other directed therapies. A recent publication from the oncology field for example illustrates the possibility of generating cytolytic viruses that specifically replicate in cells where a certain gene is expressed or pathway is induced⁷⁷.

As we explained in chapter 5, macrophage phenotype skewing *in vivo* through exposure to the right cytokines or other skewing stimuli may become a future therapeutic option for atherosclerosis⁷⁸. A thorough knowledge of the phenotypes present in the lesion, their contribution to atherogenesis and the triggers needed to generate the desired

phenotype would be required, as well as a method to specifically affect the plaque macrophages while retaining the full functionality of macrophages in other tissues. This may be achieved by exploiting the above mentioned lesion-specific genes. For example, viral delivery of constructs that provide the aP2 or ABCA1 promoter regulating the IL-10 gene could trigger the expression of this anti-inflammatory cytokine specifically in the plaque macrophages and thereby induce an auto- and paracrine macrophage phenotype skewing and an anti-atherogenic effect⁷⁹. While aP2 expression is independent of IL-10⁸⁰, ABCA1 expression is upregulated upon IL-10 signaling⁸¹ and will therefore further amplify the cytokine's expression. Experimental evidence should clarify which of these options would be most successful, or if self-limiting mechanisms should rather be pursued.

In this thesis, the importance of leukocyte (and predominantly macrophage) recruitment to the plaque is an often recurring theme. Its modulation largely affects plaque growth, presumably most in newly forming lesions and to a lesser extent in more advanced stages of the disease. Reducing this recruitment by inhibiting chemokine secretion or blocking adhesion molecules slows down plaque growth⁸²⁻⁸⁴ but it is important to remember that this recruitment is still essential in other functions of the macrophage, like infiltrating tissues upon the local detection of infectious agents. Therefore, general inhibition of recruitment is no option and more targeted solutions should be found⁸⁵.

Instead of interfering with the function of adhesion molecules and chemokines, it might be more useful to focus on the stimuli that induce their production instead, for example by skewing the plaque macrophages towards a phenotype that has a significantly reduced chemokine production. The endothelial cells' adhesion molecule production on the other hand could be reduced by targeting the source of the activation of these cells. As shown by Duewell et al⁸⁶, cholesterol crystals activate the NLRP3 inflammasome in plaque macrophages, leading to the activation and secretion of IL-1. Although the influence of this IL-1 on the surrounding cell types is still to be investigated, endothelial cells do express its receptor IL-1R and can, at least *in vitro*, be activated by IL-1⁸⁷. Indeed, plaque size was reduced in mouse models with IL-1 deletion⁸⁸, IL-1R antagonist overexpression^{89,90} or injections with this IL-1Ra⁹¹. The IL-1 production is rather specific for sterile inflammatory stimuli like cholesterol crystals, necrotic cells, asbestos and silica crystals but not for the microorganism-stimulated inflammatory response and subsequent cell recruitment⁹². It may therefore be an interesting target in future therapies aiming at the reduction of endothelial activation as a result of these stimuli while maintaining a proper response to the detection of microorganisms. Conveniently, IL-1 targeting therapies (e.g. anakinra) are already available in the clinic⁹³.

In conclusion, this thesis further illustrates the importance of macrophage recruitment in the pathogenesis of atherosclerosis. We show how an increase in the attraction of new macrophages to the lesion deteriorates the disease while specifically targeting these cells through vaccination reduces plaque size. These data may result in new therapeutic strategies for (early) atherogenesis. Also a thorough knowledge of the macrophages' inflammatory signaling and their intra-plaque heterogeneity will reveal new possibilities for the treatment of this disease, as described in this thesis.

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Supplemental Figures

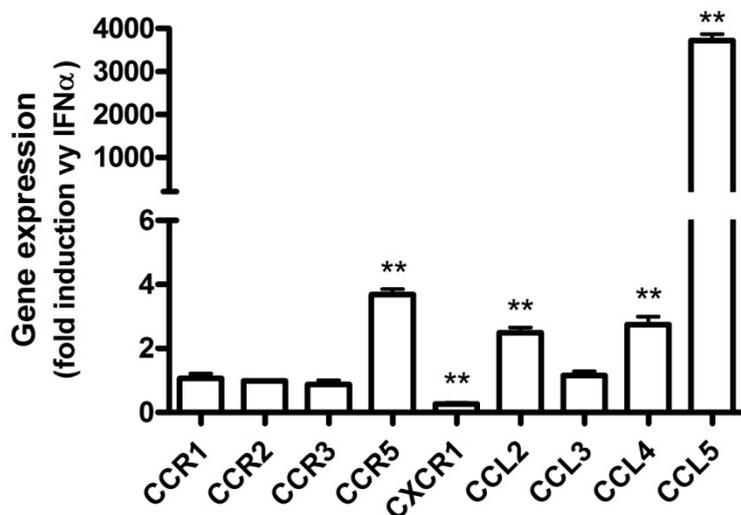


Figure S2.1: Expression of chemotactic factors after treatment with IFN α .

Indicated is the fold-induction in bone marrow macrophages after 24h treatment with IFN α compared to untreated cells. Bars represent average of triplicate wells. (** p < 0.01)

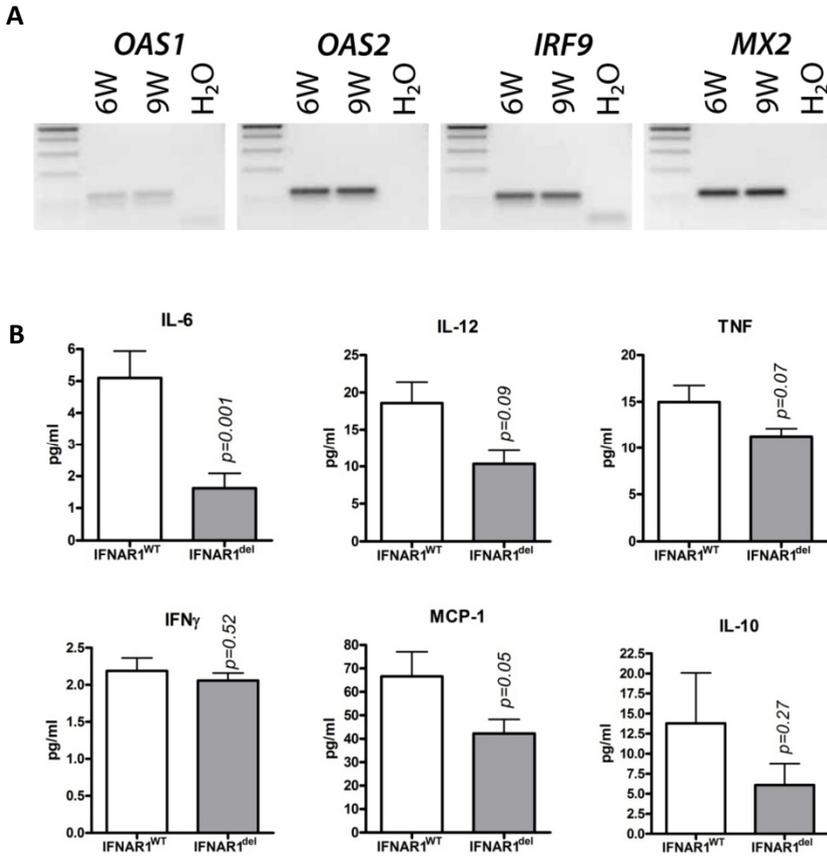


Figure S2.2: Expression of type I IFN signature genes in arches of atherosclerotic *Ildr*^{-/-} mice and cytokine levels in IFNAR1^{WT} and IFNAR1^{del} mice after 8 weeks of high fat diet.

(A) Expression of OAS1, OAS2, IRF9 and MX2 was detected in arches of *Ildr*^{-/-} mice that had been fed a high fat diet for 6 (6W) or 9 (9W) weeks. (B) IL-6 levels are reduced in IFNAR1^{del} mice compared to IFNAR1^{WT} mice, while IL-12, TNF and MCP-1 show a non-significant trend towards a reduction. N=10 animals per group, P values are indicated.

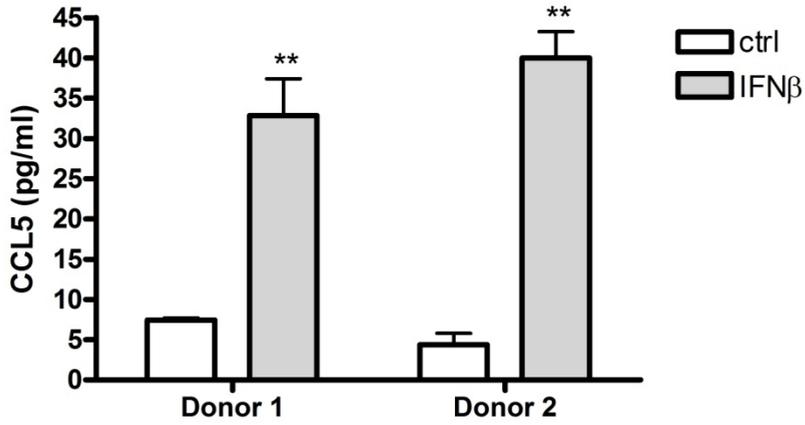


Figure S2.3: Secretion of CCL5 after treatment with IFN β .

Human primary macrophages were untreated (ctrl) or treated with IFN β for 24h (IFN β) and CCL5 was measured in the supernatant. Bars indicate average of triplicate wells. (** $P < 0.01$)

GeneSymbol	Fold Change Ruptured/Stable	Average logIntensity	Adjusted P-Value
<i>Signalling pathway</i>			
IFNAR1	1.27	9.62	4.03E-04
IFNAR2	1.45	9.00	1.99E-03
JAK1	0.99	9.05	9.29E-01
STAT1	1.92	10.42	4.91E-07
STAT2	1.17	11.57	4.12E-03
PTPN2	1.24	8.04	3.21E-06
TYK2	1.23	10.19	3.10E-04
<i>Transcription products</i>			
IFIT1	1.58	10.12	3.74E-04
IFIT3	1.72	9.38	3.08E-05
IFITM1	1.72	10.82	5.99E-04
MX1	1.51	12.19	1.50E-03
MX2	1.08	7.35	8.88E-05
OAS1	1.80	8.93	1.86E-06
OAS2	2.04	10.38	3.90E-06
PSMB8	1.36	8.83	1.69E-06
IRF9	1.14	11.20	1.24E-02
IFI35	1.47	9.88	9.85E-07
<i>Chemokines</i>			
CCR1	2.40	9.11	3.10E-08
CCR2	1.49	8.41	1.98E-06
CCR3	1.03	7.32	2.94E-03
CCR4	1.01	7.28	2.82E-01
CCR5*	5.58*		0.0040*
CX3CR1	1.00	8.02	9.78E-01
CCL2	1.88	11.85	1.60E-03
CCL3	2.33	11.06	2.67E-04
CCL4	Not detected on array		
CCL4L1	2.02	10.42	1.93E-04
CCL5	1.35	9.71	3.46E-02

Table ST2.1: Differential gene expression of Type I IFN signaling pathway and chemokines in human carotid plaques: Ruptured vs. Stable.

The Type I IFN signaling pathway is upregulated in unstable regions of human atherosclerotic lesions. Both genes involved in the Type I IFN signaling pathway as well as its target genes are mostly upregulated in ruptured human carotid plaques compared to stable regions. Fifty percent of the investigated chemokines are also upregulated in ruptured plaque regions.

* CCR5 was not detected on the array for unknown reasons, values presented are qPCR values relative to 18s rRNA of cDNA from 4 stable vs. 8 ruptured plaque lysates.

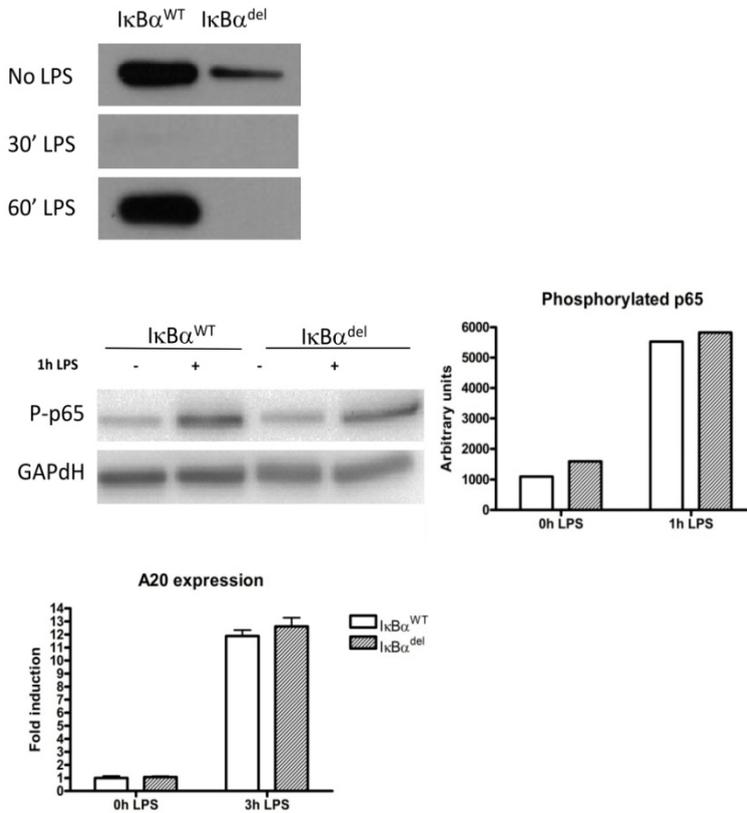


Figure S3.1: Deficiency of IκBα does not lead to increased NF-κB activity and subsequent gene expression.

(A) Western blotting for IκBα on lysates of bone marrow derived IκBα^{WT} or IκBα^{del} macrophages confirmed a reduction in IκBα production, both before and after LPS activation. (B-C) A western blot for phosphorylated NF-κB subunit p65 showed that NF-κB is not continuously activated in IκBα^{del} macrophages, (D) resulting in a lack of differential expression patterns for NF-κB dependent genes.

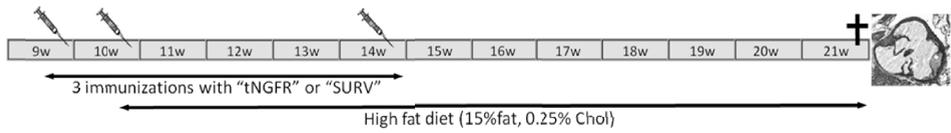


Figure S4.1: Schematic overview of the experimental setup used in the atherosclerosis vaccination study.

Each bar represents one week and indicates the age of the mice. Three immunizations with lentivirus were administered and the mice were fed a high-fat diet for 11 weeks. At the end of the experiment, mice were sacrificed and their atherosclerotic plaques were analyzed.

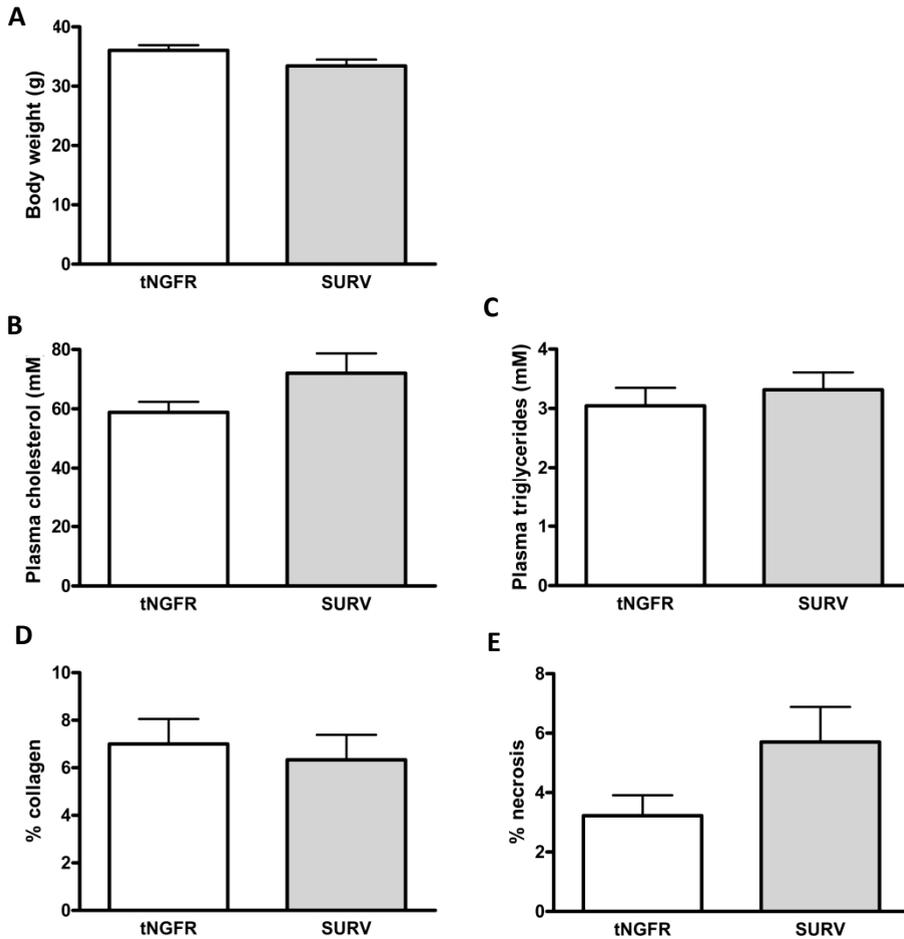


Figure S4.2: Body weight, plasma lipids and atherosclerosis parameters of vaccinated mice.

(A) Body weight of the mice after 11 weeks of high-fat diet. (B-C) At the end of the experiment, blood was drawn from the mice and plasma cholesterol (B) and triglyceride (C) levels were measured enzymatically. None of these parameters showed significant differences. (D-E) Histochemical analysis of the lesions revealed no difference in plaque collagen content (D) and necrotic area (E).

Summary

Cardiovascular events are the number one cause of death in western society and even gain quickly in importance since a disease-promoting lifestyle becomes more common. Obesity, high blood pressure, smoking and a lack of exercise are just some of the risk factors that contribute to atherosclerosis, the underlying pathogenesis of most of these cardiovascular diseases. While we have a fair idea of the cellular and molecular mechanisms that contribute to atherogenesis, treatment options are still mainly focused on reducing the risk factors.

The disease initiates when macrophages migrate to the vessel wall of an artery, where they take up modified lipids. This turns them into large, lipid-laden foam cells that cannot re-enter the blood stream but instead secrete factors that create a local inflammation, attracting even more macrophages and other immune cells. This accumulation of lipids and cells in the vessel wall is called an atherosclerotic plaque. In the chronic disease phase, the plaque will further grow until it ruptures and thereby forms a blood clot that can occlude downstream blood vessels. Depending on the location of the affected blood vessel, this results in acute manifestations such as ischemia, stroke or heart attack.

The macrophages are the most abundant cell type within the plaques and play a key role in all disease phases, from early atherogenesis to plaque rupture. This makes them potentially interesting targets for future therapies. Consequently, the experiments presented in this thesis mainly focus on the macrophages and their signaling pathways that are involved in the creation of an inflammatory environment as well as the recruitment of other cells to the plaque.

In **Chapter 2**, we investigated the effect of type I interferons (IFN) on the plaque macrophages. Although classically seen as antiviral mediators, it is becoming increasingly clear that these cytokines also play a major role in normal physiology and can act both as pro- or anti-inflammatory mediators in a variety of non-pathogen associated diseases. Using both treatment with IFN β and myeloid specific knockouts for the type I interferon (IFN) receptor (IFNAR1) we have investigated the functional role of type I IFN in atherosclerosis in experimental models. We show that IFN β enhances atherogenesis in two well established mouse models of atherosclerosis by upregulating chemokines and chemokine receptors that are necessary for atherosclerotic lesion development. In addition, we show that major targets for endogenous type I IFNs in atherosclerosis are myeloid cells, since mice with a myeloid specific deletion of IFNAR1 show reduced accumulation of myeloid cells in atherosclerotic lesions and thereby have reduced disease development. Moreover, we show that type I IFN signaling pathways are also activated in human atherosclerotic lesions and are associated with plaque rupture, making them potentially relevant for clinical outcome of disease.

It has been shown before that both the level of inflammation and the balance between pro- and anti-inflammatory mechanisms tremendously regulate the growth and composition of the atherosclerotic plaque. An important transcription factor involved in this regulation is NF- κ B, which has indeed been described, by our group and others, to affect the process of atherogenesis. Its precise role(s) in the different cell types present in the plaque however remains unclear. In **Chapter 3**, the role of the NF- κ B pathway in atherogenesis is further assessed by deletion of NF- κ B's main inhibitor, I κ B α , in the myeloid cells of a murine atherosclerosis model. It resulted in a two-fold increase in the size of the plaques. Further analysis suggests that myeloid I κ B α deletion promotes atherogenesis by an enhanced recruitment of myeloid cells to plaques. This was supported by an immunohistochemical staining of the plaques with the ER-MP58 antibody, a novel method to quantify myeloid cell recruitment to the atherosclerotic plaque that we introduce in this chapter. This effect of myeloid I κ B α deletion on murine atherogenesis is of high relevance in the further assessment of the role of inflammatory signaling pathways in the atherosclerotic plaque.

Chapter 4 features a new vaccination strategy in the treatment of experimental atherosclerosis, using a lentiviral vaccination approach to specifically target newly recruited macrophages in the atherosclerotic plaque. Evoking an immune reactivity against the antigen 'survivin' indeed resulted in a reduction of the plaque size and a local accumulation of cytotoxic T cells without causing adverse side effects.

Survivin is mainly known as a tumor-specific antigen since its expression in healthy cells is low but it is upregulated in virtually all tumors. Several survivin-based (gene) therapies targeting tumor cells have been developed in experimental models and some of them have already been applied in the clinic. Next to its tumor-specificity however, an article published a few years ago described that newly recruited macrophages in human atherosclerotic plaques also express survivin under the influence of local growth factors.

We confirm survivin expression in the newly recruited macrophages of murine plaques, target them using a vaccination strategy with a survivin-expressing lentivirus and demonstrate that this results in a significant reduction in lesion growth accompanied by an induction of recruitment of CD3⁺ T cells and CD8⁺ cytotoxic T cells to the plaque. This completely novel approach in experimental atherosclerosis may open up the potential of new therapies for this disease.

In **Chapter 5**, we describe the characteristics of different macrophage subsets and their specialized functions. While this wide range of phenotypes is already well described in other diseases with a key role for macrophages, like infection diseases and cancer, it most probably also affects atherogenesis. In this chapter we give an overview of the current relevant knowledge on macrophage heterogeneity and its putative roles in

disease progression, but also speculate on diagnostic and therapeutic possibilities that may benefit from it.

In conclusion, this thesis illustrates through different KO and treatment models the importance of macrophages and their inflammatory and chemotactic signaling pathways in the pathogenesis of atherosclerosis. Next to showing the disease promoting effect of enhanced recruitment of macrophages to the plaque, we also demonstrate that the specific targeting of these newly recruited cells may present a new strategy for plaque reducing therapies.

Samenvatting

Cerebrovasculaire accidenten zijn de voornaamste doodsoorzaak in de westerse samenleving en winnen nog steeds aan belang wegens het groeiend aantal mensen met een levensstijl die deze aandoeningen in de hand werkt. Obesitas, een verhoogde bloeddruk, roken en een gebrek aan lichaamsbeweging zijn slechts enkele van de risicofactoren die bijdragen tot atherosclerose, in de volksmond ook wel aderverkalking genoemd, de ziekte die de meeste van die accidenten veroorzaakt. En hoewel we al vrij goed op de hoogte zijn van de cellulaire en moleculaire mechanismen die verantwoordelijk zijn voor het ziekteverloop van atherosclerose, blijven de therapeutische opties voorlopig beperkt en wordt er vooral gefocust op het elimineren van de risicofactoren.

Atherogenese begint wanneer macrofagen vanuit het bloed naar de vaatwand van een slagader migreren, waar ze chemisch gemodificeerde lipiden opnemen. Hierdoor veranderen ze in zogenaamde schuimcellen. Deze grote, met vet gevulde cellen kunnen niet meer terug migreren naar de bloedbaan en zitten dus vast in de vaatwand. Van daar secreteren ze specifieke ontstekingsseiwitten die nog meer macrofagen en andere immuuncellen rekruteren. Deze lokale ontstekingshaard en de opeenstapeling van cellen in de vaatwand wordt een atherosclerotische plaque genoemd. Gedurende de chronische fase van de ziekte zal deze plaque steeds verder groeien tot ze uiteindelijk openscheurt, waarbij zich een bloedklonter vormt die stroomafwaarts kleinere slagaders kan verstoppen. Afhankelijk van de slagader waarin de bloedstroom wordt afgesloten veroorzaakt dit acute problemen zoals lokaal zuurstoftekort, een beroerte of een hartaanval.

Een plaque bestaat voor een groot deel uit macrofagen en zij spelen dan ook een hoofdrol in elke fase van de ziekte, van de vroegste atherogenese tot het scheuren van de plaque. Dit maakt ze interessante doelwitten in de zoektocht naar nieuwe therapieën. De experimenten die in deze thesis worden beschreven focussen daarom voornamelijk op de rol van macrofaag signaaltransductie in het creëren van een inflammatoire omgeving en het aantrekken van nieuwe cellen naar de plaque.

In **Hoofdstuk 2** gaan we dieper in op het effect van type I interferonen (IFN) op plaquemacrofagen. Hoewel deze cytokines vooral zijn bestudeerd omwille van hun antivirale effecten, krijgen hun fysiologische functies steeds meer aandacht en is het duidelijk dat ze ook in andere, niet-infectieuze ziektes een rol spelen die zowel pro- als anti-inflammatoir kan zijn. Onze studie van deze type I interferonen in atherosclerose bestond zowel uit KO- als behandelingsexperimenten: in twee verschillende muismodellen voor atherosclerose konden we aantonen dat een dagelijkse behandeling met IFN β de atherosclerotische plaquegroei stimuleert via een verhoogde productie van enkele chemokines en chemokinereceptoren die essentieel zijn in de

plaquevorming. Daarnaast beschrijven we hoe, binnen de plaque, myeloïde cellen het belangrijkste doelwit zijn van endogene interferonen, aangezien muizen waarin de receptor voor IFN (IFNAR) specifiek ontbreekt in de beenmerg afgeleide cellen minder accumulatie van cellen in de vaatwand vertonen en daardoor een vertraagd ziekteverloop hebben. Ten slotte bekijken we ook atherosclerotische plaques uit patiënten, waarin de IFN-signalering ook is geactiveerd en kan worden geassocieerd met het scheuren van de plaque. Dit wijst op een mogelijke klinische relevantie die verder onderzoek vereist.

Het is reeds vaak beschreven dat de groei en samenstelling van de atherosclerotische plaque zeer afhankelijk is van de mate van ontsteking alsook van de balans tussen pro- en anti-inflammatoire mechanismen. Bij de regulatie hiervan is een centrale rol weggelegd voor de transcriptiefactor NF- κ B, waarvan inderdaad eerder al door zowel onze groep als andere groepen een invloed op het atherogeneseproces is beschreven. Desondanks blijft het precieze mechanisme in de verschillende celtypes van een plaque voorlopig onduidelijk. In **Hoofdstuk 3** wordt deze rol van NF- κ B verder onderzocht door middel van een muismodel waarin I κ B α , de belangrijkste inhibitor van NF- κ B, ontbreekt in de beenmergafgeleide cellen. Het resultaat is een verdubbeling van de plaquegrootte in de muizen met I κ B α -deletie. Verdere analyse van deze plaques suggereert dat dit kan worden verklaard door een verhoogde rekrutering van myeloïde cellen naar de plaques. Dit wordt bevestigd door immunohistochemische kleuringen van de plaques met het antilichaam ER-MP58, een in dit hoofdstuk geïntroduceerde methode voor het bepalen van de hoeveelheid nieuw-gerekruteerde macrofagen in een plaque. Dit effect van myeloïde I κ B α -deletie kan van groot belang zijn in de verdere studies naar de rol van inflammatoire signalering in plaque-ontwikkeling.

In **Hoofdstuk 4** introduceren we vaccinatie met lentivirussen die een immuunreactie tegen het antigen 'survivin' induceren als een nieuwe strategie in de behandeling van experimentele atherosclerose. Door zo specifiek de nieuw-gerekruteerde macrofagen in de plaque te viseren konden we inderdaad de plaquegrootte reduceren zonder belangrijke neveneffecten te induceren. Survivin is vooral bekend als tumorspecifiek antigen aangezien het nauwelijks detecteerbaar is in gezonde cellen maar sterk tot expressie komt in zowat alle tumoren. Bij onderzoek in experimentele tumormodellen werd van deze eigenschap reeds gebruik gemaakt voor het ontwikkelen van verschillende survivin-gebaseerde (gen)therapieën, waarvan sommige intussen zelfs al succesvol in de kliniek worden toegepast. Een publicatie van enkele jaren geleden beschreef echter dat, naast deze tumorspecificiteit, nieuw-gerekruteerde macrofagen van humane plaques ook survivin tot expressie brengen onder invloed van lokale groeifactoren. Wij bevestigden de aanwezigheid van deze survivin-positieve, nieuw-gerekruteerde macrofagen in atherosclerotische plaques van de muis. Wanneer we

deze cellen gericht viseerden door middel van een vaccinatie met survivin-producerende lentivirussen, resulteerde dit in een significante vermindering van de plaquegroei en een verhoogde aanwezigheid van CD3⁺ T-cellen en CD8⁺ cytotoxische T-cellen in de plaques. Deze nieuwe behandelingsstrategie in experimentele atherosclerose zou het pad kunnen effenen voor nieuwe therapiemogelijkheden.

In **Hoofdstuk 5** beschrijven we de eigenschappen en specifieke functies van de verschillende macrofaagsubsets. Hoewel het bestaan van een brede waaier aan macrofaagfenotypes reeds uitvoerig is beschreven bij ziektes met een hoofdrol voor macrofagen, zoals infectieziektes en kanker, is dit concept hoogstwaarschijnlijk ook relevant voor de macrofagen in een atherosclerotische plaque. We geven daarom eerst een overzicht van de relevante kennis die over deze verschillende macrofagen bestaat, relateren dit aan de gekende macrofaagfuncties in een plaque en beschrijven tot slot enkele mogelijke diagnostische en therapeutische toepassingen.

Samenvattend illustreert deze thesis in verschillende KO- en behandelingsmodellen het belang van macrofagen en hun inflammatoire en chemotactische signalering in de pathogenese van atherosclerose. Behalve een sneller ziekteverloop ten gevolge van een verhoogde rekrutering van macrofagen naar de plaque, tonen we ook dat een specifieke targeting van deze nieuw-gerekrueteerde cellen een nieuwe strategie voor atherosclerose-therapieën kan zijn.

List of abbreviations

ACE	Angiotensin-converting enzyme
acLDL	Acetylated LDL
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ApoE	Apolipoprotein E
Arg1	Arginase-1
ATM	Adipose tissue macrophage
Bcl	B-cell lymphoma-encoded protein
bFGF	Basic fibroblast growth factor
BMM	Bone marrow-derived macrophage
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
COX	Cyclooxygenase
Cre	Cre recombinase
CT	Computed tomography
CTL	Cytotoxic T cell
Ctrl	Control mice/cells
CVD	Cardiovascular disease
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDG	Fluorodeoxyglucose
Flox	Flanked by loxP
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony stimulating factor
HDL	high-density lipoprotein
HSP	Heat-shock protein
IAP	Inhibitor of apoptosis protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
IFNAR	Interferon- α/β receptor
IFNGR	Interferon- γ receptor
I κ B	Inhibitor of NF- κ B

IKK	I κ B kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
iNOS	Inducible nitric oxide synthase (=NOS2)
IP3	Inositol triphosphate
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
JAK	Janus kinase
KO	Knock-out
LDL	low-density lipoprotein
LDLR	LDL receptor
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LysM	Lysozyme M
MAP	Mitogen-activated protein
MAPK	MAP kinase
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
mLDL	Modified low-density lipoprotein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MR	Mannose receptor
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
Mx	Myxovirus resistance
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear factor- κ B
NK	Natural killer cell
NKT	Natural killer T cell
NLRP3	NOD-like receptor family, pyrin domain containing 3 (=NALP3)
NO	Nitric oxide
OAS	2'-5'-oligoadenylate synthetase
oxLDL	Oxidized LDL
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PET	Positron emission tomography
PKC	Protein kinase C

PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand 1
RANTES	Regulated upon activation, normal T cell expressed and secreted
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
SPECT	Single-photon emission computed tomography
SR-A	Class A scavenger receptor
STAT	Signal transducer and activator of transcription
SURV	Survivin
sVSG	Soluble variant-specific surface glycoprotein
TAM	Tumour associated macrophage
TF	Tissue factor
TGF- β	Transforming growth factor β
Th1/2	T helper 1 or 2 cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAIL	TNF-related apoptosis-inducing ligand
T _{reg}	Regulatory T cell
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
TZD	Thiazolidinediones
UPR	Unfolded protein response
USPIO	Ultrasmall superparamagnetic particles of iron oxide
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen 4
VSG	Variant-specific surface glycoprotein

Dankwoord

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Immediately thereafter I had the opportunity to start working in the group of Dr. Michael Kalai at the Pasteur Institute of Brussels. Miki, I really appreciate your trust in me! And although our (too) short collaboration didn't result in publications, it sparked my passion for innate immunity. Dankzij onze samenwerking met het DMBR in Gent heb ik veel interessante mensen leren kennen, zoals Dr. Rudi Beyaert, Dr. Peter Vandenaabeele, Dr. Mohamed Lamkanfi en de mensen in hun groepen.

Al van mijn negende was ik elke zondagmiddag te vinden bij Chiro Ommekaar. Zeventien jaar lang heb ik mij met jullie geamuseerd in de Vlassenbroekse polders! Bedankt aan alle leiding, oud-leiding en leden voor deze fantastische tijd!

In de weekends waarin ik Maastricht ontvluchtte en naar Dendermonde ging heb ik mij nooit verveeld dankzij de Ridders van de Vettige Tafel, merci mederidders Maarten, Timo en Roel! En ook bedankt aan het Ardennenoffensief-team (Ewoud, Timmie, Steven & Blonde Nele, enniroC, Tine, Dompie, Benji & Nele, Yves & Liesbeth, Merel & Collin, Lijne, ...) voor de leuke tijden samen. En laat ik natuurlijk ook Kurt & Linda, Staaf & Marie-Antoinette, Jan, Zanne, Billy, Bram & Griet, Piet, die Mädchen,... niet vergeten!

En dan wordt het hoog tijd om mijn familie te bedanken. Hoewel, ik zou eerst even willen beginnen bij Guy en Liliane, die eigenlijk geen familie zijn maar waar ik me intussen al lang een kleinzoon van voel! Guy, ik voel me zeer vereerd dat je zo veel tijd gespendeerd hebt aan de unieke cover van dit boekje. Ik hoop dat je er even tevreden over bent als ik zelf. De originele tekening is intussen ingekaderd en krijgt een ereplaats in ons appartement in Marseille. Liliane, ik wens je al het beste met je gezondheid! Hopelijk gaan we jullie nog af en toe eens kunnen zien nu we zo ver wonen...

Heel veel dank ben ik natuurlijk verschuldigd aan mijn ouders! Al van zeer jonge leeftijd werd leergierigheid erg gestimuleerd. Zo kon ik namen van componisten opsommen voor ik zelfs nog maar deftig kon spreken, kon ik lezen (en zelfs al wat schrijven, ook dankzij meter natuurlijk) voor ik dat op school zou leren, ging ik in de bibliotheek enkel bij de boeken van een hogere leeftijdscategorie zoeken en was ik in een opstel zelden of nooit op een taalfout te betrappen. Toen ik in het zesde studiejaar de vraag kreeg wat ik in het secundair zou gaan studeren antwoordde ik dan ook met de vraag "Wat is het moeilijkste?", en ook zes jaar later heb ik me niet neergelegd bij het studieadvies maar koppig hoger gemikt (bedankt dat jullie mij toen ook het vertrouwen gegeven hebben). Ongetwijfeld pluk ik nu nog steeds de vruchten van die onverzadigbare nieuwsgierigheid, en in tijden waarin alles wat al gekend is eenvoudig op te zoeken valt ga ik dan maar op zoek naar dingen die nog niet ontdekt zijn. Die zoektocht leidde mij ook steeds verder weg van huis: van het schooltje op 't Keur naar het college in Dendermonde, van daar naar Brussel en dan via Maastricht nu naar Marseille. Gelukkig (voor ons moederke) is dit nog steeds te overbruggen met de auto, al zullen jullie mij nu ongetwijfeld nóg minder zien. Maar ooit kom ik wel weer wat dichterbij wonen hoor...

Zus, amuseer u met de Sisters maar vooral ook met Björn. En vergeet niet om regelmatig eens op bezoek te komen, kies u maar al eens een match van l'OM uit waar we samen naar toe kunnen!

Ook de rest van mijn familie is heel erg bedankt voor alle hulp en interesse! Sinds een paar jaar hoort daar ook mijn schoonfamilie bij, ik ben blij dat ik me zo welkom mag voelen bij jullie!

En zoals het hoort eindig ik dit dankwoord met de allerbelangrijkste persoon, diegene aan wie ik het allermeeft gehad heb: Gudrun, mijn schatteke, ik zou gemakkelijk nog ne keer zo'n boekje kunnen vullen als ik zou moeten beschrijven wat gij voor mij betekent, maar ik weet dat ge liever niet te veel *in the picture* staat. Toch zou ik u enorm willen bedanken voor alle hulp, plezier, steun, verdraagzaamheid en vooral liefde die ik van u gekregen heb! We hebben op de nauwelijks 3 jaar waarin we elkaar kennen al zoveel meegemaakt en nu ik je meesleur naar Frankrijk, op avontuur in het onbekende, zal dat waarschijnlijk nog niet meteen veranderen... Bedankt da ge dat ziet zitten. En hopelijk mag ik nog voor de rest van mijn leven van u genieten!

Curriculum vitae

Pieter Goossens was born on September 11, 1981, in Dendermonde (Belgium). In 1999 he acquired his secondary school diploma in Latin – Sciences at the Heilige Maagdcollege (Dendermonde, Belgium). In September of the same year, he started to study Biomedical Sciences at the VUB (dutch-speaking Free University of Brussels) in Jette, Belgium, obtaining his Bachelor (candidate) diploma in 2001. In 2003, he got his Master (licentiate) degree cum laude after performing an internship of 10 months in the Laboratory for Molecular and Cellular Therapy at the Department of Immunology and Physiology of the VUB (Jette, Belgium). Under the supervision of Dr. Karine Breckpot, Lieven Straetman and Prof. Dr. Kris Thielemans he investigated the then newly-discovered protein survivin as a target in dendritic cell-based cancer immune therapy and as a potential tool for cell immortalization. In agreement with the university, this Master's degree was further complemented with a one-year custom made program, combining selected courses from the curriculum of the 2nd and 3rd year of the Bio-Engineer studies (specialization Cell and Gene Biotechnology) of the VUB in Etterbeek, Belgium. These courses included Microbiology, Virology, Parasitology, Immunology and Molecular Biotechnology.

In 2004, Pieter started working as a research fellow at the Laboratory of Cellular Microbiology of the Pasteur Institute Brussels (Belgium), under the supervision of Dr. Michael Kalai (Pasteur Institute Brussels) and Dr. Rudi Beyaert (Department for Molecular Biomedical Research, VIB Gent, Belgium). Supported by grants of Les Amis de l'Institut Pasteur and the Jean et Rose Hoguet Foundation, he assisted in organizing this newly-established lab and started investigating the interaction of the hepatitis G virus with the antiviral and ER stress pathways of its host cells. Less than two years later however, this project was discontinued prematurely due to a lack of funding.

In 2006, Pieter joined the Macrophage Group Maastricht of Dr. Menno de Winther in the Molecular Genetics department of the Maastricht University and the Cardiovascular Research Institute Maastricht (CARIM). His research on the role of macrophages and their inflammatory signaling in mouse models of atherosclerosis led to the findings described in this thesis. These experiments were supported by a Marie Curie Research Training Fellowship and the Netherlands Heart Foundation. Next to his own research, Pieter also mentored several students during their internships in the lab. His results were published in high-impact journals and presented on conferences and in labs in the Netherlands as well as the USA, Canada, Germany, Denmark, France and Greece.

Currently, Pieter is working in Marseille (France), in the Inflammation Biology Group of Dr. Toby Lawrence at the Centre d'Immunologie de Marseille-Luminy (CIML). Here, he is studying the phenotype of tumour-associated macrophages and the regulation of their inflammatory signaling pathways.

List of publications

Goossens P*, Stöger JL*, De Winther MPJ (*equally contributing)
Macrophage heterogeneity: relevance and functional implications in atherosclerosis
Current Vascular Pharmacology 2010, Mar; 8(2): 233-48 (Review)

Goossens P, Gijbels MJJ, Zerneck A, Eijgelaar W, Vergouwe MN, Van der Made I, Beckers L, Buurman WA, Daemen MJAP, Kalinke U, Weber C, Lutghens E, De Winther MPJ
Myeloid type I interferon signaling promotes atherosclerosis by accelerating macrophage recruitment to lesions
Cell Metabolism 2010, Aug; 12(2): 142-53

Lievens D, Zerneck A, Seijkens T, Soehnlein O, Beckers L, Munnix I, Wijnands E, **Goossens P**, Van Kruchten R, Thevissen L, Boon L, Flavell RA, Noelle RJ, Gerdes N, Biessen EA, Daemen MJAP, Heemskerk JWM, Weber C, Lutgens E
Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis
Blood 2010, Nov; 116(20): 4317-27

Goossens P, Vergouwe MN, Curfs D, Van Woezik JHG, Van der Made I, Rupec RA, Hofker M, Gijbels MJJ, De Winther MPJ
Myeloid I κ B α deficiency induces macrophage accumulation in atherosclerotic lesions
PLoS ONE 2011, 6(7):e22327

Submitted publications

Goossens P, Van Puijvelde GH, Wolfs IMJ, Gijbels MJJ, Breckpot K, Straetman L, Heirman C, Donners MPC, Bot I, Thielemans K, Kuiper J, De Winther MPJ
A lentiviral vaccination strategy targeting the tumor antigen survivin reduces murine atherogenesis

Highlighted publications

Bird L
Macrophages: Interferons promote artery lesions
(Highlighting **Goossens** et al., *Cell Met* 2010)
Nature Reviews Immunology 2010 Sep; 10, 616-617

Published abstracts

Goossens P, Gijbels MJJ, Zerneck A, van der Made I, Beckers L, Buurman WA, Weber C, Kalinke U, Lutgens E, de Winther MPJ
Type I interferons promote atherosclerosis by affecting chemotaxis and cell death
Circulation 2008, 118: S 308

Goossens P, Vergouwe MN, Curfs D, Rupec RA, Gijbels MJJ, de Winther MPJ
Myeloid specific absence of I κ B α aggravates atherosclerosis without altering plaque stability
European Journal of Immunology 2009 Sep; 39-S1

Fellowships & awards

Poster prize - Maastricht Medical Student Research Conference	(2003, Maastricht, NL)
Les Amis de l'Institut Pasteur Fellowship	(2004-2005, Brussels, BE)
Jean et Rose Hogue Foundation Fellowship	(2005-2006, Brussels, BE)
Marie Curie Research Training Fellowship	(2006, European Commission)
Oral presentation award - Dutch Heart Foundation PhD meeting	(2007, Papendal, NL)
Poster prize - CARIM PhD student meeting	(2008, Maastricht, NL)
Young investigator award - Scandinavian Society for Atherosclerosis Research	(2008, Humlebæck, DK)
Invitation to present - ATVB Early Career reception	(2008, New Orleans, USA)
Oral presentation award - Dutch Atherosclerosis Society meeting	(2010, Ede, NL)

Oral presentations

Netherlands Heart Foundation PhD meeting	(2007, Papendal, NL)
11 th Dutch Atherosclerosis Society meeting	(2008, Ermelo, NL)
Gesellschaft für Mikrozirkulation und Vaskuläre Biologie meeting	(2008, Aachen, DE)
American Heart Association Scientific Sessions	(2008, New Orleans, USA)
Cardiovascular Research Institute Maastricht meeting	(2009, Maastricht, NL)
Maastricht Genetics and Cell Biology plenary sessions	(2010, Maastricht, NL)
13 th Dutch Atherosclerosis Society meeting	(2010, Ede, NL)
1 st CARIM PhD workshop on publishing in high impact journals	(2010, Maastricht, NL)
La Jolla Institute for Allergy and Immunology seminar	(2010, San Diego, USA)
The Scripps Research Institute, Bruce Beutler lab seminar	(2010, San Diego, USA)
UC Berkeley Division of Immunology & Pathogenesis seminar	(2010, Berkeley, USA)
Centre d'Immunologie de Marseille-Luminy seminar	(2011, Marseille, FR)
Alexander Fleming Biomedical Sciences Research Center seminar	(2011, Athens, GR)

Poster presentations

7 th Maastricht Medical Student Research Conference	(2003, Maastricht, NL)
Netherlands Heart Foundation PhD meeting	(2006, Papendal, NL)
10 th Dutch Atherosclerosis Society meeting	(2007, Ermelo, NL)
Netherlands Heart Foundation PhD meeting	(2007, Papendal, NL)
Keystone Macrophage (D2) meeting	(2007, Copper Mountain, USA)
CARIM symposium	(2007, Maastricht, NL)
1 st CARIM PhD student meeting	(2008, Maastricht, NL)
14 th Scandinavian Society for Atherosclerosis Research meeting	(2008, Humlebæck, DK)
Netherlands Heart Foundation PhD meeting	(2008, Papendal, NL)
CARIM symposium	(2008, Maastricht, NL)
ATVB Early Career reception	(2008, New Orleans, USA)
Maastricht Genetics and Cell Biology plenary sessions	(2009, Maastricht, NL)
12 th Dutch Atherosclerosis Society meeting	(2009, Ede, NL)
2 nd European Congress of Immunology	(2009, Berlin, DE)
Keystone Atherosclerosis & Macrophage (J7 & J8) meeting	(2010, Banff, Canada)

Other conferences attended

Life, a Noble Story	(2004, Brussel, BE)
Maastricht Medical Student Research Conference	(2004, Maastricht, NL)
9 th Dutch Atherosclerosis Society meeting	(2006, Ermelo, NL)
Immunology Research Maastricht session	(2006, Maastricht, NL)
Belgian Immunological Society meeting on macrophage biology	(2007, Gent, BE)
European Vascular Genomics Network workshop	(2008, Maastricht, NL)
CARIM PhD student weekend	(2008, Kerkrade, NL)
New Frontiers in Pattern Recognition Receptors symposium	(2009, Nijmegen, NL)
European Macrophage and Dendritic cell Society meeting	(2011, Brussel, BE)
FWO Meeting on Signaling in Inflammation and Immunity	(2011, Gent, BE)

Student's projects supervised

<i>In vitro</i> models for macrophage heterogeneity	
Struijk R & Mattheij N , Bachelor Molecular Life Sciences	(2007 – 2008)
Polarizing macrophages with helminth soluble egg antigens to combat atherosclerosis	
Wolfs IMJ , Master Biomedical Sciences	(2008 – 2009)
Unraveling the different signaling mechanisms in activated macrophage subsets	
Hoeksema MA , Master Molecular Life Sciences	(2009 – 2010)