Macrophage regulatory mechanisms in atherosclerosis
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Chapter 10

Discussion and future perspectives
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Atherosclerosis is a multifactorial disease driven by lipids and inflammation (1). Monocytes, macrophages and foam cells are the central players in atherosclerosis and contribute to all stages of lesion formation (2, 3). The overall aim of this thesis was to identify novel regulators in monocytes and macrophages to combat atherosclerosis (chapter 1).

Epigenetic pathways have been identified to play a key role in monocyte-to-macrophage differentiation and activation (chapter 2) (4-6). We hypothesized that interference with these pathways in macrophages can improve atherosclerosis outcome (chapter 3) (7). In this thesis we validated the role of the repressive H3K27me3 methyltransferase enhancer of the zeste homolog 2 (Ezh2) and lysine demethylase 6b (Kdm6b) in macrophage foam cells and atherosclerotic lesion development in mice. In humans, we studied DNA methylation in monocyte-to-macrophage differentiation and subsequent activation. Moreover, we studied the link between low-density lipoprotein (LDL) and monocyte activation in patients with familial hypercholesterolemia (FH).

The main findings of the studies in this thesis are:
- Inhibition of epigenetic enzymes, especially Hdacs, by pharmacological inhibitors can improve atherogenic macrophage functions.
- Foam cells induce a pro-fibrotic transcriptome signature, which is partly regulated by Kdm6b.
- Myeloid Kdm6b deficiency results in advanced atherosclerosis, with lesions that contain more collagen and necrosis.
- Myeloid Ezh2 deficiency limits atherosclerosis, due to impaired neutrophil migration and a partly reduced inflammatory response of foam cells.
- DNA methylation changes occur mostly during monocyte-to-macrophage differentiation, rather than upon activation of macrophages.
- Both gain and loss of DNA methylation occurs during monocyte-to-macrophage differentiation at single CpGs or small regions at enhancer sites.
- Elevated levels of LDL-cholesterol associate with a pro-atherogenic profile of circulating monocytes, which is reversed upon lipid lowering.

Targeting histone modifying enzymes in macrophages to combat atherosclerosis

We hypothesized that skewing of macrophages to cells with anti-atherogenic functions would benefit atherosclerosis outcome. Histone modifications and its corresponding enzymes are important regulators of macrophage function and might therefore be a good target for atherosclerosis intervention (chapter 2 and 3). In chapter 4 we
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Targeting histone modifying enzymes in macrophages to combat atherosclerosis
We hypothesized that skewing of macrophages to cells with anti-atherogenic functions would benefit atherosclerosis outcome. Histone modifications and its corresponding enzymes are important regulators of macrophage function and might therefore be a good target for atherosclerosis intervention (chapter 2 and 3). In chapter 4 we
performed an in vitro screening assay with inhibitors for different classes of histone modifying enzymes (HMEs) and demonstrated that broad range inhibition of histone deacetylases (HDACs) and histone methyl transferases (HMTs) had both pro- and anti-atherogenic effects in macrophages. It was already shown that broad range inhibition of Hdacs by trichostatin A (TSA) enhances atherosclerosis as a result of enhanced lipid uptake in mice (8). Since histone modifications are regulated by a wide variety of HMEs, a better approach would be to target specific HMEs. We demonstrated that inhibition of Hdac3, switched macrophages to cells with anti-atherogenic properties (9). Hdac3-deleted mouse macrophages show an enhanced IL-4 response and a diminished LPS response, making Hdac3 a favorable target for atherosclerosis management (10, 11). Within our group, we showed that myeloid Hdac3 deficiency promotes collagen deposition in atherosclerotic lesions and induces a stable plaque phenotype, confirming that Hdac3 is one of the epigenetic enzymes that can be regarded as a promising target for atherosclerosis therapy (12).

Next, we validated the role of novel HMEs in macrophages as targets for atherosclerosis. Based on the in vitro screening assay and literature we studied the enzymes involved in H3K27 methylation. H3K27me3 is a repressive histone mark catalyzed by the polycomb repressor complex 2 (PRC2) with Ezh2 containing the catalytic subunit of the complex (13). The H3K27 demethylases Kdm6b, Utx and Uty remove repressive histone marks (14). Both LPS and IL-4 induce Kdm6b expression (15, 16) and knockdown studies show that Kdm6b is involved in both the inflammatory and anti-inflammatory response of macrophages, and is not necessary associated with a specific macrophage activation state (17-19). On top of regulating inflammatory responses, in chapter 5 we showed that kdm6b also controls the expression of profibrotic genes in foam cells, describing an additional regulatory function for Kdm6b (20). Since foam cell formation, inflammatory responses and anti-inflammatory responses all occur in atherosclerosis, we studied the involvement of myeloid Kdm6b in atherosclerosis. In chapter 6 we observed that atherosclerotic lesions of myeloid Kdm6b-deleted transplanted mice were more advanced as indicated by enhanced collagen and necrosis area. Whether this is beneficial or undesirable cannot be concluded based on these results. Increased cap thickness suggests that this is favorable but enhanced necrosis area on the other hand is associated with plaque instability (21). The pharmacological Kdm6b/Utx inhibitor GSK-J4 seemed promising in human macrophages by reducing inflammatory responses (22), but our study demonstrates that myeloid kdm6b-deficiency results in pro- and anti-atherogenic properties, making kdm6b not the best target for treatment of atherosclerosis.
Because atherosclerotic lesions of Kdm6b-deleted transplanted mice were more advanced we hypothesized that inhibition of the H3K27 methyltransferase Ezh2 is a better approach, since Ezh2 has opposite effects on H3K27 methylation compared to Kdm6b. In chapter 7 we demonstrated that lesion size of myeloid Ezh2-deleted transplanted mice were smaller, contained less neutrophils and had a partly reduced inflammatory response of the foam cells. This suggests that compared to Kdm6b, Ezh2 has more potency as novel target for treatment of atherosclerosis. The beneficial effects on atherosclerosis are probably not all due to macrophage specific effects, since also neutrophils were deleted for Ezh2. These neutrophils showed reduced migration in vitro and were less present in atherosclerotic lesions.

**Ezh2 inhibition as novel drug target in atherosclerosis**

Recent years, several Ezh2 inhibitors have been developed as cancer therapeutics, but their impact on macrophages remains unknown (23). The use of these inhibitors in macrophages can help to validate the potency of Ezh2 as novel drug target in atherosclerosis. 3-deazaneplanocin A (DZNep) was the first established inhibitor and is most commonly used, yet this compound appeared to be non-specific for Ezh2 as it targets lysine methyltransferase activity in general (24). Therefore, several selective S-adenosylmethionine (SAM)-competitive Ezh2 inhibitors with high specificity have been developed, which act on the catalytic site of Ezh2 and affect tumor development (25-28). To make these drugs more valuable for clinical use, orally bioavailable inhibitors were synthesized (29-31). Phase 1/2 clinical trials are currently being performed with one of these compounds (EPZ-6438) in patients with advanced solid tumors or B cell lymphomas. Ezh2 is also described to have non-enzymatic functions (discussed in next paragraph), therefore inhibitors that block Ezh2 activity may not block all of its functions. A small molecule inhibitor that does not target the catalytic domain of Ezh2 but blocks the Ezh2-Eed interaction and thereby disrupts the PRC2 complex is now available (32). The use of these inhibitors should thus be tested on macrophages.

**Macrophage-specific targeting of compounds in atherosclerosis**

We suggest that the use of Ezh2 inhibitors should be tested in experimental settings of atherosclerosis, however we cannot estimate the outcome on atherosclerosis when inhibiting Ezh2 in non-myeloid cells. Previously we have shown that Hdac3 deletion in macrophages is beneficial in a mouse model of atherosclerosis (12), but blocking Hdac3 in endothelial cells increases plaque development (33). Therefore, cell-specific targeting of compounds to macrophages is of great interest. Statin-loaded HDL-nanoparticles accumulate in atherosclerotic lesions of mice, where it reduced macrophages numbers and inflammation (34). Loading of HDL-nanoparticles with
inhibitors against HMEs might thus serve as a novel approach to specifically deliver compounds. Another approach is to develop drugs with an esterase-sensitive chemical motif (ESM). Carboxylestrase-1 is predominantly expressed in human monocytes and macrophages and drugs with this ESM motif can be hydrolyzed from inactive to active drugs. Hda inhibitors with this ESM motif are anti-inflammatory and are another novel macrophage-specific drug targeting tool for the future (35).

**Upstream regulators of Ezh2 and functions beyond epigenetics**

The upstream regulators of Ezh2 in monocytes and macrophages are not yet identified. In cancer, many regulators, including transcription factors (TFs) and non-coding RNAs (ncRNAs mainly microRNAs) control Ezh2 expression (36). We showed that LPS downregulated Ezh2 expression and upregulated Kdm6b expression in macrophages, suggesting that TLR signaling regulates the expression of these HMEs. Besides the induction, Ezh2 can bind to other proteins and ncRNAs, resulting in its recruitment to specific loci, thereby regulating gene expression (36). Ezh2 activity and stability is also regulated by post-translational modifications, like phosphorylation (37-41). Besides the direct epigenetic function of Ezh2 and other HMEs, also histone methylation- and demethylation-independent functions of these enzymes exist. In various cancers, Ezh2 acts as a transcriptional activator rather than a direct repressor, an effect which is independent of the PRC2 complex (42). Furthermore, Ezh2 can also methylate non-histones, like STAT3, thereby regulating its recruitment to the DNA (43, 44). In addition, methylation of non-histones can also result in degradation of methylated proteins via ubiquitination (45). It should thus be noted that Ezh2 is not only a H3K27 methyltransferase and transcriptional repressor, since it can methylate non-histones and have PRC2-independent effects as a co-activator. Therefore, future studies should shed a light on the direct and indirect effects of HMEs in immunological settings. Ideally, the performed RNA-sequencing analysis should be overlaid with ChIP analysis for H3K27me3 and its enzymes, to gain more insight in direct and indirect effects of these enzymes.

**Regulation of macrophage cell-identity by DNA methylation**

Next to histone modifications, DNA methylation is another important epigenetic regulator. In chapter 8 we revealed that DNA methylation changes mainly occur during monocyte-to-macrophage differentiation and less during macrophage activation. While previous studies showed a role for DNA methylation in monocyte-to-macrophage differentiation in differentially methylated regions (DMRs) (46), we identified that DNA methylation changes occur at single CpGs or very small regions that contribute to monocyte-to-macrophage differentiation. In addition, it was
previously reported that mainly loss of methylation occurs during differentiation, while we also identified substantial gain of methylation (46, 47). We found that DNA methylation changes occur at positions that are enriched for enhancers and identified that gain of methylation occurs at positions that contain motifs, which can be bound by the lineage determining transcription factors (LDTFs) CEBP and ETS, while loss of methylation is enriched for motifs that bind AP1 factors. During monocyte-to-macrophage differentiation many epigenetic changes occur, of which enhancers are central regulatory regions necessary for macrophage identity (48). Gene transcription is regulated by multiple enhancers and their activity is cell-type specific (49). The selection and activity of enhancers is regulated by both LDTFs and stimuli-dependent TFs (SDTFs). The LDTFs are cell type-specific and regulate cellular identity, while SDTF are turned on based on environmental triggers thereby activating enhancers (50, 51).

While DNA methylation in general is associated with gene repression, evidence is now showing that DNA methylation can influence TF binding, both positively and negatively, depending on the methylated motif (52). Binding of the TFs bLHL, bZIP and ETS is inhibited by CpG methylation, while transcription factors such as homeodomain, POU and NFAT preferred to bind methylated CpGs (52). This suggests that in our study, binding sites for LDTFs are switched off during monocyte-to-macrophage differentiation (ETS motifs), while positions that bind SDTFs are turned on (bZIP motifs; AP1 factors). The question remains whether differential methylation is a cause or consequence of TF-binding. DNA methylation is catalyzed by methylcytosine dioxygenases (DNMTs) and removed by ten-eleven translocation proteins (TETs), but recent studies suggest that also non-enzymatic TFs regulate DNA methylation (53). DNA methylation can thus influence TF binding, but TF-binding can also influence DNA methylation.

**LDL and the inflammatory response of monocytes**

Elevated LDL levels are associated with increased risk for atherosclerosis and lipid lowering by statins effectively lowers the risk for cardiovascular disease (54). Because atherosclerosis is driven by both lipids and inflammation, we studied the link between the two. We hypothesized that LDL has inflammatory effects on circulating monocytes, which can be reversed upon lipid lowering. Whether the cardiovascular benefit of statins is due to their direct lipid lowering actions or their anti-inflammatory pleiotropic effects is under debate (55). We therefore used PCSK9 monoclonal antibodies which selectively lower LDL and in contrast to statins do not lower the inflammation marker c-reactive protein (CRP) (56, 57).
In chapter 9, we showed that in patients with familial hypercholesterolemia (FH), not using statins, elevated levels of circulating LDL induce pro-inflammatory and migratory changes in monocytes, which coincide with an increase in cytoplasmic lipid droplets compared to controls (58). This implies that there is a relation between intracellular lipid accumulation and inflammatory changes in monocytes. We showed that treatment with PCSK9 monoclonal antibodies in these FH patients reversed the migratory capacity, inflammatory response and intracellular lipid content, similar to treatment with PCSK9 monoclonal antibodies in these FH patients reversed the effects, contributes to anti-inflammatory effects on monocytes. Since PCSK9 antibody treatment selectively lowers LDL without decreasing CRP, these data imply that LDL lowering itself, rather than the pleiotropic effects, contributes to anti-inflammatory effects on monocytes.

**Foamy monocytes**
The idea that also monocytes encounter lipids in circulation, a concept called “foamy monocytes” was already described in mice (59, 60). We here show that this is also true in humans with hyperlipidemia and that lipid lowering reduces the amount of lipid droplets in monocytes. Questions that still need to be answered from a mechanistic point of view are: What type of lipids accumulate in these monocytes and how are these lipids taken up by the cell (61). Data from our study suggest that lipid uptake is not directly regulated via the LDLR, since the expression was virtually absent on circulating monocytes. Furthermore, PCSK9 antibodies enhance LDLR surface expression, but the monocytes of PCKS9 antibody treated-patients showed reduced accumulation of lipids. Homozygous FH-patients, characterized by the absence of functional LDLRs also show accumulation of lipids in monocytes and in addition, foamy monocytes were seen in Ldlr-deficient mice (59, 60, 62). We and others observed that scavenger receptors like CD36 are increased and thus might play a role in lipid uptake (59, 62). Furthermore, LDL can also be engulfed by macrophages via micropinocytosis, independent of receptor-mediated phagocytosis, a process which might also occur in monocytes (63). It is also possible that lipid synthesis is affected rather than the actual lipid uptake. We also observed a correlation between LDL and CCR2 surface expression, suggesting that LDL influences CCR2 expression. The exact mechanism via which LDL regulates CCR2 expression also needs to be addressed.

**Trained immunity**
In vitro, it has been shown that oxLDL priming of monocytes results in an enhanced cytokine response after re-stimulation with TLR ligands that was accompanied by epigenetic changes, a concept called trained immunity (64). These data support the idea that pro-atherogenic mediators can have long lasting effects on monocytes. In our study, we observed that LDL lowering by PCSK9 monoclonal antibodies reverse the
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pro-atherogenic properties of monocytes, suggesting that the memory induced by lipids can be partly reduced. The question remains whether this is true for all inflammatory and atherosclerosis-related genes and whether epigenetic modifications are altered in our study. RNA-sequencing and ChIP-sequencing could help to identify the complete transcriptional profile and epigenetic landscape induced by lipids. It can also help to answer the question whether epigenetic changes are reversed upon lipid lowering.

Targeting inflammation
Besides these lipid-lowering actions, patients still have a strong residual risk for cardiovascular events (65). As hypothesized before, this might be due to some kind of epigenetic memory, but can also be due to the residual inflammatory risk. Recently it was shown that anti-inflammatory therapy targeting IL-1β in cardiovascular patients, significantly lowered recurrent cardiovascular events, independent of lipid lowering, highlighting the importance of inflammation in cardiovascular disease (66). Targeting both lipids and inflammation can thus improve atherosclerosis outcome.

Lesional macrophage activation
Shifting monocytes and macrophages to cells with anti-atherogenic properties as treatment for atherosclerosis also highlights the importance of understanding the process of macrophage activation in atherosclerotic lesions. Activation of macrophages and combinations of activation triggers results in a spectrum of macrophage activation states in humans (67). Furthermore, macrophages from different tissues have a specific transcriptional and epigenetic profile, dependent on the environment in mice (68). Although the concept of classical (M1) and alternatively (M2) activation can be used as a reductionist tool to study macrophage activation, it should be taken into account that these states are at the ends of the macrophage activation spectrum, and that wide variety of macrophage activation states are observed based on their origin and environmental triggers (69). It is thus of great interest to know what is the origin of monocytes and macrophages present in atherosclerotic lesions, what is their lifespan and which activation states are present. Although efforts have been made to answer some of these questions, by use of reporter mice, the question remains whether the same holds true in human atherosclerotic lesions. In mice, monocyte-derived cells can replenish tissue resident macrophages, based on environmental triggers. Whether monocyte-derived cells turn into tissue resident macrophages in atherosclerotic lesions remains to be answered. Novel technologies like single-cell RNA sequencing and Mass cytometry (CyTOF)
analysis could help to identify different transcriptional macrophage activation states present in atherosclerotic lesions for both mice and humans.

Summary
In conclusion, we identified the H3K27me3 methyltransferase Ezh2 as a novel target in myeloid cells to combat atherosclerosis. Inhibitors against Ezh2 should be tested in macrophages and experimental atherosclerosis, to validate the potency of Ezh2 inhibition as treatment for atherosclerosis. In addition to histone modifications, we highlight that also DNA methylation is an important regulator in human monocyte-to-macrophage differentiation. Finally, by use of PCSK9 monoclonal antibodies we showed that LDL lowering itself contributes to anti-inflammatory effects on monocytes.

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