Bone marrow transplantation in mice as a tool to study M2 macrophage activation in atherogenesis

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General introduction

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1 Atherosclerosis

Atherosclerosis is a blood vessel narrowing and hardening disease characterized by the deposition of cholesterol locally in the arterial wall, leading to a low-grade chronic inflammation.[1] Atherosclerotic lesions take decades to become large enough as to have a significant effect and cause cardiovascular complications in humans.[2] Atherosclerosis can eventually lead to serious problems such as chest pain (angina), heart failure, heart attack, stroke, ischemic attack, aneurysms, or even death.[3] Nowadays, atherosclerotic cardiovascular disease is the leading cause of mortality worldwide, accounting for 31% of global deaths in 2015.[4] Although it is closely related to contemporary lifestyles, atherosclerosis is not only found in modern human beings.[5] In contrast, the disease has been found in 4000 years old mummies and the earliest literature that described the pathological changes of atherosclerosis can be traced back to 442 years ago. In the year 1575, the Italian anatomist Gabriel Fallopius had already described calcification of the arterial wall, a pathological phenomenon of atherosclerotic plaques.[6] Later on, several researchers and medical doctors had observed atherosclerosis in large arteries,[7-9] and proposed a connection of atherosclerosis with angina and ischemic heart disease.[10] The first description of plaque rupture was reported in 1844.[10] In 1829, Jean Lobstein for the first time introduced the word "arteriosclerosis" in his unfinished book "*Traité d' Anatomie Pathologique*" which was ultimately published in the year 1933,[11,12] almost 100 years after his death. However, in general the German pathologist Felix Marchand (1904) is recognized as the first using the term "atherosclerosis", stemming from the Greek words of porridge "athero" and hardening "sclerosis", to describe the fat-rich materials that accumulated inside a hardened artery.[13,14]

Since its discovery, researchers have been trying to uncover the etiopathogenesis of atherosclerosis. At the end of 18th century, theories underlying atherosclerosis development were proposed by Carl von Rokitansky and Rudolf Virchow[15] who both recognized the presence of inflammation. However, it was unclear whether inflammation played a causative role. Carl von Rokitansky considered atherosclerosis to be the result of the buildup of fibrin or other blood elements, which subsequently was modified to a lipid-rich plaque in the arterial wall.[14] In contrast, Rudolf Virchow suggested that the inflammatory response to lipid insudation or intimal injury is the cause of atherosclerosis.[15] The exact pathogenesis of atherosclerosis remained unclear until the "response to injury hypothesis", initially proposed by Rudolf Virchow and revived by Russell Ross in 1999, became a widely accepted theory.[16]

1.1 Lipoproteins: metabolism and association with atherosclerosis

Hyperlipidemia plays a leading role in triggering and promoting atherosclerosis development.[17] Lipoproteins, being the main carrier of cholesterol in the circulation, were firstly associated to cardiovascular disease in 1949 by John Gofman and his colleagues. They found that increased levels of low-density lipoprotein (LDL) cholesterol are associated with an increased cardiovascular risk, and that patients with familial hypercholesterolemia are predisposed to the development of premature atherosclerosis.[18] These patients have an overall cholesterol elevation in their plasma, which can mainly be attributed to an increase in LDL and intermediate-density lipoprotein (IDL).[14] The hypothesis that high plasma lipid levels are associated with increased cardiovascular risk was further supported by a cooperative study performed in the 1950s and 1960s, which confirmed the connection of cardiovascular risk to plasma cholesterol levels using patient cohorts from seven different countries.[19] Circulating lipids are transported by lipoproteins, particles composed of a shell of a monolayer of phospholipids with free cholesterol and apolipoproteins (Apo) and a lipid-rich core constituted of esterified cholesterol and triglycerides.[20] Based on the proportion of each component as well as particle density, lipoproteins are classed into 5 groups: high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very-low-density lipoprotein (VLDL), and chylomicrons. Except for HDL, all these subclasses contain apolipoprotein B (ApoB) as their major apolipoprotein and are considered athero-promoting lipoproteins. In contrast, HDL has apolipoprotein A as its primary apolipoprotein and is considered to act as an athero-protective lipoprotein.

1.1.1 ApoB-containing lipoproteins and atherosclerosis

Chylomicrons, ApoB48-containing lipoproteins, are the major carriers for lipids absorbed from the diet in the intestine, and represent a major source of triglycerides (TG) for various tissues. In addition to TG, chylomicrons transport cholesterol, but only a limited amount.[21] Therefore, for quite a long time chylomicrons were believed not to contribute to atherogenesis.[22,23] However, later on ApoB48 was found in atherosclerotic plaques[24,25] and an ApoB 48-specific receptor was detected in human and murine macrophages,[26,27] thereby highlighting the contribution of chylomicrons to atherosclerosis development. In general, increased chylomicron levels seem to generate a pro-atherogenic profile, however, there is still not much evidence showing a direct link between high chylomicron levels and atherogenesis.[28]

In humans, and specifically in women with elevated TG levels, increased risk for cardiovascular events was shown in a 11.4 years follow-up study in America.[29] Interestingly, both fasting and non-fasting TG levels are associated with cardiovascular disease, with postprandial TG levels showing the strongest association with future cardiovascular risk.[29-31] Interestingly, the strong linear correlation between plasma TG and atherosclerosis is most likely gender-dependent, of which TG levels in females have shown to be the best predictor for cardiovascular risk in both human and mice.[29,32]

Fasting TG levels are determined by the amount of TG transported by VLDL particles. VLDL is synthesized by hepatocytes,[33] and serves as the precursor for IDL and LDL. Similar to chylomicrons, VLDL is also a TG-rich lipoprotein. In contrast to chylomicrons that carry exogenous (dietary) lipids, VLDL transports endogenous lipid products to peripheral tissues. High VLDL levels are considered a risk factor for coronary artery disease. Likely the lipid composition of the VLDL particle determines cardiovascular risk. For example, VLDL is a strong predictor for cardiovascular risk in females with a significantly higher VLDL cholesterol/TG ratio than males.[32]

IDL, as the remnant of VLDL and precursor of LDL, is also considered a causal factor for the development of atherosclerosis.[34] The IDL concentration has been associated with the incidence of coronary artery disease, especially in patients with normal cholesterol levels.[35-38] However, as an intermediate form between VLDL and LDL, the exact role of IDL in atherosclerosis is less-well defined. The circulating IDL particles are quickly taken up by the liver, or converted to LDL by undergoing triglyceride hydrolysis in peripheral tissues.

In humans, LDL is the primary carrier of cholesterol, accounting for 70-80% of the total cholesterol concentration in the circulation. Importantly, each LDL particle contains a single copy of ApoB100.[39] Hence, by analyzing the ApoB100 concentration, LDL particle numbers in the circulation can be calculated. Plasma LDL cholesterol is highly associated with atherosclerosis development. Noteworthy, not only the concentration of LDL cholesterol, but also the heterogeneity of the LDL particles, plays an important role in atherogenesis. LDL is comprised of multiple subclasses that differ in size and density and each contribute distinctly to the susceptibility for cardiovascular disease.[40,41] The size and density of LDL varies with its lipid content.[42] Small and dense LDL particles are more atherogenic, due to their higher penetration capabilities of the endothelial barrier[43,44] and greater oxidation potential compared to the larger, less dense LDL particles.[45] Epidemiological studies showed that the small and dense LDL particles are associated with a cluster of cardiovascular disease risk factors, including elevated levels of plasma TG and ApoB, reduced concentrations of HDL cholesterol, and impaired insulin sensitivity.[46,47] Thus, atherogenesis is not only affected by the amount of cholesterol transported by LDL, but also by the characteristics and heterogeneity of the LDL particles.[48]

1.1.2 High-density lipoproteins and atherosclerosis

In contrast to LDL that promotes atherogenesis, high-density lipoprotein (HDL) is considered to protect against atherosclerosis. HDL removes cholesterol out of lipid-rich tissues and transports it to the liver, a process commonly referred to as reverse cholesterol transport (RCT).[49,50] RCT is a complicated process involving various steps. First, cholesterol efflux from peripheral cells, including foam cells in the arterial wall, is facilitated by the ATP-binding cassette (ABC) transporters ABCA1 or ABCG1, which mediate the efflux of intracellular lipids to lipid-poor ApoA1 (nascent HDL) and mature HDL in the circulation, respectively.[51,52] Upon uptake by HDL, the effluxed cholesterol is esterified, via lecithin cholesterol acyltransferase (LCAT), and transferred to the core of HDL, resulting in the remodeling and maturation of the HDL particle. Next, the cholesterol in HDL is transferred to the liver, either via selective uptake of HDL-cholesterol by scavenger receptor BI (SR-BI), holoparticle uptake via the LDLr, or indirectly, via the transfer of cholesterol to other, TG-rich, lipoproteins, through cholesteryl ester transfer protein (CETP) which are subsequently also fluxed back to the liver. Here, the cholesterol taken up by the liver is excreted into bile and feces or used as substrate for *de novo* cholesterol synthesis. Importantly, mice and rats naturally lack CETP activity, and hence CETP-induced cholesterol transfer does not occur in these animals.[53]

Since HDL can mediate cholesterol efflux from lipid-rich macrophages, HDL has been identified as an important anti-atherogenic particle.[54] The anti-atherogenic properties of HDL, however, extend beyond the removal of excess lipid from the vascular wall.[54,55] For example, HDL can also exert antioxidant effects, as under oxidizing conditions, the presence of HDL can significantly decrease lipid peroxide concentrations within the LDL particle.[56,57] In addition, HDL inhibits monocyte adhesion to the vessel wall, by suppressing the expression of endothelial adhesion and migration molecules.[58] Finally, HDL also protects against damage inflicted by inflammatory mediators to the endothelium, and prevents thrombosis by upregulating nitric oxide (NO) production in endothelial cells.[59-61]

Considering the wide array of atheroprotective functions of HDL, high HDL concentrations were long thought to be associated with a reduction in coronary artery disease (CAD) risk. Indeed, epidemiological studies have indicated that low HDL cholesterol is associated with an increased risk. However, pharmacological induction of plasma HDL cholesterol levels did not reduce CAD risk.[62] In line, increased macrophage cholesterol efflux capability of human serum is also independent of the HDL cholesterol level.[63,64] HDL cholesterol efflux capacity, however, did strongly correlate with the concentration of lipid-poor ApoAI (pre-β HDL).[65,66] To explore the development of a novel HDL based-atheroprotective therapy against it is thus important to focus on modulating nascent HDL (with high cholesterol efflux capacity) rather than HDL cholesterol levels.[67]

1.2 General pathogenesis underlying atherosclerotic lesion development

The development of atherosclerosis is the consequence of a chronic inflammatory reaction of the vascular wall, in response to dyslipidemia and endothelial distress, involving the inflammatory recruitment of leukocytes and the activation of resident vascular cells.[68] According the response-to-injury hypothesis, the development of atherosclerosis is initiated by dysfunction of the arterial endothelium.[69] Cardiovascular risk factors, such as smoking, hypertension, inflammation, age, and lipids (in particular LDL), are known to aggravate endothelium dysfunction and activation. This causes the activated endothelial cells to start expressing surface factors that stimulate the infiltration of monocytes from the blood stream into the intima and subintimal space, where they differentiate into macrophages.[70] LDL retained in the arterial wall, mostly after extensive oxidative modification[71], is phagocytized by the monocyte-derived macrophages, leading to the formation of lipid-rich foam cells as well the start of a chronic inflammatory process. Monocytes, T cells and mast cells all migrate to the site of action in response to inflammatory signals produced at the site of the early atherosclerotic lesion. These cells in turn will contribute to the immune reaction, creating a progressive inflammatory environment in the developing plaque which further accelerates atherosclerosis development. When the plaque macrophages are unable to sufficiently efflux their excess cholesterol, they become heavily lipid-laden foam cells. Ultimately, these foam cells grow in size and die, thereby releasing all cellular cholesterol into the plaque, causing intraplaque cytotoxicity and further aggravation of the inflammatory response. At this stage, the atherosclerotic plaque consists of a lipid core which contains cholesterol, cellular debris and infiltrated immune cells, covered by a fibrous cap. This collagen-rich fibrous cap overlying the lipid-core of the plaque is the consequence of vascular smooth muscle cells (VSMC) proliferation and provides stability to the plaque. However, thinning of this fibrous cap by mediators secreted by inflammatory cells can ultimately result in plaque rupture.[71] In this advanced stage of lesion development, plaque stability and correlated susceptibility to plaque rupture, is determined by the balance between VSMCs that protect the plaque, and cytotoxic factors released by immune cells/endothelial cells that damage the fibrous cap.[72] A stable plaque is usually rich in extracellular matrix and smooth muscle cells and in most cases does not cause acute clinical symptoms. Rupture of the atherosclerotic plaque, or erosion of the endothelial layer lead to the formation of a thrombus on top of the atherosclerotic lesion, the culprit for the development of acute cardiovascular events (**Figure 1**).

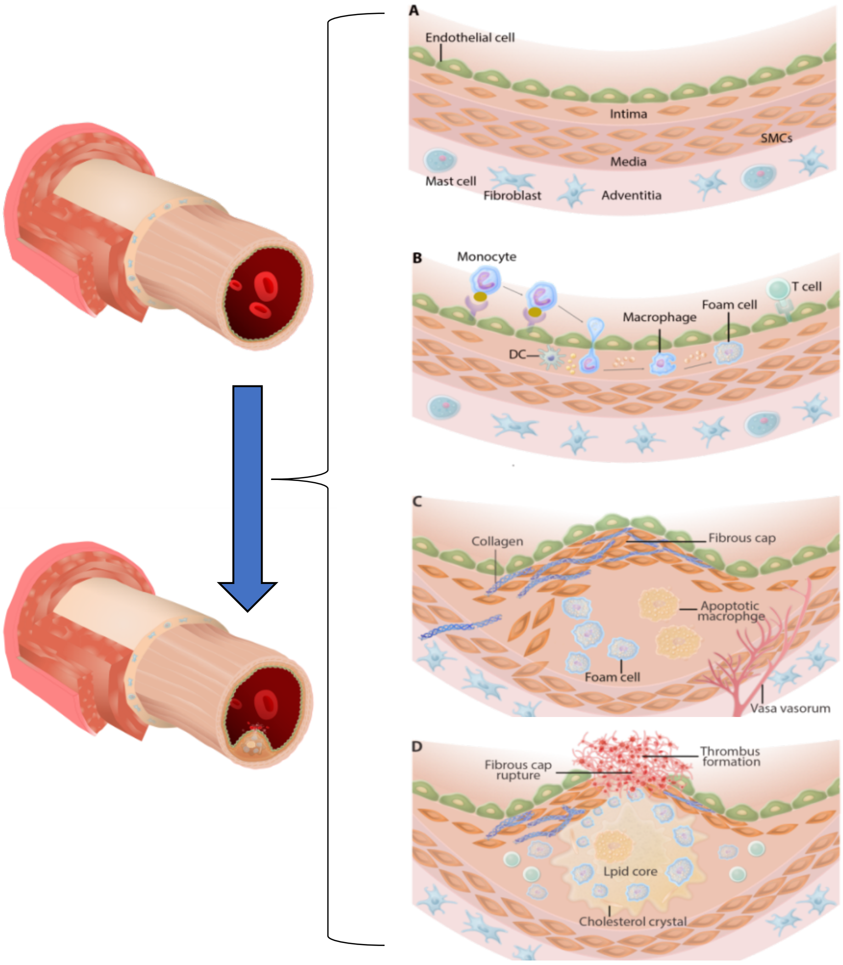

Figure 1 Progression of atherosclerosis development.

This schematic diagram shows the changes occurring in the arterial wall from a normal healthy artery to an artery with advanced atherosclerosis, and the development of atherothrombosis. a) A healthy artery, composed of three layers, the intima, media and adventitia. The inner layer of the artery, the intima, is formed by a monolayer of endothelial cells. The middle layer of the artery, the media, is principally made up of smooth muscle cells (SMCs). The outer layer, the adventitia is composed of elastic collagen fibers and fibroblasts. b) Upon initiation of atherosclerosis, blood leukocytes, including monocytes are recruited to the activated endothelium. Monocytes, as the largest population during recruitment, differentiate into macrophages, take up oxLDL, and become foam cells. c) During lesion progression, vascular SMCs migrate from the media to the intima and start producing collagen, elastin and proteoglycans that form a fibrous cap overlying the lesion. In advanced lesions, apoptosis of cells in the lipid core contribute to the formation of a necrotic core. d) Once atherosclerotic lesions rupture, a thrombus rapidly develops on top of the lesions. Thrombosis impedes blood flow to a varying extent, but could totally block the blood flow and cause an acute infarction or cerebral stroke. **Painting by Mengyin An, adapted from Libby *et al.*** [73]

2 The role of monocytes and macrophages in the pathology of atherosclerosis

2.1 Monocyte heterogeneity

The bone marrow is a hematopoietic organ responsible for generating new blood cell components, including monocytes. Monocytes are the precursors of macrophages which represent the primary cell type in atherosclerosis.[74] Monocytes originate from bone marrow myeloid progenitor cells, which are also the progenitor cells of neutrophils.[75] Notably, circulating monocytes show morphological heterogeneity. In humans, monocytes are identified by the expression of surface receptors CD14 and CD16. Based on the expression levels of these two markers, monocytes are divided into two main subtypes. The first subtype is a so-called 'classical' monocyte, expressing high levels of CD14 and very low levels of CD16 ($CD14^{hi}CD16^{-}$). This classical monocyte population also has a high producing capacity for pro-inflammatory cytokines. The second subtype is the 'non-classical' monocyte, which is characterized by low expression levels of CD14 and high expression levels of CD16 ($CD14^{+}CD16^{++}$). These monocytes are also called patrolling monocytes, as they constantly patrol the vasculature, and are involved in the early responses to pathogens and tissue repair.[76,77]

In mice, monocytes are classified into two subtypes based on the expression of Ly6C and CCR2, with $Ly6C^{hi}CCR2^{+}$ monocytes representing the equivalent of human $CD14^{hi}CD16^{-}$ monocytes, and $Ly6C^{low}CCR2^{-}$ being equivalent to human $CD14^{+}CD16^{++}$ monocytes.[78-80] After infiltration into the arterial wall, the monocytes are exposed to specific environmental factors, triggering their differentiation into different types of macrophages that differentially contribute to the progression of atherosclerosis.[81]

2.2 M1/M2 macrophages and atherosclerosis

Similar as their monocyte precursors, multiple types of macrophages can be distinguished.[82] Since different macrophage activation forms were proposed by Mackness[83] in 1962 and Gordon[84] in 1992, for M1 and M2 macrophages, respectively, more and more researchers have started investigating the relationship between macrophage phenotypes and atherosclerosis. Both M1 and M2 macrophages have been implicated in atherosclerosis. In vitro, the M1 macrophage phenotype can be induced by incubation with LPS or IFN-gamma.[85] Upon LPS activation, macrophages secrete high levels of pro-inflammatory cytokines, including interleukin 1β (IL-1β), IL-6, IL-12, tumor necrosis factor α (TNF-α), but low levels of anti-inflammatory cytokines such as IL-10. As such, M1 macrophages actively contribute to the persistent inflammatory environment in the atherosclerotic plaque, and thereby accelerate atherosclerosis development. In contrast, M2 macrophages are known to protect against atherosclerosis. M2 macrophages can be induced by IL-4, IL-10 and IL-13 and, upon activation, produce high levels of anti-inflammatory cytokines, including IL-10, and low levels of pro-inflammatory cytokines, such as IL-12. Besides producing anti-inflammatory cytokines, M2 macrophages also enhance the production of pro-fibrotic factors, including collagen, and thereby promote tissue repair and remodeling.[86] Hence, M2 macrophages protect against atherosclerosis not only by decreasing the local inflammatory status of the plaque but also by increasing plaque stability. In mice, M1 macrophages express high levels of inducible nitric oxide synthase (iNOS), which renders iNOS a murine M1 marker gene. In addition to iNOS, high expression levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-12 are also considered as M1 markers. In contrast, murine M2 macrophages are known to express high levels of arginase 1 (Arg1). Additionally, YM1 and FIZZ1 and scavenger receptors (CD204,[87] CD163[88]) are also considered M2 macrophage markers.[81]

2.3 Macrophage phenotype and foam cell susceptibility

Besides playing a role in the immune response, another important function of macrophages is to engulf foreign agents, including oxidized LDL (oxLDL).[89] Macrophages take up oxLDL and become foam cells, a process which is considered to be one of the hallmarks of atherosclerosis. SR-A[90] and CD36 (scavenger receptor class B member 3) are the main receptors involved in foam cell formation, being responsible for up to 90% of the oxLDL uptake by macrophages *in vitro*.[91] Interestingly, both SR-A[92] and CD36[93] are upregulated during M2 macrophage differentiation, suggesting an increased susceptibility of M2 macrophages to become foam cells. Indeed, van Tits *et al.* found that compared to M1 macrophages, M2 macrophages are more prone to take up oxLDL and become foam cells.[94] This suggests that, in contrast to their atheroprotective anti-inflammatory role, M2 macrophages are also likely to play a pro-atherogenic role by promoting macrophage foam cell formation.

2.4 Macrophage phenotype switch

The process of macrophage polarization is dynamic, as macrophages can rapidly switch from one phenotype to another in response to a changing microenvironment.[95-97] In atherosclerosis, numerous factors affect the lesional microenvironment, including cholesterol oxidation, inflammation mediators, infiltrated immune cells, growth factors, dead cells and other substances.[98] Therefore, the lesional microenvironment changes with the different stages of atherosclerosis development, thereby further influencing macrophage polarization.[99] Da Silva and colleagues found that cholesterol loading of human macrophages limited their capability to be primed to M1 macrophages, but not to M2 macrophages, suggesting an anti-inflammatory property of foam cells.[100] Furthermore, in response to oxidized phospholipids, a product of lipid oxidation, macrophages are primed to a so-called Mox phenotype.[101] Finally, in response to haem and haemoglobin exposure after intraplaque hemorrhage, macrophages can be polarized towards an Mhem phenotype.[102] Noteworthy, both Mox and Mhem macrophages display a reduced capacity to engulf oxLDL, and are thus considered less prone to foam cell formation.[101,103] All M1, M2, Mox, and Mhem macrophage phenotypes have been demonstrated in atherosclerotic lesions. However, M1 and M2 macrophages are suggested to act as the main precursors for macrophage foam cells.[104]

3 Experimental mouse models and strategies for studying atherosclerosis

3.1 Mice and atherosclerosis susceptibility

Multiple animal species have been used as experimental models to study atherosclerotic lesion development, including pigs, rabbits, monkeys, non-human primates and mice.[105-110] Among these non-human models, mice now are considered the best choice for studying atherogenesis, due to their low cost, high reproduction rate and short time frame for disease development.[106] Although C57BL/6 (hereafter referred to as WT mice) is the mouse strain most sensitive to the development of atherosclerosis, as compared to other murine strains, C57Bl/6 mice are still relatively resistant to diet-induced atherosclerosis.[106] Persistent hypercholesterolemia, reflecting plasma cholesterol levels exceeding 300 mg/dL, is needed to induce atherosclerosis development in mice.[111] The main reason that mice in general are resistant to atherosclerosis is their distinct plasma lipoprotein profile, as compared to humans.[112] The fact that mice lack the cholesterol ester transfer protein (CETP) and exert a low ability to absorb dietary cholesterol causes a cardiometabolic lipid profile, reflected by consistently high plasma levels of HDL cholesterol and low plasma levels of LDL cholesterol.[112] Therefore, exposing mice to high concentrations of dietary cholesterol alone is not sufficient to induce atherosclerosis development. To enhance the atherosclerosis susceptibility of mice, genetic modification is required for induction of a sufficiently high pro-atherogenic lipoprotein profile. Since the 1990s, the technique of homologous recombination in embryonic stem cells made it possible to selectively knock out genes involved in the metabolism of pro-atherogenic lipoproteins.[113] Currently, the most frequently used mouse models to study atherosclerosis are LDL receptor (LDLr) knockout (KO) and ApoE KO mice.[112]

3.2 ApoE KO mice and LDLr KO mice

ApoE is a constituent of non-LDL lipoproteins and serves as an essential ligand for the uptake of these lipoproteins by the liver.[114] Therefore, mice lacking ApoE show impaired clearance of plasma cholesterol, resulting in severe hypercholesterolemia. ApoE KO mice fed a regular chow diet display plasma cholesterol levels of > 500 mg/dL, which can mainly be attributed to increased levels of chylomicrons and VLDL, whereas plasma HDL-cholesterol is decreased.[115] ApoE KO mice develop extensive atherosclerotic lesions.[116,117] Under normal chow conditions, spontaneous atherosclerotic lesion development is observed in the aortic sinus within 3-4 months of age. In the older mice, atherosclerotic lesions are visible throughout the aorta at locations of principal branches.[118,119] Importantly, this process can be accelerated by feeding ApoE KO mice a high fat/high cholesterol diet.[120]

The LDL-receptor regulates plasma cholesterol levels by removing IDL and LDL from plasma. Mice lacking the LDLr, as compared to WT mice, display a 2-fold higher plasma cholesterol level (~230 mg/dL) when fed a regular chow diet, as compared to WT mice. This increase can mainly be attributed to an increase in cholesterol within the IDL/LDL fraction.[121,122] Furthermore, LDLr deficiency also leads to a small increase in VLDL-cholesterol levels. Moreover, HDL-cholesterol levels are increased.[122] The mild hypercholesterolemia induced by LDLr deficiency however, is not sufficient to effectively induce atherosclerosis development in mice on chow. Interestingly, plasma cholesterol levels of LDLr KO mice are highly responsive to dietary interventions,[123,124] and a high-fat/high-cholesterol (Western-type) diet is known to induce severe hypercholesterolemia and rapid atherosclerosis development.[122,125,126] Similar to ApoE KO mice, atherosclerotic lesion development in LDLr KO mice on Western-type diet is initiated in the aortic root.[127] Intermediate aortic lesion development occurs within 3 months of Western-type diet feeding, and advanced lesions are present in the aorta after 5 months of dietary challenge.[123,124,126] A commonly used approach to investigate the function of a specific gene in atherosclerosis, is crossbreeding of mice deficient for the gene of interest with the hypercholesterolemic LDLr KO or ApoE KO mice. Since generating double knockout mice is a time consuming and costly approach, there is an ongoing search for alternative methods to induce hypercholesterolemia in mice.

3.3 Bone marrow transplantation

Hematopoietic stem cell transplantation to an established mouse model of atherosclerosis, such as the apoE or LDLr KO mice, is an effective strategy to generate chimeric mice with target gene alterations in bone marrow-derived cells of an atherosclerosis-prone background. One of the strengths of this model is that in the recipients specifically the genotype of the bone marrow-derived cells, which represent the major players in atherosclerosis, is altered and thus allows analysis of the specific contribution of a gene of interest in blood cells. Hematopoietic cell transplantation not only helps to ease the time and money needed for the generation of sophisticated cell type-specific knockout mouse models, but also allows a closer mechanistic insight into the cellular biology underlying atherosclerosis development.[128]

Before birth, blood cells are derived from the fetal liver and spleen, however, after birth the bone marrow becomes the primary origin for the generation of blood cells. Therefore, bone marrow is normally the source of hematopoietic cells for transplantation, especially in murine models. In bone marrow transplantation studies (**Figure 2**), LDLr KO mice are normally chosen over ApoE KO mice as the atherosclerosis-prone recipients. This is because 1) the lipid profile of LDLr KO mice, characterized by a high IDL/LDL cholesterol fraction, resembles strongly the plasma lipid profiles of human dyslipidemic patients,[129] 2) the morphology of the atherosclerotic lesions in LDLr KO mice resemble human atherosclerotic plaques,[130,131] and, most importantly, 3) the presence of the LDLr in the donor bone marrow does not affect the pro-atherogenic lipoprotein profile and atherosclerosis susceptibility of the LDLr KO recipients.[132-134] In contrast, several studies have demonstrated that restoration of ApoE in bone marrow-derived cells normalizes serum cholesterol to WT levels and reduces atherosclerosis development in ApoE KO recipients.[135-138]

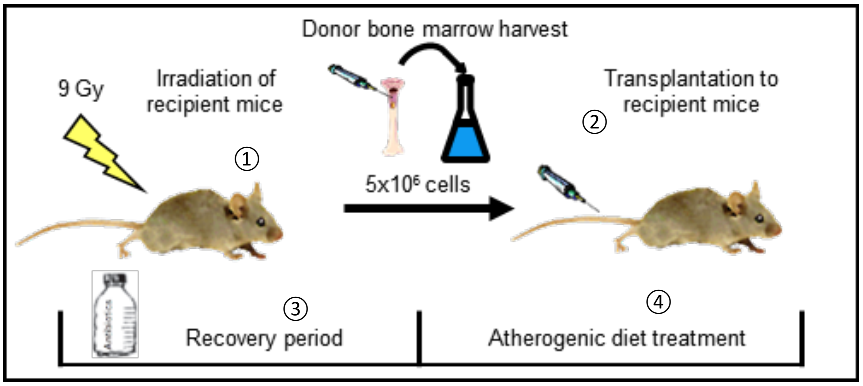

Figure 2 Schematic diagram of a bone marrow transplantation (BMT) procedure in mice for studying atherosclerosis.

1) Original bone marrow of atherosclerosis-prone recipient mice (often LDLr KO mice) is destroyed by a lethal dose of irradiation (9 Gy). 2) The recipients receive five-million donor bone marrow cells, lacking or overexpressing the gene of interest. 3) Recipients are allowed to recover for 8 weeks on a chow diet. One week before the irradiation, and throughout the complete recovery period, recipient mice receive antibiotics via drinking water. 4) After recovery, the recipients are challenged with an atherogenic Western-type diet to induce atherosclerosis development.

4 Signaling pathways and atherosclerosis

As mentioned before, macrophages are the main cell type in atherosclerotic lesions and their phenotype and activation status influence their exact role in the pathogenesis of the disease. Importantly, macrophage phenotype and activation are highly dependent on the lesional microenvironment and the intracellular signaling pathways that are activated within the macrophages.

Macrophage activation is a very complicated process. From receiving an initial stimulus to the point that eventually the macrophage's phenotypic functional protein production is altered, this process entails activation of a complex set of signaling pathways, and transcriptional and post-transcriptional regulatory networks.[96,139] In a simplified summary, as shown in **figure 3** the following key steps can be distinguished: 1) macrophage surface receptors recognize environmental stimuli; 2) the signals are amplified and transmitted to the nucleus by protein kinase transducers; and 3) the nuclear transcriptional/post-transcriptional factors regulate macrophage-specific gene expression and thus dictate macrophage polarization and functions.[139] This complicated activation process provides multiple possibilities to design novel strategies, addressing the different activation steps, to reprogram specific macrophage phenotypes for therapeutic benefit. In this thesis, four genes involved in macrophage activation at different levels, were investigated. Macrophage polarization to the M1 and M2 phenotype has long served as a paradigm for studying atherosclerosis. Inducing new effector activities by activated macrophages is considered as an attractive therapeutic approach for atherosclerosis treatment.[139-143] To explore potential novel therapeutic targets, we first evaluated and discussed the role of the M2 macrophage signature gene Arg1, a key player in the nitric oxide/L-arginine pathway, in atherosclerosis development. Protein kinases play essential role in the transcriptional and epigenetic regulation of macrophage polarization,[139, 144,145] and are the most intensively studied protein targets in pharmacology research.[146-150] In this thesis the atherosclerotic role of Akt2 and MKP2, key members of the protein kinase B and mitogen-activated protein kinases family respectively, are addressed. Furthermore, transcription factors, critical regulators of gene expression, have long been proposed to execute essential regulatory functions in the pathogenesis of atherosclerosis.[151] In addition, in this thesis, we focus on the upstream stimulatory factors (Usfs), recently identified lipid-related transcription factors,[152] that are regulators of several important cellular processes[153] and hence are expected to influence atherosclerosis development. Below the background of 1) the Nitric oxide/L-arginine and Arg1, 2) protein kinases and their inhibitors Akt2 and MKP2, and 3) Usfs in atherosclerosis is described in more detail below.

4.1 Nitric oxide/L-arginine pathway in atherosclerosis

Nitric oxide (NO) is an important signaling molecule that influences many cellular processes.[154] The cardiometabolic related functions of this molecule include: 1) prevention of endothelial cell apoptosis,[155,156] 2) reduction of oxidative stress, induced by reactive oxygen species (ROS),[157] 3) inhibition of smooth muscle cell proliferation, and 4) inhibition of vascular cell adhesion molecule-1 (VCAM-1) expression and, hence, inhibition of monocyte recruitment.[158] In line with these athero-protective functions of NO, several animal studies have confirmed that decreasing NO production induces atherosclerosis,[159] while increasing NO production attenuates atherosclerosis.[160,161]

Important to note is that there are indications that the protective role of NO is both tissue-specific[159,160,162-164] and dose-dependent.[160,162,165] NO is a product of nitric oxide synthases (NOSs). The NOS family has three members, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).[154] eNOS and nNOS are constitutively expressed.[166] As indicated by the name, eNOS is primarily produced by endothelial cells and, nNOS by neurons. The expression of iNOS can be induced by stimulators, especially inflammatory cytokines.[167] More importantly, iNOS is expressed by macrophages, and highly upregulated in response to lipopolysaccharide and inflammatory cytokines,[168] leading to an enhanced production of NO.

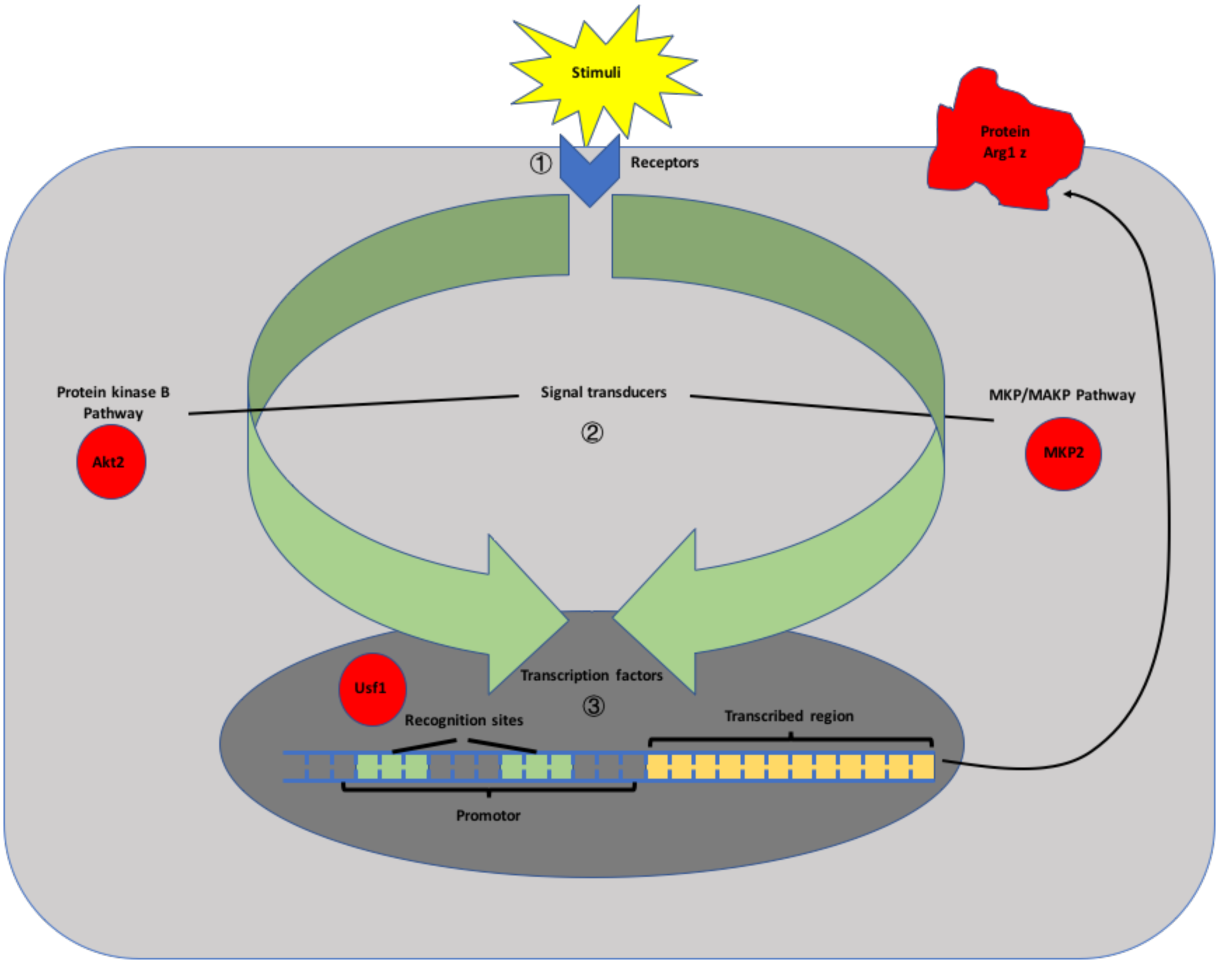

Figure 3 Regulation in macrophage activation and target factors Arg1, Akt2, MKP2 and Usf1 studied in more detail in this thesis.

1) Environmental stimuli activate cellular effector receptors. 2) The signals are amplified and transmitted to the nucleus by protein kinase transducers, including Akt2 or MKP2. 3) Transcription factors, such as Usf1, regulate the stimulus-specific gene expression, eventually leading to phenotypic functional protein production, such as Arg1.

Increasing evidence indicates that NO produced by nNOS and eNOS is atheroprotective, while NO produced by iNOS is more likely pro-atherogenic.[169] Diminished levels of bioavailable NO are associated with endothelial dysfunction,[170] the indicator of early atherosclerosis. Deletion of eNOS in ApoE deficient mice leads to increased atherosclerosis development,[159] and eNOS gene transfer improves atherosclerosis,[160,161] further evidencing the athero-protective role of NO derived from eNOS. The beneficial effects of nNOS in atherosclerosis described by Kuhlencordt *et al.*,[171] were surprising at first, since nNOS is primarily detected outside of cardiovascular system.[172-174] However, in their study, nNOS expression was demonstrated in the atherosclerotic lesion, predominantly in smooth muscle cells, and to a lesser extent in macrophages, but not in endothelial cells.[175] nNOS-derived hydrogen peroxide induces endothelial dysfunction, but the mechanism underlying the protective effects of nNOS in atherosclerosis is not clear yet.[169,176] NO produced by iNOS of lesional macrophages, is pro-atherogenic in humans.[177] In support, deletion of iNOS in mice reduces their susceptibility to the development of atherosclerosis.[163,164,178] In addition to its cell type-specific effects on atherosclerosis, NO also influences atherogenesis in a dose-dependent manner: low concentrations of NO improves atherosclerosis,[160,161] but massive NO production worsens atherosclerosis.[162]

iNOS, a signature marker of M1 macrophages, uses the substrate L-arginine, to produce NO. Interestingly, this same substrate is also used by Arginase-1 (Arg1), an important M2 macrophage marker, which catalyzes the conversion of L-arginine to urea and ornithine, the latter being a precursor for collagen production. Deletion of iNOS is known to cause a decrease in NO production, resulting in an increased availability of its substrate L-arginine for Arg1 mediated-convertion into ornithine. Hence, iNOS deletion can indirectly lead to an enhanced production of collagen,[179] thereby improving atherosclerotic plaque stability. Conversely, deletion of Arg1 might lead to increased substrate availability for the production of NO. As mentioned above, NO in atherosclerosis is well-studied, however, the role of Arg1 in atherosclerotic plaque development is currently still unknown.

4.2 Protein kinase B (Akt) in atherosclerosis

Protein kinase B is a serine/threonine-specific protein kinase, of which three isoforms can be distinguished: Akt1, Akt2, and Akt3.[180] Akt plays an important role in many cellular processes, such as apoptosis,[181] proliferation,[182] migration,[183] transcription,[184] and insulin responsiveness.[185] Importantly, Akt signaling is also known to influence atherosclerotic lesion development.

Akt1 is widely expressed in all tissues, whereas Akt2 expression is limited to metabolic tissues, such as adipose tissue, liver, and skeletal muscle, and Akt3 is preferentially expressed in brain.[180,186,187] Interestingly, macrophages express all three Akt isoforms.[188] Recent studies showed that Akt isoforms differentially contribute to macrophage polarization.[189,190] For example, upon Staphylococcus aureus infection, Akt1 deficient macrophages showed upregulated expression of the M1 signature gene iNOS.[189] Conversely, upon LPS stimulation, Akt2 knockout macrophages display an M2-like phenotype, as evidenced by augmented expression of the M2 macrophage markers Arg1, YM-1, and FIZZ-1.[190,191] So far, the role of Akt3 in macrophage polarization remains unknown. In addition to their roles in macrophage polarization, Akts also differentially influence macrophage foam cell formation. Previous studies have shown that Akt1 does not affect oxLDL-induced cholesterol accumulation in macrophages,[192] whereas Akt2 promotes acLDL-induced foam cell formation,[191] and Akt3 protects macrophages against acLDL-induced foam cell formation.[193]

In line with the different roles of the Akt isoforms in macrophage polarization and foam cell formation, Akt isoforms also distinctly contribute to atherosclerosis development. Akt1 has been reported to have an atheroprotective role.[192] However, this effect is likely due to Akt1 of vascular origin and not macrophage Akt1. Indeed, bone marrow-specific deletion of Akt1 did not influence atherosclerosis susceptibility.[192] Moreover, Fernandez-Hernanode and colleagues showed that whole body Akt1 deletion inhibits the proliferation and migration of vascular smooth muscle cells (VSMCs), leading to the development of vulnerable atherosclerotic plaques with increased necrosis and a smaller collagen-rich fibrous cap.[194] Akt3 is barely detectable in the healthy vasculature.[195] However, in line with the importance of Akt3 to limit macrophage foam cell formation, an increased susceptibility to atherosclerosis development was observed in Akt3 total body and bone marrow-specific knockout mice.[193]

The role of Akt2 in atherosclerosis is more complex. Rensing and colleagues found that total body deletion of Akt2 induces smaller but unstable atherosclerotic lesions, with a major causative role for VSMC derived Akt2 in the decreased lesional collagen content and increased necrotic core formation.[196] The unstable phenotype induced by Akt2 loss is likely due to disturbances in VSMCs migration, proliferation, and metalloproteinase production.[196] However, a study from another group found that total body Akt2 deletion does not affect atherosclerosis development.[191] To exclude the contribution of smooth muscle cell Akt2, Babaev *et al.* used fetal liver cell transplantation and Rotllan *et al.* used bone marrow transplantation to specifically delete Akt2 in hematopoietic cells of LDLr KO mice.[188,191] Both studies indicated that hematopoietic Akt2 deficiency protects LDLr KO mice against diet-induced atherosclerosis. Akt2 deletion in macrophages induced M2 macrophage polarization, decreased macrophage migration and inhibited macrophage foam cell formation; processes that are likely responsible for the observed decrease in atherosclerosis susceptibility of the LDLr KO recipients.[191,197] These findings indicate that both VSMC Akt2 and hematopoietic Akt2 play a role in atherogenesis. Importantly, the protective effect of hematopoietic Akt2 deficiency was independent of Akt1 and Akt3, because bone marrow-specific deletion of Akt1 and Akt3 led to unchanged or increased atherosclerosis development, while the phenotype of the reduced susceptibility to atherosclerosis persisted upon combined Akt2/Akt1 or Akt2/Akt3 deletion in bone marrow.[192,193,198-200]

4.3 MAPKs/MKPs pathway in atherosclerosis

4.3.1 MAPKs and atherosclerosis

MAPKs are a family of protein kinases that specifically phosphorylate serine/threonine residues. Three major subfamilies can be distinguished: extra cellular regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. All these subfamilies have been reported to participate in atherosclerosis development.

4.3.1.1 ERK and atherosclerosis

In vivo, ERK expression is increased in atherosclerotic lesions of cholesterol-fed rabbits.[201] *In vitro*, ERK is rapidly activated upon oxLDL stimulation in macrophages.[202] These findings indicate a role for ERK in foam cell formation. Indeed, inhibition of ERK1/2, by their upstream MEK1/2 inhibitor U0126, significantly decreased foam cell formation both *in vivo* and *in vitro*, which is most likely the consequence of upregulated cholesterol efflux transporters ABCA1 and ABCG1.[203,204] In agreement with the decreased susceptibility to foam cell formation, atherosclerotic lesion development was reduced in ApoE knockout mice treated with the inhibitor U0126. These findings suggested an anti-atherosclerotic role for ERK1/2 inhibition.

ERK also influences macrophage polarization. Inhibition of ERK by the inhibitors U0126, or PD0325901 led to an M2-like macrophage phenotype, reflected by increased M2 marker gene expression.[205,206] Furthermore, the ERK inhibitor–dependent increase of M2 macrophage marker gene expression is likely independent of the pre-existing polarization state of the macrophage,[206] as re-priming of LPS-polarized M1 macrophages by IL-4/IL-13 still induced a shift towards the M2 phenotype. These findings suggest that skewing of macrophages towards an M2 phenotype might also contribute to the observed athero-protective effect of ERK inhibition.

4.3.1.2 P38 and atherosclerosis

P38, also called mitogen-activated protein kinase 11 (MAPK11), has 4 isoforms: p38α, p38β, p38γ, and p38δ.[207] p38α, the most well-studied isoform of p38, is rapidly phosphorylated in macrophages in response to LPS and is responsible for the subsequent induction in the production of pro-inflammatory cytokines.[208-211] Genetic deletion of p38α in macrophages results in an impaired TLR4-mediated LPS-induced innate immune response, reflected by a decreased production of the pro-inflammatory cytokines TNF-α and IL-12.[212] Furthermore, p38 activation is enhanced in IL-4-induced alternatively activated macrophages, and inactivation of p38 led to decreased IL-4-induced M2 marker expression.[213] Collectively, these findings suggest that p38 activation is likely needed for both M1 and M2 macrophage polarization and function.

The role of p38 in foam cell formation is not clear yet. Inactivation of pan p38 by a pharmaceutical inhibitor prevents foam cell formation *in vitro*.[214,215] However, genetic deficiency of p38α in macrophages does not affect foam cell susceptibility,[216] albeit it does enhance macrophage apoptosis.[217] Several studies have been performed to address the role of p38 in atherosclerosis. Systemic p38 inhibition, either via pharmaceutical inhibition or genetic deletion of the p38 substrate MK2, protects ApoE KO mice against atherosclerosis,[218,219] indicating a pro-atherogenic role of p38. Interestingly, Seimon *et al.* showed that macrophage-specific deletion of p38α promotes atherosclerosis development.[217] In contrast, Kardakaris *et al.*, using the same experimental set up, found that p38α deficiency had no effect on atherosclerosis development.[216] Hence, the role of macrophage p38 in atherosclerosis remains controversial.

4.3.1.3 JNK and atherosclerosis

c-Jun N-terminal kinase (JNK), also named mitogen-activated protein kinase 8 (MAPK8), is present in three isoforms: JNK1, JNK2, and JNK3.[220] Of these three isoforms, JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is mainly expressed in brain, and to a lesser extend in the heart and testes.[220] JNK1 and JNK2 are both expressed in macrophages,[221] and their activation is known to regulate various macrophage functions, including polarization, foam cell formation, and programed cell death.

Inhibition of JNK activation leads to impaired macrophage development, proliferation, and survival, [222] suggesting a broad function of JNK in macrophages. In vitro, JNK1 was shown to be responsible for cytokine and NO production by LPS-stimulated M1 macrophages.[223,224] Furthermore, *in vivo*, deletion of JNK1 reduced macrophage migration and infiltration in a murine arthritis model.[225] On the other hand, JNK2 was shown to stimulate oxLDL-induced foam cell formation.[202] Conversely, oxLDL triggers JNK2 activation in macrophages and facilitates scavenger-mediated foam cell formation in a CD36-JNK-SR-A loop manner.[226] In accordance, enhanced activation of JNK2 is observed in macrophage-rich atherosclerotic lesions.[227,228]

In line with the distinct effects of JNK isoforms on macrophage function, JNK isoforms also contribute to atherosclerosis differently. Genetic deletion of JNK2 protects ApoE KO mice against high-fat diet induced atherosclerosis, and this is likely attributed to the lack of JNK2-induced foam cell formation.[227] Interestingly, hematopoietic JNK1 deficiency promotes atherosclerosis development in LDLr KO mice, likely caused by the lack of JNK1-mediated regulation of macrophage survival.[221]

4.3.1.4 Interactions between MAKP members

MAPK cascades can be activated by either intra- or extracellular stimulators and signaling molecules. Depending on the cell type, stimulus signal strength and dynamics, MAPK can be activated differently and serve distinct functions. Importantly, each member of MAPK family closely interacts with the other members, and in most cases is subject to negative feedback regulation.[229]

4.3.2 MAP kinase phosphatases and atherosclerosis

The activity of MAPKs is tightly regulated by MAP kinase phosphatases (MKPs) that belong to the group of dual-specificity phosphatases (DUSPs), which includes at least 10 members.[230] MKPs are normally referred to as the typical DUSPs.[231] Atypical DUSPs lack the MAPK-binding motif or kinase-interacting motif (MKB/KIM), which determines the dephosphorylation activity of DUSPs.[232,233] Based on their subcellular localization, MKPs are divided into three groups. The first group consists of MKP1 (DUSP1), PAC1 (DUSP2), MKP2 (DUSP4), and DUSP5, which are all inducible MKPs that are located in the nucleus. The second group is represented by MKP3 (DUSP6), MKP-X (DUSP7) and MKP4 (DUSP9), which are all located in the cytoplasm. Finally, the third group is comprised of DUSP8, MKP5 (DUSP10) and MKP-7 (DUSP16), which are located in both the nucleus and the cytoplasm of a cell.[230,234,235] MKPs inactivate MAPKs by dephosphorylating their phosphoserine/threonine and phosphotyrosine residues.[236,237] Noteworthy, the expression of group 1 members, being inducible MKPs,[230,234,235] is induced in response to stimulation with LPS,[238] a potent activator of MAPKs. Therefore, this group of MKPs is likely to act as MAPKs feedback loop regulator.[230,239]

All three members of the MAPK family are involved in macrophage polarization and foam cell formation, indicating that MKPs are most likely also involved in the regulation of these processes and thereby influence atherosclerosis development. Indeed, several studies have demonstrated that various macrophage functions are affected by depletion of MKPs, especially the inducible MKPs. For instance, deletion of MKP1 led to the upregulation of several M1 signature genes, including TNF-α, Il-6, IL-1β, and CCL2.[240,241] In addition, PAC1 deficient macrophages displayed an increased production of pro-inflammatory cytokines in response to LPS stimulation.[242] These data suggest an important role for MKP1 and PAC1 in regulating LPS-induced M1 polarization. Unlike the clear roles of MKP1 and PAC1 in the regulation of M1 polarization, the current findings on the role of MKP2 in macrophage polarization remain contradictory. Al-Mutairi *et al.* found that upon LPS stimulation, genetic loss of MKP2 in bone marrow-derived macrophages (BMDM) led to enhanced production of pro-inflammatory cytokines, while the production of anti-inflammatory cytokines was decreased.[243] In contrast, Cornell and colleagues found that upon LPS stimulation, MKP2 deletion in BMDMs resulted in decreased TNF-α and IL-10 production.[244] DUSP5 deficient macrophages showed no altered cytokine or chemokine production in response to LPS stimulation.[245] However, despite some indications for a role of MKPs in macrophage polarization and function, so far only limited research has been done addressing the role of MKPs in atherosclerosis.[246-248]

4.4 Usf transcription factors and atherosclerosis

Upstream stimulatory factors (Usfs) are DNA-binding proteins, featured as a helix-loop-helix motif and leucine repeat, that serve as transcription factors.[249] By binding to target DNA as Usf homo- and heterodimers, Usfs regulate target gene expression.[139,250] Accumulating evidence showed that disturbed Usf signaling normally leads to metabolic disorders, especially in the case of Usf1.[251-254] Usf1 is ubiquitously expressed, and has a broad range of target genes, of which the genes involved in lipid and glucose metabolism are most widely studied.[255] Usf1 is also known to regulate gene expression in response to stressors, such as ultraviolet (UV) irradiation,[256] insulin,[257] and growth factor.[258,259] Moreover, in response to PI3 kinase/Akt signaling, Usf1 regulates the transcription of genes implicated in cellular apoptosis and cell cycle arrest.[260]

Usf1 is a direct target of p38 and AMPK, which both have been shown to be involved in macrophage polarization.[254,256,261-263] Furthermore, Usf1 is phosphorylated by protein kinase CK2.[264] Protein kinases are important for regulation of various cellular functions. However, the exact role of Usf1 in cell function regulation is still unclear. Importantly, mutations in Usf1 are strongly associated with familial combined hyperlipidemia, a common hereditary dyslipidemia with a prevalence of 20% in CVD patients.[265] In support of this association, a strong pro-atherogenic role for Usf1 is found in LDLr KO mice lacking Usf-1, which display a remarkable decreased in their susceptibility to atherosclerotic lesion development.[152] The mechanism behind this effect, however, is poorly defined.

5 Outline of this Thesis

Currently, therapeutic strategies to prevent atherosclerosis are primarily based on the use of cholesterol lowering drugs, e.g. statins. However, the morbidity and mortality of cardiovascular disease caused by atherosclerosis remains high. Therefore, identification of novel pharmaceutical targets suitable for the development of novel drugs is strongly needed. The aim of this thesis is to unravel the role of various candidate genes in macrophage activation and their subsequent role in atherosclerosis.

In **Chapter 2**, we determined the role of hematopoietic Arg1, a classic marker of M2 macrophage activation, in atherosclerosis. We show that although Arg1 deficiency promotes macrophage foam cell formation, it does not impact on atherosclerosis development.

Akt2 is a key player in the PI3K/Akt transduction pathway that regulates M2 macrophage polarization.[190] In **Chapter 3,** we addressed the effects of bone marrow Akt2-reconstitution, in Akt2/LDLr dKO mice. We show that hematopoietic Akt2 promotes foam cell formation, but does not alter atherosclerosis development in Akt2/LDLr dKO mice.

Next, we determined the effects of the upstream regulators MKP2, of the MAPK transduction pathway, in atherosclerosis. MKP2 was found to play an important role in regulating macrophage function, and in **Chapter 4** we show that MKP2 deficiency skews macrophages to an M2 phenotype associated with an enhanced susceptibility to foam cell formation. In line, deletion of MKP2 in bone marrow-derived cells leads to increased atherosclerosis development.

Additionally, we focused on the bone marrow-specific effects of Usf1, an upstream transcription stress sensor in atherosclerosis. In **Chapter 5,** we show that Usf1 in bone marrow-derived cells protects against atherosclerosis.

Finally, the overall conclusions and future perspectives of this thesis are discussed in **Chapter 6**.

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