Macrophage regulatory mechanisms in atherosclerosis

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Chapter 1

General introduction
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Atherosclerosis

Cardiovascular disease is still the leading cause of mortality worldwide (1). The main underlying cause is atherosclerosis, a lipid driven chronic inflammatory disorder of the arteries. Elevated levels of circulating low density lipoprotein (LDL) are associated with increased risk for atherosclerosis and cardiovascular disease (2). Within the arterial wall LDL can be modified (e.g. oxidized) resulting in immune cell activation and initiation of inflammatory responses. In response, blood monocytes are attracted to the site of inflammation (3). Once monocytes enter the arterial intima they differentiate into macrophages which make up the majority of immune cells present in atherosclerotic lesions and are important regulators of the disease (3). Atherosclerotic macrophages engulf modified lipids and turn into foam cells (Figure 1A). Besides foam cell characteristics, macrophages can adopt to a range of activation states (4). Foam cell formation and macrophage activation results in ongoing inflammation and disease progression. Also neutrophils, mast cells, dendritic cells, B cells and T cells are present in atherosclerotic lesions and contribute to lesion inflammation (5, 6). Excessive accumulation of lipids results in foam cell cytotoxicity, cell death and necrotic core formation. Atherosclerotic lesion growth causes smooth muscle cells (SMCs) to migrate from the media to the intima where activated SMCs produce collagen, elastin and proteoglycans, which together make up the fibrous cap that stabilize lesions (Figure 1B). Macrophages also contribute to plaque stabilisation by the production of matrix metalloproteinases that degrade the fibrous cap matrix components (7). Atherosclerotic lesion growth results in narrowing of the lumen, which can cause an occlusion. Unstable lesions are characterized by a large necrotic core and thin fibrous cap and these can ultimately rupture and initiate thrombosis (Figure 1C). This can result in a myocardial infarction or stroke. Patients at high cardiovascular risk are currently mainly treated with lipid-lowering agents, but besides these lipid-lowering actions, patients still have a strong residual risk for cardiovascular events (8). Since atherosclerosis is an inflammatory disorder, clinical trials with anti-inflammatory therapeutics (e.g. CANTOS and CIRT trial) have been performed in cardiovascular patients and the first results are considered to be predominantly beneficial (9, 10). Atherosclerosis is thus a multifactorial disease driven by lipids and inflammation. A better understanding of both the lipid metabolism and inflammatory processes in atherosclerosis (and the link between the two) is necessary to identify regulatory pathways and novel targets for atherosclerosis treatment.
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LDL as a risk factor in atherosclerosis

It is well established that elevated levels of LDL and other apolipoprotein B (ApoB) containing lipoproteins, like very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and lipoprotein(a) (Lp(a)) are associated with increased risk for atherosclerosis (12). ApoB100 is the main structural apolipoprotein of LDL and is mainly produced by the liver. ApoB100 is necessary for the synthesis and secretion of VLDL, which consist mainly of triglycerides (TG). The triglycerides of VLDL particles are hydrolyzed by lipoprotein lipase and hepatic lipase, which results in the formation of cholesteryl ester enriched particles IDL and LDL. LDL particles make up the majority of ApoB containing lipids in the blood and are cleared by the liver via the LDL receptor (LDLR). LDL, but also VLDL, IDL and Lp(a) from the bloodstream can enter the arterial intima (13). Having normal LDL-levels in the blood, this retention of lipids in the intima is of relative low risk to cardiovascular disease. However, increased levels of circulating LDL cause increased retention of LDL in the arterial intima contributing to the initiation of lesion formation (13). Recently, the European Atherosclerosis Society Consensus Panel stated that there is a causal link between LDL and atherosclerosis cardiovascular disease.
disease based on evidence from genetic, epidemiologic and clinical studies (2). They state that there is a consistent dose-dependent log-linear association between the absolute magnitude of LDL exposure and the risk of atherosclerosis.

Familial hypercholesterolemia (FH) is a genetic disorder associated with increased risk of atherosclerosis. FH-patients have elevated LDL-levels in circulation and are marked by premature atherosclerosis (14). Loss-of-function mutations in the LDLR are the most common mutations causing FH. Other mutations include loss-of-function mutations in the APOB gene or gain-of-function mutation in the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene, all resulting in elevated circulating LDL-levels (14). FH-patients are mainly treated with statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that block the synthesis of cholesterol in the liver, thereby effectively lowering plasma LDL levels and the risk for cardiovascular disease (15). Nevertheless, a part of these patients will not reach effective LDL lowering by statins (16). Besides, there are also patients that are statin-intolerant, mainly due to statin-associated muscle symptoms (SAMS) (17). In recent years, monoclonal antibodies against PCSK9 have emerged as novel drugs to lower LDL cholesterol levels (18). LDL is cleared from the blood via hepatocyte endocytosis by the LDLR and these receptors are recycled to the surface. PCSK9 binds to the LDLR resulting in lysosomal degradation (Figure 2A). Using antibodies that inhibit PCSK9, LDL receptors are more recycled to the cell surface enhancing clearance of LDL from the circulation (Figure 2B) (19). PCSK9 monoclonal antibodies significantly lower LDL cholesterol, alone or added on top of standard statin therapy (20-24). Recently it was also shown that PCSK9 antibody treatment combined with statins effectively lowers cardiovascular events (25) making them valuable for patients who do not reach desired LDL lowering by statins alone. The link between lipids and immune cell activation should be further addressed since both play a key role in atherosclerosis development.
Monocytes, macrophages and foam cells in atherosclerosis

Monocytes develop from myeloid precursors in the bone marrow and fetal liver, where after they circulate in the blood (26). In addition, monocytes can be mobilized from the splenic hematopoietic stem and progenitor cells under inflammatory conditions like atherosclerosis (27, 28). Two major monocyte subsets are described in mice and three in humans. In mice these are the classical (Ly6C<sup>hi</sup>) and non-classical (Ly6C<sup>low</sup>) monocytes, whereas in humans these are the classical (CD14<sup>++</sup>, CD16<sup>-</sup>), intermediate (CD14<sup>+</sup>, CD16<sup>+</sup>) and non-classical (CD14<sup>low</sup>, CD16<sup>++</sup>) monocytes (29). In both mice and humans, classical monocytes are characterized by high C-C chemokine receptor type 2 (CCR2) expression, whereas non-classical monocytes are characterized by high CX3C chemokine receptor 1 (CX<sub>3</sub>CR1) expression (30). The classical monocytes are described to be inflammatory monocytes that respond strongly to bacterial stimuli via toll like receptor (TLR) 4 and are recruited to sites of inflammation where they extravasate and differentiate into macrophages. Non-classical monocytes on the other hand respond to viral stimuli via TLR7 and TLR8 (31) and crawl and patrol the endothelium (32, 33).
In atherosclerosis, especially classical monocytes are recruited to sites of inflammation (34). During lesion progression there is continuous recruitment of monocytes that differentiate into macrophages in the presence of colony-stimulating factor 1 (CSF1; M-CSF). Besides by recruitment of blood monocytes, lesional macrophages increase in number by local proliferation, thereby contributing to the pool of macrophages present in atherosclerotic lesions (35). The lesional macrophages take up modified LDL (oxLDL) mainly via the scavenger receptors cluster of differentiation 36 (CD36) and SR-A1 (36, 37). OxLDL is intracellularly hydrolyzed into free cholesterol and fatty acids. Free cholesterol is subsequently re-esterified into cholesterol-esters via acyl-CoA cholesterol ester transferase (ACAT) eventually turning macrophages into foam cells (Figure 3). Excessive cholesterol uptake can lead to lipotoxicity resulting in foam cell apoptosis and combined with defective efferocytosis (clearance of apoptotic cells by macrophages), this results in the formation of a necrotic core. Lipid accumulation in macrophages also induces the expression of the cholesterol efflux genes ATP binding cassette transporters A1 and G1 (ABCA1 and ABCG1). Both promote cholesterol efflux to HDL particles, directly (ABCG1) and indirectly (ABCA1) for reverse cholesterol transport (Figure 3).

Besides obtaining foam cell characteristics, macrophages also contribute to atherosclerotic lesion inflammation by responding to danger signals (e.g. cholesterol crystals and oxLDL) and inflammatory triggers such as cytokines (Figure 3). OxLDL and cholesterol crystals can induce the NLR family pyrin domain containing 3 (NLRP3) inflammasome, which results in the secretion of the pro-inflammatory cytokine IL-1β (38, 39). In addition, oxLDL is recognized by various TLR receptors causing secretion of cytokines like TNF, IL-6 and IL-10 (40, 41). In contrast, oxLDL blocks the cytokine response via TLR2/4 signaling in human monocytes and macrophages (42, 43). In a mouse model of peritoneal foam cell formation it was further shown that foam cell formation dampens inflammatory responses, an effect which is mediated by desmosterol and liver X receptor (LXR) ligand (44). Macrophage foam cell formation is thus associated with various inflammatory responses. In human atherosclerotic lesions both inflammatory as well as anti-inflammatory macrophages are present (45). Pro-inflammatory macrophages are mainly found in rupture-prone shoulder regions. On the other hand, adventitial tissue contains more anti-inflammatory macrophages (45).

Accordingly, monocytes, macrophages and foam cells are the central players in atherosclerosis where they contribute to lesion development in all stages of atherosclerosis.
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Figure 3: Macrophage foam cell formation. Macrophages internalize LDL, VLDL and oxLDL via macropinocytosis, phagocytosis and scavenger receptor-mediated uptake. OxLDL is intracellularly hydrolyzed into free cholesterol and fatty acids. Free cholesterol is then re-esterfucrated into cholesterol-esters via ACAT turning macrophages into foam cells. The accumulation of cellular cholesterol upregulates expression of the cholesterol efflux genes ABCA1 and ABCG1. This mediates the transfer of free cholesterol to lipid-HDL. Excessive free cholesterol accumulation can induce cholesterol crystal formation to activate the NLRP3 inflammasome. OxLDL is not only a ligand for the scavenger receptors, but can also be recognized by various TLR receptors. This signaling results in the activation of nuclear factor-κB (NF-κB) and in the production of pro-inflammatory cytokines and chemokines. from Moore KJ, Sheedy FJ, Fisher EA. Nat rev Immunol. 2013;13(10):709-21.(4).

Epigenetic mechanisms in macrophages as targets for atherosclerosis

Since macrophages are central regulators in atherosclerosis, skewing monocytes and macrophages to cells with anti-atherogenic properties could be envisaged as an athero-protective treatment. It is therefore essential to identify regulatory pathways that are important for macrophage functioning in atherosclerosis. Epigenetic pathways are now identified to play a key role in monocyte-to-macrophage differentiation and activation (46, 47). Epigenetic regulation of gene transcription refers to changes in DNA accessibility without changing the DNA itself. Several modes of epigenetic regulation are described where DNA methylation and histone modifications are most common. Histone modifications are modifications at histone tails and the type and
position of modification determines whether genes are transcribed or repressed. Histone modifications, histone modifying enzymes, their role in macrophage function and their potential as a novel therapeutic target for atherosclerosis are discussed and reviewed in detail in chapter 2 and chapter 3.

Aim and outline of the thesis
The overall aim of this thesis is to identify novel regulators in macrophages to combat atherosclerosis. The first part of my thesis focuses on the identification and validation of histone modifying enzymes in macrophages as a target for atherosclerosis in mice. The second part of this thesis focuses on regulatory mechanisms in human monocytes and macrophages (Figure 4).

We hypothesize that epigenetic regulators and histone modifications regulate the transcriptional profile of macrophages. Chapter 2 is a review on epigenetic mechanisms controlling macrophage activation and polarization. This review highlights histone modifications and histone modifying enzymes as important regulators of macrophage function. As macrophages are key immune cells in atherosclerosis, interfering with epigenetic mechanisms in these cells is an interesting approach to treat atherosclerosis. In chapter 3 we further discuss epigenetic mechanisms as a novel target for atherosclerosis. In chapter 4 we performed an in vitro screening assay with pharmacological inhibitors for histone modifying enzymes to identify epigenetic enzymes and their classes that modulate macrophage activation.

Kdm6b (also known as Jmjd3) is a histone H3K27 demethylase, which removes the repressive H3K27 methyl marks and has been a well-studied enzyme in macrophages. It was shown in literature that Kdm6b controls both the inflammatory and anti-inflammatory properties of macrophages. In chapter 5 we therefore studied the role of Kdm6b in foam cells, an important macrophage subset in atherosclerosis. We found that Kdm6b regulates the pro-fibrotic transcriptional profile of foam cells. To study the functional consequence for disease, we further studied macrophage Kdm6b in atherosclerosis. In chapter 6 we found that kdm6b deficiency in myeloid cells results in more advanced atherosclerosis. Since the H3K27 methyltransferases have opposite effects on this histone mark, we hypothesized that inhibition of the H3K27me3 methyltransferase Ezh2 in myeloid cells might be beneficial in case of atherosclerosis which is studied in chapter 7.
Next to histone modifications, DNA methylation is another important epigenetic regulator. In chapter 8 we studied the DNA methylation profile in human monocyte-to-macrophage differentiation and subsequent activation.

In chapter 9 we studied the link between LDL and monocyte activation, two key players in atherosclerosis. We hypothesize that high levels of circulating LDL are associated with monocyte activation, which can be reversed by lipid lowering. We tested this hypothesis using novel PCSK9 monoclonal antibodies in patients with FH.

Chapter 10 is a general discussion of the thesis, which summarizes the findings and discusses future research lines.

Figure 4: Schematic overview of the chapters in this thesis.
References