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Ren, B.

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Baoyan (Olive) Ren

任保彦

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Prof. dr. H. Irth	LACDR, Leiden	(voorzitter)
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路漫漫其修远兮，吾将上下而求索。

--屈原 （Quyuan，c. 340－278 BC）

Sixty years ago I knew everything; now I know nothing; education is a progressive discovery of our own ignorance.

-- Will Durant

For my family

献给我的家人

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Table of contents

1

General introduction

Table of contents

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**).

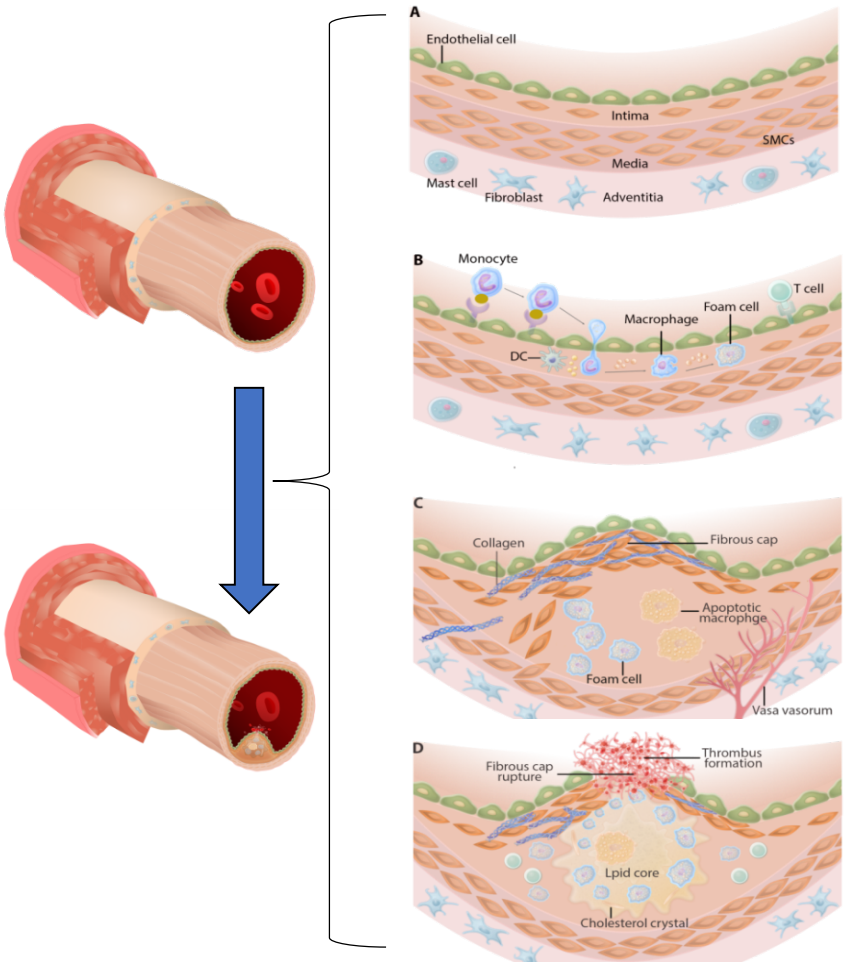

Figure 1 Progression of atherosclerosis development.

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]

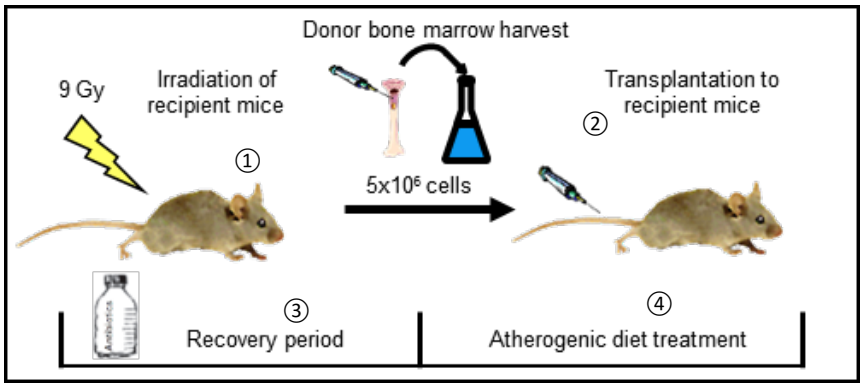

Figure 2 Schematic diagram of a bone marrow transplantation (BMT) procedure in mice for studying atherosclerosis.

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.

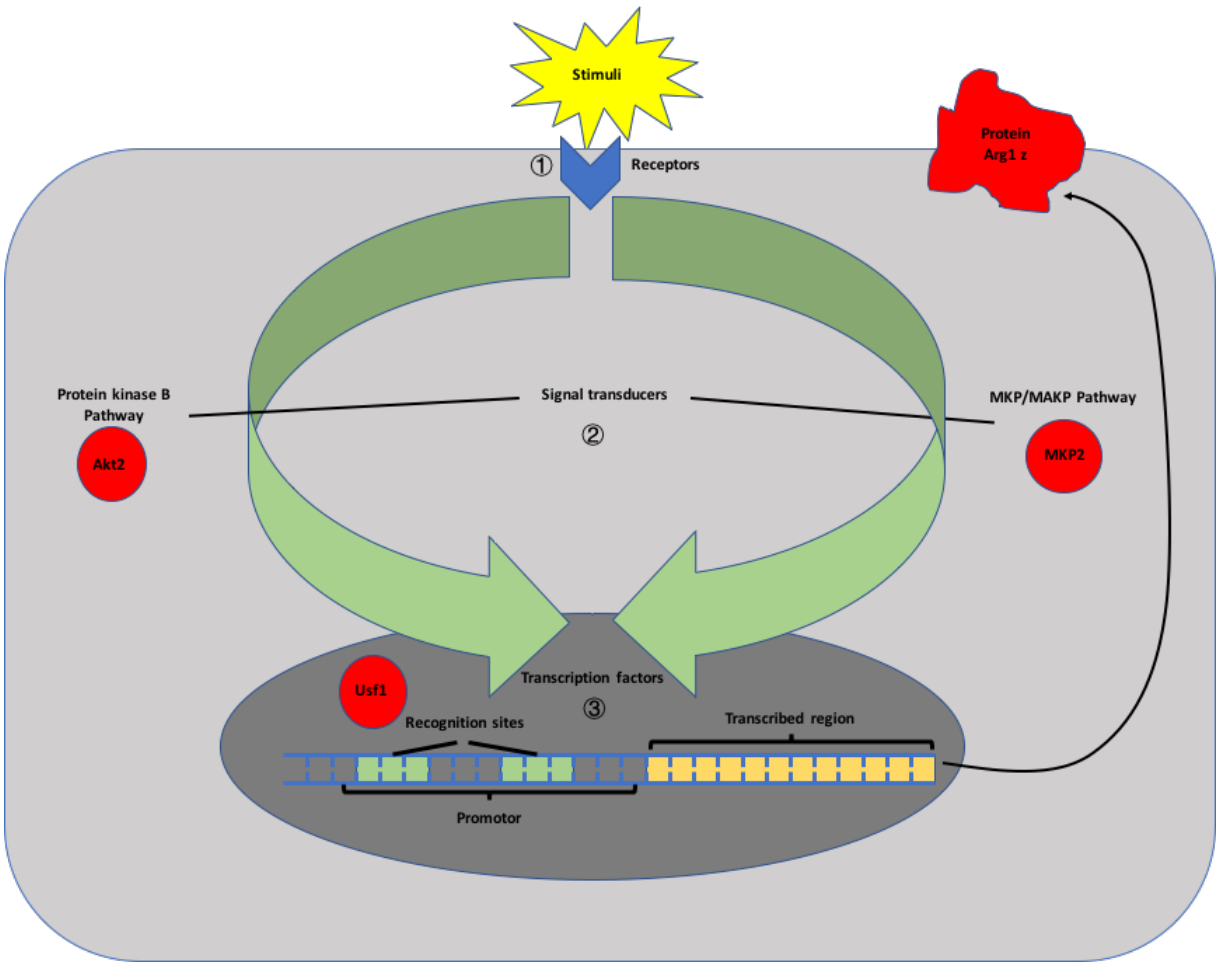

Figure 3 Regulation in macrophage activation and target factors Arg1, Akt2, MKP2 and Usf1 studied in more detail in this thesis.

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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2

Hematopoietic Arginase 1 deficiency results in decreased leukocytosis and increased foam cell formation but does not affect atherosclerosis

Baoyan Ren[a]*, Erik Van Kampen[a]*, Theo J.C. Van Berkel[a], Sheena M. Cruickshank[b], Miranda Van Eck[a]

* Both Authors contributed equally to this work

[a]Division of Biopharmaceutics, Cluster BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

[b]Manchester Immunology Group, Faculty of Life Sciences, The University of Manchester, Manchester, UK

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Arginase1 (Arg1), a M2 macrophage marker, plays a critical role in a number of immunological functions in macrophages, which are the main cell type facilitating atherosclerotic lesion development. Arg1 uses the substrate L-arginine to create L-ornithine, a precursor molecule required for collagen formation and vascular smooth muscle cell differentiation. By reducing L-arginine availability, Arg1 limits the production of Nitric Oxide (NO), a pro-atherogenic factor in macrophages. In endothelial cells, conversely, NO is strongly anti-atherogenic. However, until now, the role of Arg1 in atherosclerosis is largely unknown. The aim of this study is to specifically investigate the effect of Arg1 deletion in hematopoietic cells on atherosclerosis susceptibility.

LDLr KO mice were transplanted with *Arg1$^{flox/flox}$;Tie2-Cre* (Arg1 KO) bone marrow (BM) or wildtype (WT) BM. After 8 weeks recovery on chow diet, recipient mice were fed a Western-Type Diet (WTD) for 10 weeks to induce atherosclerosis. After 10 weeks WTD challenge, blood leukocyte counts were decreased by 25% (p<0.001), and spleen leukocytes were decreased by 35% (p=0.05) in LDLr KO mice transplanted with Arg1 KO BM, compared to mice transplanted with WT BM. The decrease in leukocytes was due to lower B lymphocyte counts. However, oxLDL-specific antibodies were increased in plasma of LDLr KO mice transplanted with Arg1 KO BM compared to WT BM transplanted controls, whereas oxLDL-specific IgM was not affected. On the other hand, peritoneal foam cells in Arg1 KO BM recipients were increased 3-fold ($p<0.001$) compared to WT BM recipients. No change in blood cholesterol was found. Despite changes in leukocyte counts and macrophage foam cell formation, we did not observe differences in atherosclerotic plaque size or plaque macrophage content in the aortic root. Surprisingly, there was also no difference in plaque collagen content, indicating that absence of macrophage Arg1 function does not reduce plaque stability.

Deletion of Arg1 in hematopoietic cells adversely affects blood leukocyte counts and increases foam cell formation. However, no effects on atherosclerosis could be demonstrated, indicating that hematopoietic Arg1 function is not a decisive factor in atherosclerotic plaque formation.

Introduction

Inhibition of the activity of the enzyme Arg1 is considered a promising novel therapeutic strategy for the treatment of cardiovascular disease.[1] In line, arginase inhibition by N(omega)-hydroxy-nor-l-arginine (nor-NOHA) improves endothelial function in familial hypercholesterolemia patients and reduces atherosclerotic lesion development in carotid arteries of apolipoprotein E (ApoE) knockout mice exposed to low shear stress.[2-4] Arg1 influences a number of processes implicated in the pathogenesis of atherosclerosis.[5-8] It is expressed in endothelial cells, vascular smooth muscle cells (VSMCs) and macrophages, which are all important cellular components of the atherosclerotic plaque.[1] Depending on the cell type it is expressed in, Arg1 function is expected to exert different effects on atherosclerotic plaque formation.

The primary function of Arg1 is production of urea and L-ornithine from L-arginine.[9] L-arginine, however, is also used as a substrate by the enzymes inducible- and endothelial Nitric Oxide Synthase (iNOS and eNOS) for the production of the endothelial-protective signalling molecule nitric oxide (NO).[2,10] By competition for the common substrate L-arginine, Arg1 can thus indirectly inhibit the synthesis of NO.[11,12] In line, endothelial Arg1 contributes to endothelial activation and vascular stiffness by reducing the L-arginine pool, leading to eNOS uncoupling and reduced NO production.[10,13] This results in endothelial activation and increased recruitment of immune cells to the plaque.[10,13] However, atheroprotective effects have also been described for Arg1 in

macrophages and VSMCs. By producing L-ornithine, Arg1 contributes to the synthesis of L-proline by the enzyme Ornithine Amino Transferase (OAT), which is a precursor for collagen biosynthesis. Ornithine can also be metabolised into polyamines, which leads to increased VSMC differentiation and decreased inflammation.[14-16] In agreement, lentiviral-mediated upregulation of Arg1 in a balloon-injury rabbit model inhibited plaque inflammation and augmented VSMC proliferation. Plaque size was, however, not affected.[8]

In macrophages Arg1 is found in the alternatively activated M2 cells, a macrophage subtype with an anti-inflammatory and wound healing function.[6] Downregulation of Arg1 expression and inhibition of Arg1 activity in Raw264.7 macrophages resulted in augmented LPS-induced TNF-α and IL-6 secretion.[8] On the other hand, Arg1 in macrophages suppresses Th2 dependent inflammation by dampening the production of anti-inflammatory cytokines by $CD4^+$ T cells and suppressing T-cell proliferation in mice infected with the trematode Schistosoma mansoni.[17] Differential gene expression analysis in macrophages of atherosclerosis-susceptible and -resistant rabbits suggested that high macrophage Arg1 expression was associated with low atherosclerosis susceptibility.[18] Furthermore, M2 macrophages are found predominantly in carotid plaques of asymptomatic patients that have more stable plaques,[19] indicating the positive association between macrophages of the M2 phenotype and atherosclerotic plaque stabilization. However, the functional role of macrophage Arg1 in atherosclerotic plaque development is currently still unknown.

In this study we specifically assessed the contribution of hematopoietic Arg1 to the development of atherosclerosis, by transplanting bone marrow from *Arg1$^{flox/flox}$;Tie2Cre* mice into atherosclerosis-susceptible LDL receptor knockout (LDLr KO) mice.

Material and Methods

Animals

LDLr KO mice and WT C57Bl/6 were obtained from the Jackson Laboratory and expanded at the Faculty of Science, Leiden University. *Arg1$^{flox/flox}$;Tie2Cre* (Arg1 KO) mice [20] were bred at the Faculty of Life Sciences, University of Manchester. All animal studies in the Netherlands were approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. All animal work in the United Kingdom was performed in accordance with Home Office regulations.

mRNA Expression Analysis by Real Time PCR

Thioglycollate-elicited peritoneal macrophages (PMs) from 12-week old male C57Bl/6 mice were obtained after injection of 1 mL of 3% thioglycollate solution 5 days prior to the experiment. After adherence and washing, the macrophages were incubated with/without 10 μg/mL oxidized low density lipoprotein (oxLDL, prepared as described previously[21]) for 24 hours. After that, cells were collected for total RNA isolation.[22] Subsequently, RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada) was used to transcribe RNA to cDNA. Quantitative PCR (qPCR, ABI PRISM 7500 system, Foster City, CA) was used to access the mRNA expression levels of genes interested using SYBR Green reagents (Applied Biosystems). *RPL27* and *36B4* were used as housekeeping genes.

Microarray analysis

Twelve-week old female LDLr KO mice were first fed Western-type Diet (WTD; Special Diet Services) that contains 15% cacao butter and 0.25% cholesterol for a run-in period of 2 weeks before bilateral perivascular collar placement in the carotid arteries. Then the mice were challenged with WTD for another 2 weeks to induce early atherosclerotic lesion development. The carotid arteries were isolated directly after the run-in WTD period (baseline group) or 2 weeks after collar-placement (atherosclerotic plaque group) for microarray analysis as previously described.[23]

Bone Marrow Transplantation

Bone marrow from male C57Bl/6 WT controls and Arg1 KO mice (around 12 weeks old) was prepared for bone marrow transplantation (BMT) to 12 weeks old female LDLr KO recipient mice. In brief, lethally irradiated recipients received $5x10^6$ bone marrow cells via tail vein injection. The mice were allowed to recover for 8 weeks on chow diet (RM3; Special Diet Services), after which they were fed WTD to induce atherosclerosis. After the 10-week WTD challenge, the mice were anaesthetized by a lethal dose of anesthetic mixture that contained rompun, ketamine and atropine. Mice were bled and perfused with PBS, after which organs were isolated. The hematologic chimerism was confirmed in genomic DNA of recipients bone marrow using the PCR method (Supplementary Figure 1A-B).

Generation of Bone Marrow-Derived Macrophages (BMDMs)

Bone marrow from LDLr KO recipients transplanted with WT BM or Arg1 KO BM was isolated at sacrifice for the *in vitro* experiments. Bone marrow-derived macrophages were obtained as described previously.[24] Macrophages were cultured for 24 hours with or without 100µg/mL acetylated-low density lipoprotein (acLDL). The preparation of acLDL is described previously.[25] Subsequently the cells were analyzed by an automated veterinary haematology analyzer (Sysmex Corporation, XT-2000iV, Japan) for foam cell formation as described previously.[26,27] Briefly, the Sysmex XT-2000iV analyzer applies a similar principle for cell differential analysis as patented fluorescent flow cytometric analysis.[28] Laser side scatter and side fluorescence lights were used for separating cell clusters. Lipid-rich macrophages (foam cells) are larger and contain more abundant granules compared to the non-foam cells.[26,27] Thus in a differential scattergram, the lipid-rich macrophage population shifts to a larger scale on the side scatter axis and side fluorescent light axis, enabling gating of a separate, shifted population of macrophage foam cells.

Flow Cytometry Analysis and WBC Differential Analysis

Blood samples, anti-coagulated with EDTA, as well as single splenic cell suspensions, were obtained using a 70µm cell strainer (734-0003, VWR), and used for FACS analysis. Erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH=7.3) was used to lyse red blood cells in the blood and splenocyte preparations. Consecutively, the cells were analyzed on a FACS Canto II (BD Biosciences, Mountain View, CA) using the relevant FACS antibodies (all obtained from eBioscience).

An automated Haematology analyzer (XT-2000iV, Sysmex Corporation, Japan) was used to analyse leukocyte counts in spleen and blood samples. Furthermore, peritoneal leukocytes collected at sacrifice from the bone marrow transplanted animals, were analyzed for quantification of macrophage foam cells formation.

Serum Cholesterol Level Determination

Total and free cholesterol concentrations in serum were determined using an enzymatic colorimetric method as described previously.[29] Absorbance was read at 490 nm.

ELISA assay for anti-oxLDL antibodies

Copper-oxLDL was prepared as previously described.[21] The mouse immunoglobulin isotyping ELISA kit was obtained from BD Biosciences (Catalog No. 550487). HRP labelled polyclonal rabbit anti-Rat immunoglobulins (Ig) were obtained from DAKO (Product No. P045001). Total Ig and IgM antibodies against oxLDL were measured by a modified ELISA. In brief, oxLDL was coated in an ELISA plate (Corning, NY, 14831) in a concentration of 10µg/mL overnight at 4°C in coating buffer (0.42% w/v $NaHCO_3$, 0.53% Na_2CO_3, pH of 9.6). After washing with 0.05% tween-20-PBS, wells were blocked for 30 minutes in 1% BSA blocking buffer. After washing, 4uL plasma was added and plates were incubated at room temperature for an hour, then washed. For determination of total Ig, the HRP-labelled rat anti-mouse Ig antibody included in the BD ELISA kit was added. After incubation for 1 hour at room temperature and subsequent washing, the plate is ready for colour development. For the IgM determination, after incubation with IgM-specific rat anti-mouse antibody for 1 hour, wells were incubated with HRP-labeled rabbit anti-rat Ig for another hour before colour development, upon following the manufacturer's instruction. Optical density (OD) was obtained by reading the plate in a plate reader (Biotek, poowerWave 340) at 450nm and 570nm. Wavelength correction was performed by subtracting the values obtained at 570nm from the values at 450nm.

Histological Analysis of the Aortic Root

After 10-week WTD feeding, mice were sacrificed. Seven-µm serial sections of the aortic root were cut using a Leica cryostat. Oil red-O staining, MoMa2 staining and Masson's Trichrome kit were used for visualization of plaque area, macrophage positive area and collagen content respectively as described previously.[29] Quantification was performed using the Leica image analysis system (Leica Ltd, Cambridge, UK).[29]

Statistical Analysis

Student's T-test or two-way ANOVA were used to determine the statistically significant differences (Graphpad Prism software). A Welch correction was applied to the T-test in the case of unequal variances in the dataset. The statistical significance was set at 0.05. Results are shown as the mean ± SEM.

Results

Arg1 expression is induced in macrophages by oxLDL loading and in carotid arteries upon induction of collar-induced atherosclerosis

The early stages of atherosclerotic lesion development are characterized by the accumulation of lipid-laden macrophages. Therefore, first the effects of incubation of macrophages with oxLDL, and induction of collar-induced atherosclerosis in the carotid artery on the expression of Arg1 were determined. In agreement with a previous study from Gallardo-Soler *et al.*,[30] *Arg1* mRNA expression was increased 4.3-fold in thioglycollate-elicited peritoneal macrophages after 24 hours oxLDL (10µg/mL) loading (p<0.001; figure 1A) after a qPCR analysis. Oil-red O staining confirmed that after oxLDL incubation, foam cells were successfully induced (Figure 1B). Moreover, microarray analysis showed that *Arg1* expression was significantly increased in collar-induced early atherosclerotic lesions in the carotid artery of LDLr KO mice (4.5-fold, p<0.01; figure 1C).

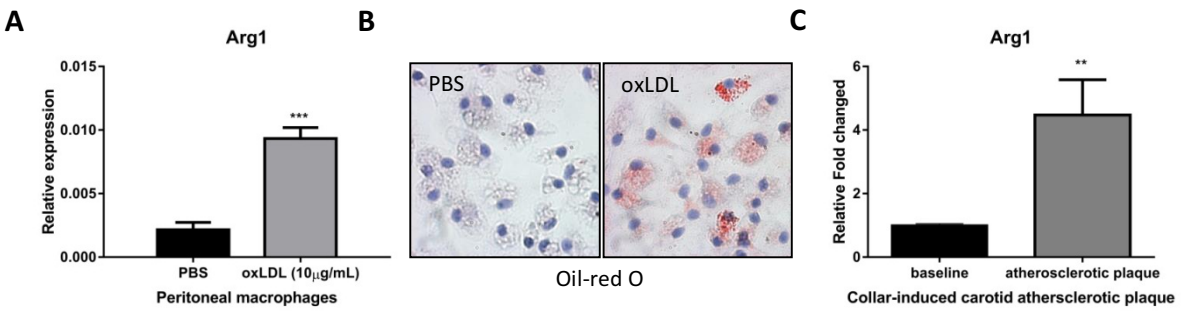

Figure 1 *Arg1* expression was induced by oxLDL loading in wild-type (WT) thioglycollate-elicited peritoneal macrophages (PMs) or Western-type diet (WTD) and collar-induced atherosclerosis in the carotid artery.
A) Thioglycollate-elicited PMs were incubated with/without 10 µg/mL oxLDL for 24 hours, and then qPCR was used for analysis of *Arg1* gene expression. (n=6). B) Oil-red O staining of PMs after incubation with/without 10 µg/mL oxLDL for 24 hours. C) LDLr KO mice were challenged with WTD 2 weeks before the collar placement (baseline). The experimental group was fed WTD for another 2 weeks after collar placement (atherosclerotic plaque). The collar-induced atherosclerotic plaques in carotid arteries of LDLr KO mice were used for microarray analysis of which the effects on*Arg1* expression are shown. (n=6). Results are expressed as mean ± SEM, significance was assessed by student T-test. **p<0.01; ***p<0.001.

Arg1 deletion in bone marrow-derived cells of LDLr KO mice increases foam cell accumulation in the peritoneum in absence of effects on serum total cholesterol levels

To generate a mouse model that specifically lacks Arg1 in bone marrow-derived cells, bone marrow (BM) from *Arg1*$^{flox/flox}$*;Tie2Cre* (Arg1 KO) mice and WT controls was transplanted into LDLr KO recipients. In *Arg1*$^{flox/flox}$*;Tie2Cre* mice Arg1 has been deleted in cells of the hematopoietic lineages and in endothelial cells.[19,31]

Niese *et al.* previously showed that Arg1 deficiency does not affect bone marrow engraftment.[32] In agreement PCR analysis on genomic DNA isolated from bone marrow after 10 weeks of WTD feeding confirmed successful disruption of Arg1 functionality in the bone marrow and peritoneal cells of the LDLr KO recipient mice (supplementary figure A-C).

Differential haematology analysis was performed on peritoneal cells harvested from LDLr KO mice, transplanted with Arg1 KO or WT bone marrow after 10 weeks of WTD feeding. No difference in total number of peritoneal leukocytes (data not shown) or the percentage of macrophages within

the peritoneal leukocyte population were found between Arg1 KO BM recipients and WT BM recipients (p>0.05, figure 2A). Interestingly, a 3-fold larger fraction of foam cells in the peritoneum of Arg1 KO BM recipients was found when compared to the WT BM recipients (p<0.001, figure 2B). This increased foam cell formation could not be attributed to increased serum cholesterol levels, as no difference in serum free cholesterol (data not shown) or total cholesterol was found (p>0.05; figure 2C).

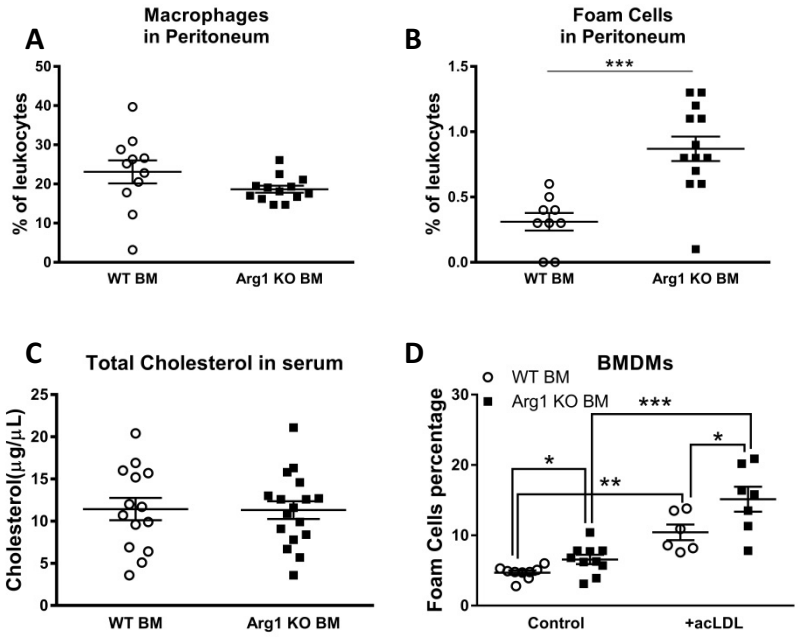

Figure 2 Increased *in vivo* peritoneal foam cell formation in LDLr KO mice transplanted with Arg1 KO BM in absence of effects on serum cholesterol and augmented *in vitro* foam cell formation in bone marrow-derived macrophages (BMDM) lacking Arg1.

Serum and peritoneal leukocytes were collected at sacrifice after 10 weeks of high-fat, high-cholesterol Western-type diet feeding. A) Macrophage percentage of total peritoneal leukocytes in BMT recipients (n=9-14). B) Foam cell percentage of total peritoneal leukocytes in the BMT recipients (n=9-14). C) Total cholesterol level in the BMT recipients (n=9-14). D) *In vitro*, BMDMs from WT BM or Arg1 KO BM recipients were incubated 24 hours in the presence or absence of 100µg/mL acLDL. Foam cell percentage was detected using a haematology analyser (n=6-10). Results are expressed as mean ± SEM, significance was assessed by student T-test or 2-way ANOVA. *p<0.05; **p<0.01; ***p<0.001.

Arg1 KO BMDMs show increased foam cell formation and tend to differentiate to an M2 macrophage phenotype upon acLDL stimulation.

BMDMs from WT and Arg1 KO mice were treated with 100µg/mL acLDL for 24 hours to gain mechanistic insight into the observed increased foam cell formation *in vivo*. AcLDL-induced lipid loading led to foam cell formation in both WT BMDMs and Arg1 KO BMDMs (4.7 ± 0.3% to 10.4 ± 1.1% for WT BMDMs, p<0.01; and 6.5 ± 0.66% to 15.1 ± 1.8% for Arg1 KO BMDMs, p<0.001, respectively; figure 2D). Interestingly, both before and after lipid loading Arg1 KO BMDMs displayed a 50% increase in foam cell formation compared to WT BMDMs (p<0.05, figure 2D). The mRNA expression of the genes related to foam cell formation was also assessed by qPCR analysis in BMDMs incubated with/without 100 µg/mL acetylated LDL for 24 hours. Upon acLDL loading, the expression of *SR-B1,* a receptor for native and modified lipoproteins, was effectively downregulated in WT BMDMs (p<0.05 as compared to non-loaded cells; figure 3A), whereas Arg1 KO BMDMs failed to downregulate *SR-B1* upon acLDL loading (p>0.05, figure 3A). No differences

were observed in *LDLr* expression between the 2 genotypes either with or without acLDL loading (p>0.05; figure 3B). Foam cell formation is determined by the balance between cholesterol uptake and synthesis on the one hand and cholesterol efflux on the other hand. Although the expression of *ABCA1*, the primary cholesterol efflux transporter, was increased in the BMDMs loaded with acLDL (WT, 15.7-fold, p<0.001; Arg1 KO, 3.7-fold, p<0.01; figure 3C), no difference in *ABCA1* expression was found upon comparison of the Arg1 KO and the WT BMDMs (p>0.05, figure 3C). Notably, expression of *SREBP1* was 2.45-fold higher in Arg1 KO BMDMs compare to WT BMDMS in response to acLDL loading, whereas no difference was found under control non-loaded conditions (Control, p>0.05; +acLDL, p<0.05; figure 3D). The difference in *SREBP1* expression between acLDL loaded Arg1 KO and WT macrophages is explained by a failure of the Arg1 KO macrophages to downregulate *SREBP-1* in response to acLDL loading (WT, p<0.01; Arg1 KO, p>0.05; figure 3D). Furthermore, the M1 and M2 markers *iNOS* and *FIZZ-1* were determined in both genotypes of BMDMs before and after acLDL loading. Under control non-loaded conditions, no differences were found in the expression of *iNOS* or *FIZZ-1*. However, in response to acLDL loading, Arg1 deficiency in BMDMs led to an M2-like phenotype, as evidenced by significantly downregulated iNOS expression (67% decrease, p<0.05; figure 3E) and extremely upregulated *FIZZ-1* expression (19-fold, p<0.001; figure 3F), whereas no such changes were observed in WT BMDMs.

Furthermore, NO production was determined in the culture medium of WT and Arg1 KO BMDMs, both under control conditions and after acLDL lipid loading. Two-way ANOVA showed an increased NO production in Arg1 KO BMDMs compared to the WT BMDMs (p=<0.05, figure 3G). NO production was significantly increased (1.6-fold, p<0.05, figure 3G) in Arg1 KO BMDMs, but not in WT BMDMs in response to acLDL loading, leading to 2.2-fold (p<0.01, figure 3G) higher NO concentrations in the supernatant of Arg1 KO BMDMs under these conditions.

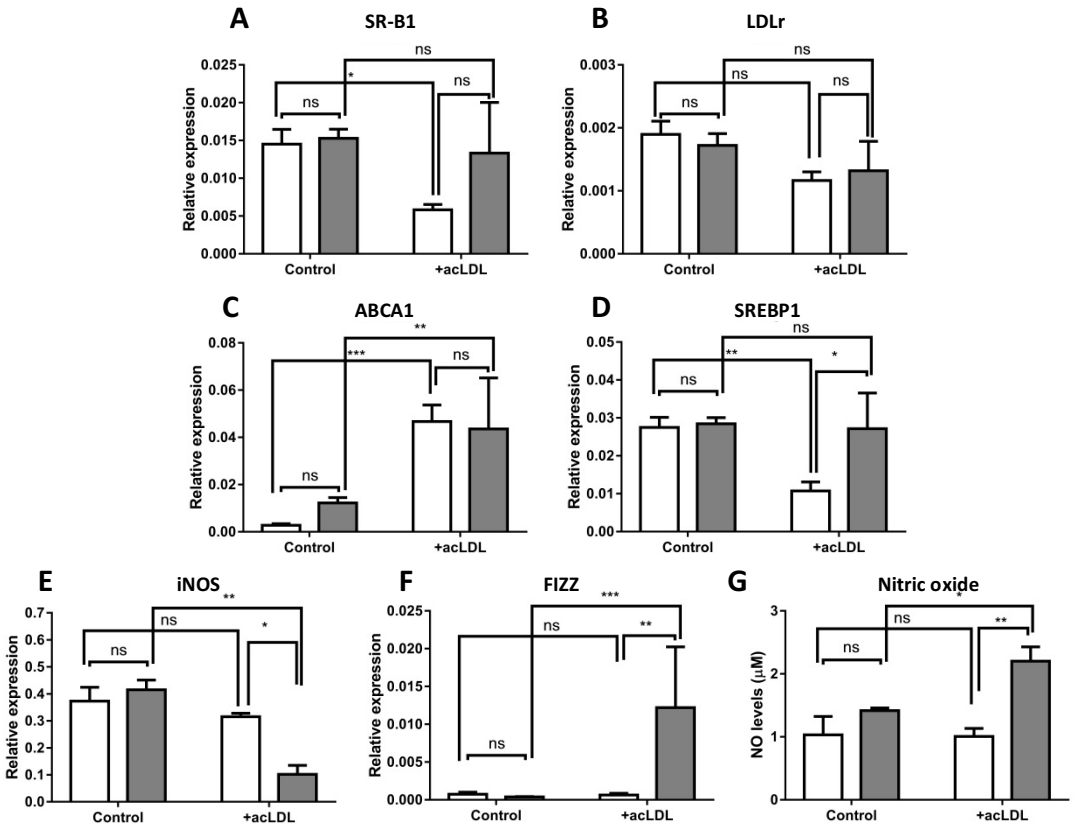

Figure 3 Gene expression and nitric oxide production in WT and Arg1 KO bone marrow-derived macrophages (BMDMs) after incubation with or without 100µg/mL acLDL for 24 hours.

Relative mRNA expression of A) *SR-B1,* B) *LDLr,* C) *ABCA1,* D) *SREBP1,* E) *iNOS*, and F) *FIZZ-1.* (Control group, n=9-12; +acLDL group, n=3-4)**.** G) Nitric oxide concentrations in the supernatant of BMDMs incubated with or without 100µg/mL acLDL 24 hours. (Control group, n=3; +acLDL group, n=4). Results are expressed as mean ± SEM, significance was assessed by student T-test or 2-way ANOVA. **p*<0.05; ***p*<0.01; ****p*<0.001.

Bone marrow Arg1 deficiency affects neither atherosclerotic plaque size nor plaque composition in LDLr KO mice.

After 10 weeks WTD feeding, the aortic root was sectioned and stained with Oil red-O to analyze atherosclerotic lesion development. Despite the observed increase in macrophage foam cell formation upon deletion of Arg1, no difference in plaque size was found between the two experimental groups (647±29 x 10^3 µm^2 for WT BM recipients vs. 634±26 x 10^3 µm^2 for Arg1 KO BM recipients, figure 4A). Macrophages in the plaque were visualized by MOMA-2 staining. No difference in plaque macrophage content as a fraction of total plaque size was observed (0.171 ±0.011 WT BM vs. 0.169 ±0.016 Arg1 KO BM, figure 4B). Collagen in the plaque was stained using a Masson's Trichrome method and Picrosirius Red staining. A trend towards a reduction in the collagen content of plaques of Arg1 KO BM recipients was found in Masson's Trichrome-stained sections (0.102 ±0.009 WT BM vs. 0.084 ±0.004 Arg1 KO BM, *p*=0.06, figure 4C). However, analysis of Picrosirius Red staining did not indicate any difference between the groups (0.124 ±0.013 WT BM vs. 0.105 ±0.012 Arg1 KO BM, figure 4D).

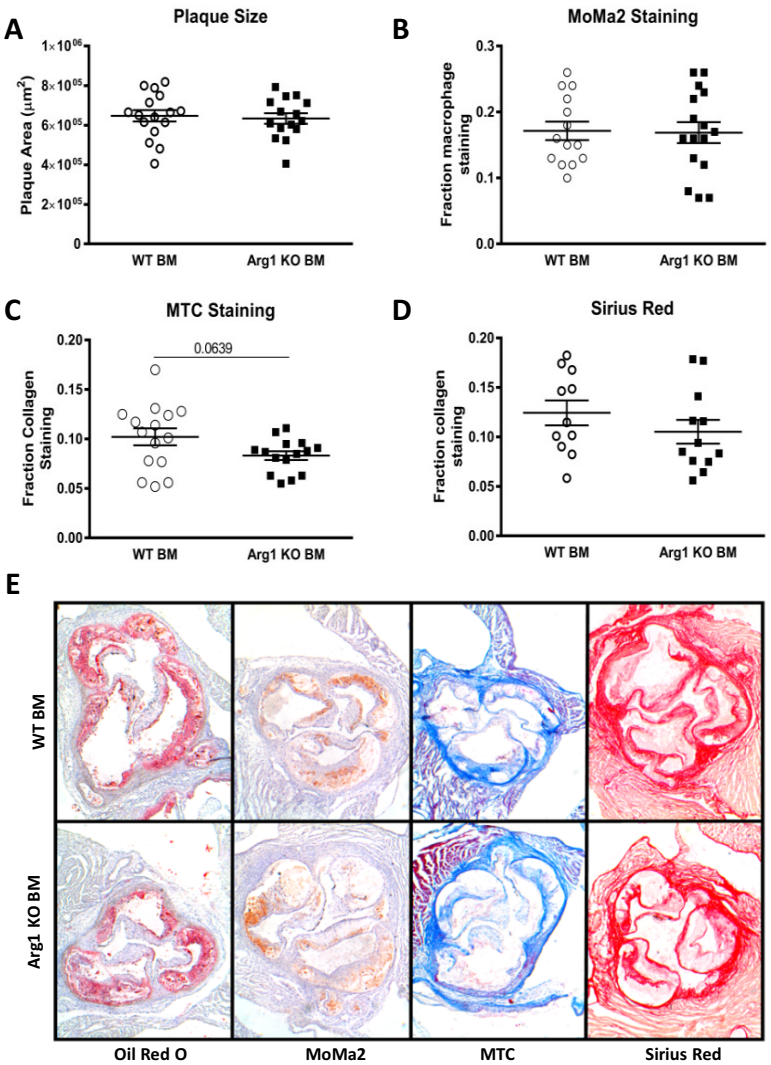

Figure 4 Deletion of Arg1 in bone marrow-derived cells does not influence atherosclerotic lesion development after 10 weeks WTD feeding.

A) Plaques were stained with Oil-Red O and plaque area was quantified. (p>0.5; WT BMT, n=16; Arg1 KO BMT, n=15). B) Plaque area stained positively with MOMA-2 antibody was measured and normalized for total lesion size. (p>0.5; WT BMT, n=14; Arg1 KO BMT, n=16). C) Plaque collagen was visualized using Masson's Trichrome (MTC) staining and plaque collagen content was analysed. (p=0.064; WT BMT, n=15; Arg1 KO BMT, n=15). D) A Sirius Red staining was performed to examine plaque collagen content further. Collagen content was analysed (p>0.5; WT BMT, n=11; Arg1 KO BMT, n=12). E) Representative images of the stainings described above. Results are expressed as mean ± SEM, significance was assessed by student's T-test.

Transplantation of Arg1 KO bone marrow into LDLr KO recipients results in reduced splenocyte and blood leukocyte counts

Flow cytometry was used to assess whether loss of Arg1 functionality in bone marrow-derived cells in the transplanted LDLr KO mice affected leukocyte numbers in the circulation or the spleen. On chow diet, no difference in total blood leukocyte numbers was detected (p>0.05, figure 5A) between the recipient mice with WT BM and the mice with Arg1 KO BM. WTD feeding for 10 weeks increased total leukocyte counts from in blood of LDLr KO mice transplanted with WT BM (chow diet: 11410 ± 708 cells/µl; WTD: 15672 ±689 cells/µl; p<0.01; figure 5A). Mice transplanted with Arg1 KO bone marrow, however, failed to show an increase in blood leukocyte counts upon WTD

feeding (chow diet: 12092 ± 906 cells/µl; WTD: 11573 ±491 cells/µl; p>0.05; figure 5A). Therefore, it led to lower total leukocyte counts in the blood of Arg1 KO BM recipients as compared WT BM recipients after 10 weeks of WTD feeding (p<0.001, figure 5A). Comparison of the subtypes of WBC, showed that, in contrast to WT BM recipients in which lymphocytes were 1.6-fold increases (p<0.001) after 10 weeks WTD feeding, lymphocytes did not increase in LDLr KO mice transplanted with Arg1 KO BM (Figure 5B). No differences were found in neutrophils, monocytes, and eosinophils (data not shown). Next, flow cytometric analysis was used to determine the effects on the different cellular subsets in blood at sacrifice. No differences were found in the absolute amounts of $CD11b^+$ cells, $CD11b^+/Ly6C^{hi}$ inflammatory, $CD11b^+/Ly6C^{low+med}$ patrolling monocytes and $CD11b^+/Ly6G^+$ neutrophils, nor the amounts of $CD4^+$ T helper cells, $CD25^+/CD4^+$ activated T helper cells and $CD8^+$ cytotoxic T-cells (figure 5C-H). Unexpectedly, the decrease in total blood leukocytes appeared to be driven by a 2-fold decrease in circulating $CD19^+$ B cells (p<0.01, figure 5I). Representative flow cytometry plots are shown in figure 5J.

At the time of sacrifice, spleens were taken and weighed. Organ weight was normalized for total body weight. A small but significant 10% decrease in spleen weight was found in LDLr KO mice transplanted with Arg1 KO BM (p<0.05, figure 6A), while there were no differences in total body weight (data not shown). Correspondingly, spleens from the Arg1 KO BM recipients contained 35% less splenocytes (p=0.052, figure 6B). Next, splenocyte composition was assessed by flow cytometry. The absolute numbers of leukocyte subtypes, including $CD11b^+$ cells, $CD11b^+/Ly6C^{hi}$ pro-inflammatory monocytes, $CD11b^+/Ly6C^{low+med}$ patrolling cells, $CD11b^+/Ly6G^+$ neutrophils, $CD19^+$ B cells and $CD8^+$ T cells in the spleen of Arg 1 KO recipients showed a significant decrease or a trend towards a decrease, suggesting that the decrease in splenocyte number was not attributable to one specific cell type (p<0.05, figure 6C-D). However, fractional analysis showed a 23% increase in $CD4^+$ and $CD25^+/CD4^+$ T cells in the spleen of LDLr KO mice that received Arg1 KO BM compared to the recipients that received WT BM (*p<0.05; $^{\$}p$ <0.05, respectively. Figure 6E). Representative flow cytometry plots are shown in figure 6F.

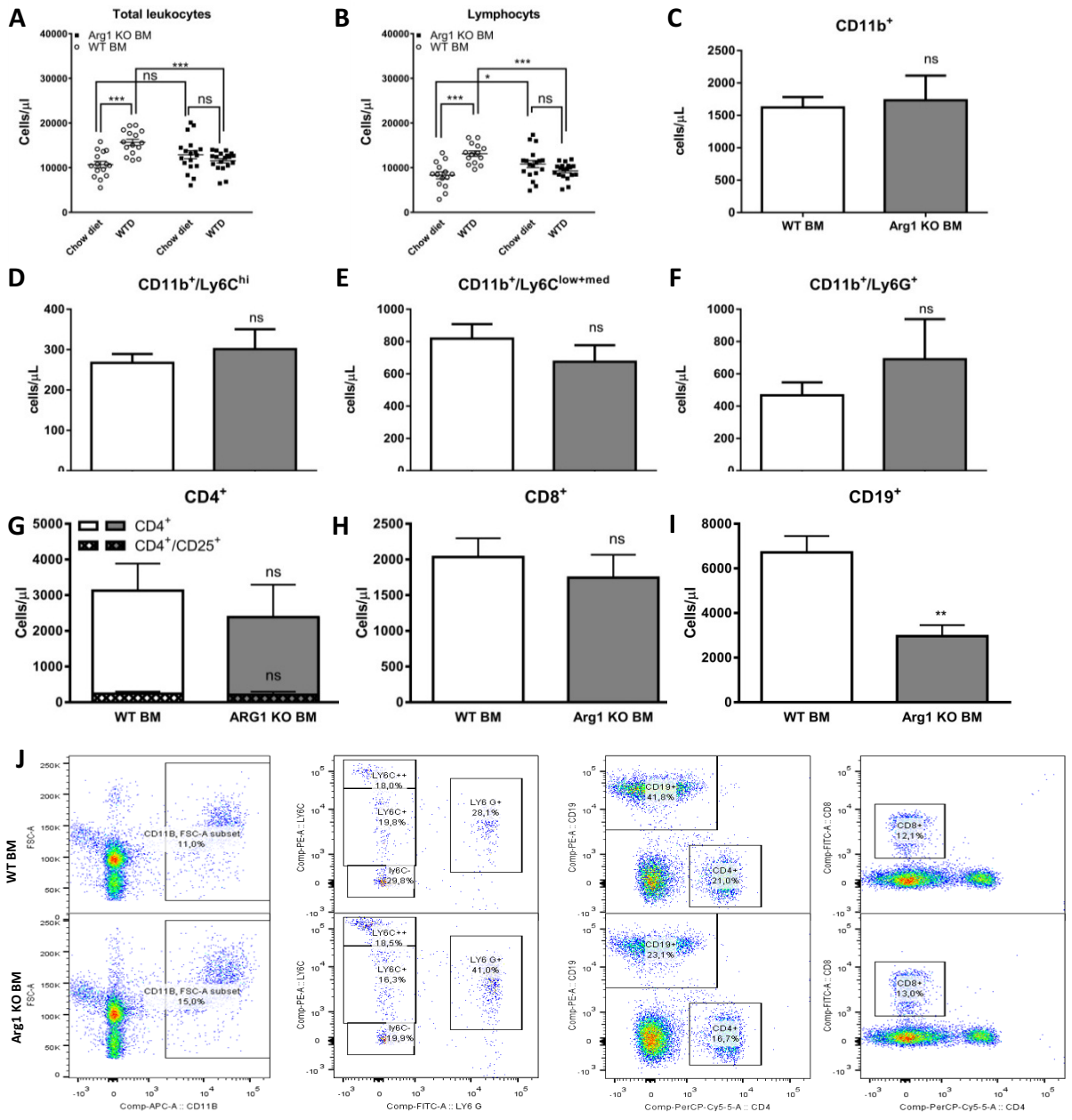

Figure 5 Decreased leukocytes in blood of LDLr KO mice transplanted with Arg1 KO BM after 10 weeks feeding a high-fat, high-cholesterol Western-type diet (WTD).

After BMT, recipient mice were fed chow diet for 8 weeks (shown as "chow diet" in figures) before the switch to WTD for an additional 10 weeks (shown as "WTD" in figures) to induce atherosclerosis development. Circulating A) leukocytes and B) lymphocytes on chow diet shown at 8 weeks after transplantation and after 10 weeks on WTD at 18 weeks after transplantation. (n=15-19). B) Circulating lymphocytes shown at 8 weeks after transplantation on chow diet and 10 weeks on WTD. (n=15-19). C-I) Circulating leukocytes were analyzed by flow cytometry for the expression of several immune cell markers. (n=5). I) Total $CD19^+$ cells in circulation (n=5). J) Representative flow cytometry plots. Results are expressed as mean ± SEM, significance was assessed by student T-test or 2-way ANOVA. *p<0.05; **p<0.01; ***p<0.001; ns, non-significant.

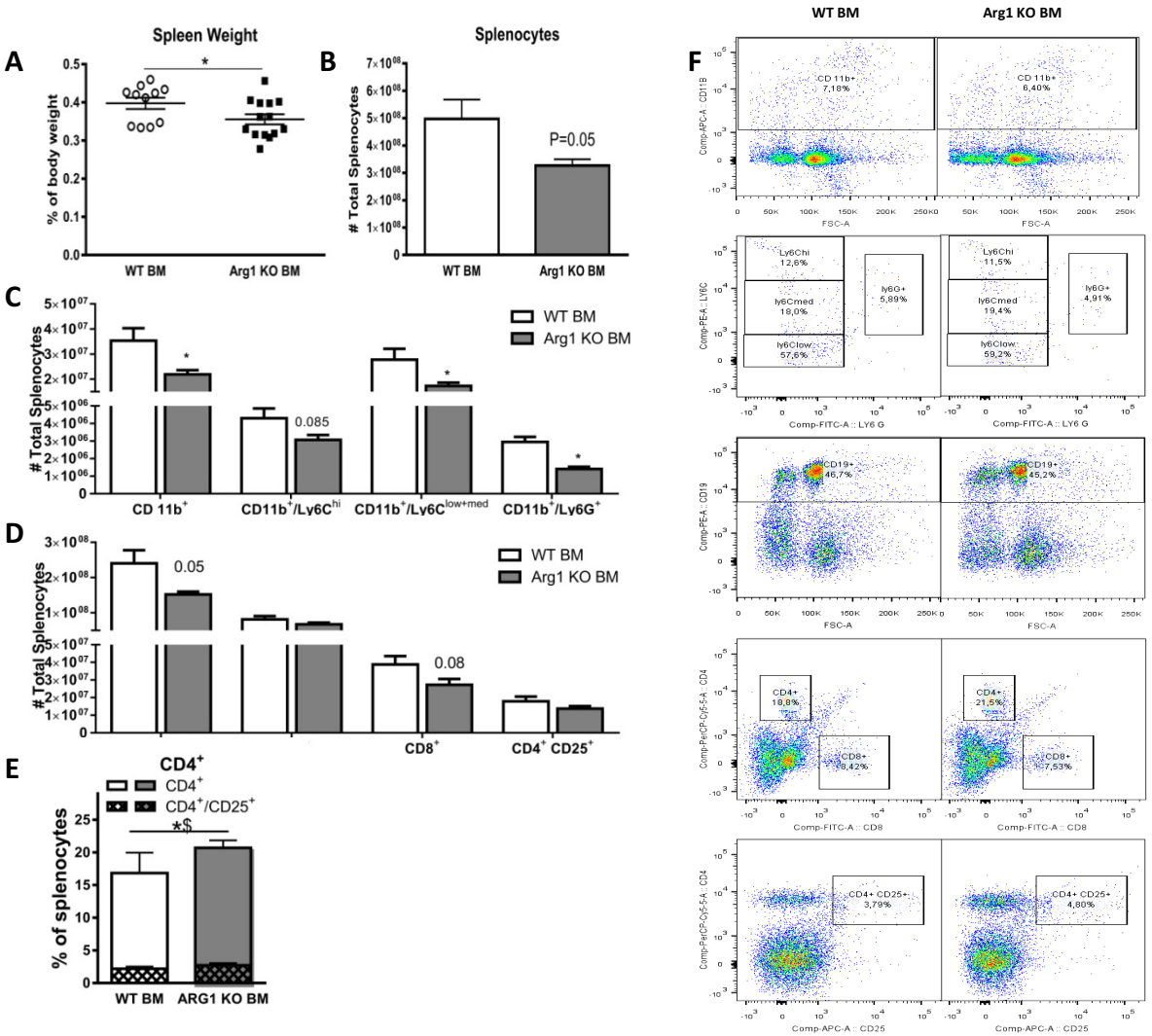

Figure 6 Decreased spleen weight in LDLr KO mice transplanted with Arg1 KO BM.

Spleens were isolated at 10 weeks after feeding a high-fat, high-cholesterol Western-type diet. A) A reduction in relative spleen weight was measured in the Arg1 KO BM recipients. The readout was corrected for the total body weight of the mice (**p*<0.05; WT BMT, n=11; Arg1 KO BMT, n=14). B) Reduction observed in the total amount of splenocytes in the Arg1 KO BM recipients (*p*=0.05; WT BMT, n=5; Arg1 KO BMT, n=5). C) Flow cytometry analysis of splenocytes showing that the amount of $CD11b^+$, $CD11b^+/Ly6C^{hi}$, $CD11b^+/Ly6C^{low+med}$ monocytes and $CD11b^+/Ly6G^+$ neutrophils were significantly decreased or trended to a decrease, in spleens of Arg1 KO recipients (**p*<0.05; WT BMT, n=5; Arg1 KO BMT, n=5). D-E) A trend to a decrease, in the amount of $CD19^+$ and $CD8^+$ splenocytes. The fraction of $CD4^+$ and $CD4^+CD25^+$ cells in the spleen was increased (**p*<0.05; $*p*<0.05, respectively; WT BMT, n=5; Arg1 KO BMT, n=5), though the total amount of $CD4^+$ and $CD4^+CD25^+$ cells in the spleen was unchanged (*p*>0.05; WT BMT, n=5; Arg1 KO BMT, n=5). F) Representative flow cytometry plots. Results are expressed as mean ± SEM, significance was assessed by student T-test. **p*<0.05; $*p*<0.05.

Arg1 deficiency in the bone marrow of LDLr KO mice induces circulating oxLDL-specific antibody levels.

The total level of ox-LDL specific immunoglobulins (Ig) and its isotype IgM were determined in plasma of the LDLr KO mice transplanted with WT or Arg1 KO BM after 10 weeks WTD challenge. As shown in figure 7, deletion of Arg1 in the bone marrow of LDLr KO mice led to increased total oxLDL-specific Ig levels (1.2-fold, $p<0.05$ compared to the WT BM LDLr KO recipients after 10 weeks WTD challenge, which was not driven by IgM.

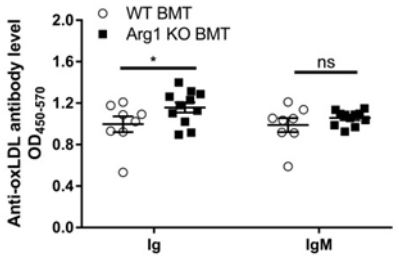

Figure 7 Increased oxLDL-specific antibody levels in LDLr KO mice transplanted with Arg1 KO BM after 10 weeks WTD feeding.

Total oxLDL-specific antibody and isotype IgM levels were assessed using ELISA. Results are expressed as mean ± SEM, significance was determined by student's T-test. *$p<0.05$ as compared between LDLr KO recipient with WT BM and recipients with Arg1 KO BM in the level of total oxLDL-Specific antibody (Ig). ns, non-significant.

Discussion

In the current study we determined for the first time the effects of Arg1 deletion in bone marrow-derived cells on macrophage foam cell formation and atherosclerosis susceptibility. We show that *Arg1* expression is increased in elicited peritoneal macrophages upon oxLDL-induced lipid loading and during the development of early murine atherosclerotic lesions in the carotid-artery. In agreement, Gallardo-Soler, Alejandro, *et al.*, previously demonstrated that *Arg1* expression is highly induced in bone marrow-derived macrophage foam cells upon oxLDL or acLDL stimulation.[30] Inhibition of PPAR-γ/δ suppresses the lipid-induced increase in macrophage Arg1 expression,[30] indicating that *Arg1* expression is increased during macrophage foam cell formation probably due to PPAR-γ/δ activation. Combined, these results indicate that Arg1 might participate in macrophage foam cell formation, thereby influencing atherosclerosis development. However, so far there had been no reports on the effects of Arg1 function on macrophage foam cell formation. Interestingly, Arg1 KO BMDMs showed increased foam cell formation both under control conditions and after acLDL-induced lipid-loading compared to the WT BMDMs. Genes involved in foam cell formation were investigated to gain insight into the mechanism behind the enhanced foam cell formation in absence of Arg1. No difference was found in the expression of *ABCA1*, the primary cholesterol export transporter, between WT and Arg1 KO macrophages. In response to acLDL loading, Arg1 deficient macrophages however did display an impaired suppression of *SREBP1* and *SR-B1* compared to WT macrophages. SREBP1 is an important regulator of cellular lipid biosynthesis,[33] while *SR-B1* facilitates the uptake of native and modified lipoproteins.[34] Enhanced foam cell formation of macrophages lacking Arg1 might thus be explained by defective suppression of cellular lipid biosynthesis and modified LDL uptake. Moreover, upon lipid loading, macrophages lacking Arg1 seem more likely to skew towards the

M2 phenotype, as evidenced by the significantly downregulated M1 marker *iNOS* and strongly upregulated M2 marker *FIZZ-1* expression in Arg1 KO macrophages. M2 macrophages exhibit increased cholesterol loading by oxLDL compared to M1 macrophages, providing one possible mechanism for the increased foam cell formation in our model.[35,36] Collectively, these findings suggest a link between Arg1 expression and macrophage foam cell formation. As Arg1 and iNOS compete for the common substrate L-arginine, macrophage Arg1 deficiency indirectly leads to enhanced synthesis of NO upon lipid loading. The observed downregulation of iNOS in the Arg1 deficient macrophages could thus be due to negative feedback by NO.[10,37-41] Notably, in agreement with our findings, excess NO has recently been shown to induce oxLDL-induced macrophage foam cell formation by inhibition of the LXR-ABCA1-cholesterol efflux axis.[42]

Next we investigated whether the observed increased foam cell formation *in vitro* also translated into increased macrophage foam cell formation *in vivo* and augmented atherosclerosis susceptibility. Hereto, bone marrow from *Arg1$^{flox/flox}$;Tie2Cre* mice was transplanted into LDLr KO mice, a commonly used model to study atherosclerosis. We decided to use *Arg1$^{flox/flox}$;Tie2Cre* mice as donors as the Tie2-cre deleter has previously been shown to lead to a complete ablation of Arg1 activity in macrophages, while in *Arg1$^{flox/flox}$;LysMCre* only ~80% reduction in macrophage Arg1 activity was achieved.[20] In line with the observed increase in lipid accumulation in Arg1 KO macrophages *in vitro*, foam cell formation in the peritoneal cavity of LDLr KO mice transplanted with Arg1 KO bone marrow was increased after 10 weeks WTD feeding.

Foam cell formation in the peritoneal cavity is a marker for atherosclerotic plaque development.[43,44] However, no differences in plaque size or plaque macrophage content were detected between the LDLr KO mice transplanted with Arg1 KO or WT bone marrow. Increased expression of Arg1 in balloon-injured rabbits results in augmented plaque stability as a consequence of enhanced VSMC proliferation.[8] Moreover, *Arg1$^{flox/flox}$;Tie2Cre* (Arg1 KO) mice exhibit impaired cutaneous wound healing, to some extent due to impaired collagen deposition.[43] The reduced collagen content in wounds of *Arg1$^{flox/flox}$;Tie2Cre* (Arg1 KO) mice was attributed to increased collagen degradation by neutrophils and macrophages lacking Arg1.[45] In atherosclerosis, collagen deposition provides stability to the atherosclerotic plaque. However, no significant effect on the collagen-content of atherosclerotic plaques of LDLr KO mice transplanted with Arg1 KO bone marrow was found. In the aortic root lesions, smooth muscle cells, the main producers of collagen deposited in atherosclerotic lesions,[46] were not affected by deletion of Arg1 in bone marrow-derived cells.[47] Conclusively, deletion of Arg1 in bone marrow-derived cells does not affect atherosclerosis susceptibility despite a clear increase in macrophage foam cell formation, indicating other compensatory mechanisms.

Tie2 is expressed in all hematopoietic lineage cells.[20] Hence, in our bone marrow transplantation model using the *Arg1$^{flox/flox}$;Tie2Cre* mice as donors, Arg1 is not only deleted in macrophages, but in all hematopoietic cells.[48] For long it has been thought that Arg1 is only expressed in the myeloid lineage and not in the lymphoid lineages.[49] Indeed Arg1 is expressed primarily in anti-inflammatory alternatively activated macrophages, however it can also be detected in neutrophils,[50,51] and innate lymphoid cells II (ILC2).[52]

To investigate whether bone marrow Arg1 deficiency had any atheroprotective effects to counteract the observed increase in foam cell formation, the leukocytes in blood and spleen, the major hematopoietic organ and an important reservoir for monocytes, were analysed in the BM transplanted mice. In agreement with previous studies by Niese *et al.*,[32] deletion of Arg1 in bone

marrow-derived cells did not affect circulating leukocytes on regular chow diet. Hypercholesterolemia is commonly known to induce leukocytosis in animal models.[53,54] and humans.[55,56] Upon challenge with a high fat/high cholesterol WTD, LDLr KO mice transplanted with Arg1 KO bone marrow failed to increase leukocyte counts in the circulation. The inflammatory and pro-atherogenic $CD11b^{+}/Ly6C^{hi}$ subset of monocytes gives rise to classically activated M1 macrophages in the atherosclerotic plaque.[57,58] However, no difference was found in the numbers of $CD11b^{+}/Ly6C^{hi}$ monocytes in blood, nor in the amount of circulating neutrophils between the 2 groups of bone marrow recipients after 10 weeks WTD challenge. Hypercholesterolemia-induced monocytosis and neutrophilia was thus not affected in the blood by deletion of Arg1 in bone marrow. In addition to myeloid cell mobilization from bone marrow, high fat diet-induced inflammatory conditions like atherosclerosis could also induce hematopoietic stem and progenitor cells (HSPCs) settlement in the spleen and leading to local production of monocytes and neutrophils.[59,60] Spleen-derived monocytes and neutrophils eventually infiltrate into the growing atherosclerotic lesion, giving rise to foam cell formation and pro-inflammatory cytokine production.[60] Notably, 30% of the total number of aortic monocytes were spleen-derived $Ly\text{-}6C^{hi}$ monocytes, which are also reported to contribute to foam cell formation in the lesions.[60] Arg1 depletion in bone marrow leads to a significant reduction of the $CD11b^{+}$ cells, $CD11b^{+}/ly6C^{low+med}$ monocytes and neutrophils in the spleen of LDLr KO mice and a tendency towards a decrease in pro-inflammatory $CD11b^{+}/Ly6C^{hi}$ monocytes. This might provide an atheroprotective mechanism counteracting the increased foam cell in LDLr KO mice transplanted with Arg1 KO BM.

As L-arginine is required for $CD4^{+}$ T-cell function and maturation, Arg1-mediated depletion of L-arginine by leukocytes results in decreased T cell proliferation.[17,61] T cell counts and activation status were therefore also investigated. However, there was no difference in the amount of $CD4^{+}$ cells and $CD4^{+}/CD25^{+}$ in blood and spleen, although a modest fractional increase in both subtypes was found in the spleen. This indicates that leukocyte Arg1 is not a strong regulating factor of T-cell proliferation in the spleen. Notably, strikingly lower amounts of $CD19^{+}$ B cells were found in both the circulation and the spleen of Arg1 KO transplanted LDLr KO mice as compared to WT transplanted animals after 10 weeks WTD challenge, explaining the decrease in total blood leukocyte counts. L-arginine is an essential amino acid for B cell maturation in the bone marrow and arginase-mediated L-arginine depletion leads to reduced B cell emigration from the bone marrow and reduced B cell numbers in the spleen and lymph nodes.[62] If anything, leukocyte Arg1 deletion is thus anticipated to enhance B cell emigration from bone marrow, which clearly cannot explain the reduced B cell numbers in blood. B cells, as the antibody producing cells of the immune system, play an important role in atherosclerosis. Anti-oxLDL antibodies, especially IgM anti-oxLDL, are inversely related to atherosclerotic plaque size in experimental studies.[63] Therefore, oxLDL antibody levels were determined in plasma obtained from recipient mice after 10 weeks WTD feeding. To our surprise, despite the reduced numbers of B cells in the LDLr KO recipients transplanted with Arg1 KO BM, total oxLDL-specific antibody levels were increased. OxLDL-specific IgM that is suggested to be atheroprotective,[63] however, was not changed. Although in clinical cardiovascular disease diverging results have been described on the association between oxLDL-specific antibodies and atherosclerosis, animal studies consistently suggest an atheroprotective role for oxLDL antibodies.[64] Whatever the mechanism behind the reduction in B cells and increased circulating levels of oxLDL-specific antibodies, it might counteract the pro-atherogenic effects of enhanced foam cell formation in the absence of Arg1.

We conclude that despite leading to an increase in foam cell formation and a decrease in circulating B cells, Arg1 deficiency in bone marrow-derived cells does not significantly affect atherosclerotic plaque development.

Acknowledgments

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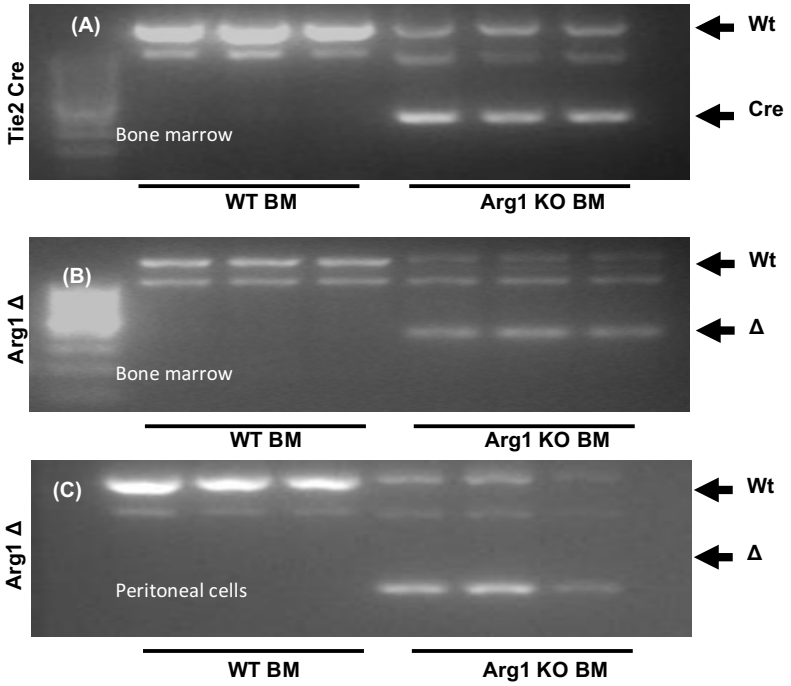
Supplementary material

Genotyping of expression of Tie2Cre and the correct excision of exons 7 and 8 from the Arg1 gene. At 18 weeks after transplantation and after 10 weeks of WTD feeding the recipient animals were sacrificed and genomic DNA was isolated from the bone marrow and peritoneal cavity of the recipients and subjected to genotyping PCR analysis. A) Genotyping by PCR shows presence of Tie2Cre in representative mice having received the Arg1flox/flox;Tie2Cre BM. B-C)The presence of the Arg1Δ construct, i.e. the successful Cre-mediated excision of exons 7 and 8 from the Arg1 gene, was also detected using a set of 3 primers. Genotyping by PCR shows positive bands for the Arg1Δ product in Arg1flox/flox Tie2Cre transplanted mice, indicating successful deletion of exons 7 and 8 in bone marrow and peritoneal cells. A faint band indicating the presence of WT DNA can still be seen, this is in accordance with previous studies where a transplantation efficiency of 95% was demonstrated[24].



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Hematopoietic Akt2 restoration enhances foam cell formation but does not affect atherosclerosis in Akt2/LDL receptor double knockout mice

Baoyan Ren, Menno Hoekstra, Ronald J. van der Sluis, Mara Kröner, Janine G. Geerling, Ilze Bot, Miranda Van Eck

Leiden Academic Centre for Drug Research, Cluster BioTherapeutics, Division of Biopharmaceutics, Leiden, The Netherlands

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AKT serine/threonine Kinase 2 (Akt2) plays a key role in insulin signaling and cardiovascular disease. LDL receptor knockout (LDLr KO) mice lacking Akt2 are glucose intolerant, but atherosclerosis susceptibility is not affected or even slightly decreased. Disruption of Akt2 function specifically in bone marrow-derived cells leads to a dramatic reduction in atherosclerotic lesion development in LDLr KO mice, indicating a potent pro-atherogenic effect of hematopoietic Akt2. The contribution of Akt2 in bone marrow-derived cells to the effects on atherosclerosis susceptibility in Akt2/LDLr double KO (dKO) mice, however, is currently unclear.

In this study, we restored bone marrow Akt2 in Akt2/LDLr dKO mice by transplanting functional Akt2 containing LDLr KO bone marrow into Akt2/LDLr dKO recipients. Akt2/LDLr dKO recipients transplanted with Akt2/LDLr dKO bone marrow served as controls. In line with a pro-atherogenic role for macrophage Akt2, enhanced foam cell formation was observed in the peritoneal cavity of Akt2/LDLr dKO mice transplanted with LDLr KO BM accumulation (3 weeks WTD, +88.0 %, $P<0.05$; 5 weeks WTD, +59.5%, $P=0.07$). Surprisingly, bone marrow Akt2 restoration in Akt2/LDLr dKO mice did not affect glucose tolerance or atherosclerosis development. The null effect on atherosclerosis can likely be explained by the fact that the pro-atherogenic increase in foam cell formation was counteracted by a beneficial change in the inflammatory status, since Akt2 restoration suppressed LPS-induced M1 macrophage polarization (evidenced by -77.8% iNOS/Arg1 ($P<0.001$) expression, -20.9% TNF-alpha ($P<0.01$) secretion, and +90% IL-10 ($P<0.001$) secretion).

In conclusion, restoration of Akt2 in Akt2/LDLr dKO macrophages suppresses M1 polarization and increases macrophage foam cell formation, but does not affect atherosclerosis susceptibility of in Akt2/LDLr dKO mice reconstituted with Akt2 positive bone marrow.

Introduction

Protein kinase B (PKB or Akt) is a family of serine/threonine-specific protein kinases, discovered by Staal and colleagues in 1977.[1] Akt plays critical roles in various cellular processes, including proliferation, apoptosis, migration, angiogenesis and glucose metabolism.[2-6] Three highly homologous isoforms have been identified: Akt1, Akt2, and Akt3. Each isoform has a distinct tissue distribution and function.[7-10] Whereas Akt1 is ubiquitously expressed and plays a key role in the modulation of cell survival,[8,9,11] Akt3 is predominantly expressed in the brain and is essential for postnatal brain development.[10] In addition, Akt2 is highly expressed in insulin-responsive tissues and plays a crucial role in the regulation of insulin signaling.[12-14] Akt2 deficiency in mice induces insulin resistance and a type 2 diabetic phenotype.[13-15] Although type 2 diabetes is generally associated with an increased risk for the development of cardiovascular disease,[16] Akt2 deficiency does not augment atherosclerosis development in the LDL receptor knockout background.[15-21] Thus, Akt2 is likely to exert an anti-atherosclerotic function that compensates for the pro-atherogenic effects of the glucose intolerant phenotype.

Macrophages play an essential role in all stages of atherosclerotic lesion development and atherosclerosis susceptibility is influenced by the phenotype of these immune cells. Grossly, two distinct types of macrophages can be distinguished: 1) pro-inflammatory M1 macrophages and 2) anti-inflammatory, healing M2 macrophages. Importantly, Akt2 deficiency is known to skew macrophages to a phenotype hallmarked by: 1) attenuated inflammatory cytokine production,[22] 2) impaired migration capability,[23] and 3) reduced foam cell formation.[17] In line with this pro-atherogenic function of Akt2, disruption of Akt2 function specifically in bone marrow-derived cells reduces atherosclerotic lesion development in LDLr KO mice.[17,23] The contribution of macrophage

Akt2 to atherosclerosis susceptibility in glucose intolerant Akt2/LDLr double KO (dKO) mice, however, is currently unclear.

Insulin resistance and glucose intolerance both affect macrophage activation and polarization. Whereas some studies have established that high glucose levels induce an M1 macrophage phenotype, Chenming and colleagues reported that peritoneal exudate macrophages and bone marrow-derived macrophages from streptozotocin-induced diabetic mice exhibit a more M2-like macrophage phenotype.[24] This phenotype could also be induced in vitro by exposing macrophages to high glucose levels in the medium. Moreover, the macrophages from diabetic mice showed an impaired inflammatory response to LPS/IFN-γ stimulation, which could be reversed by insulin treatment. Importantly, similar observations were made after treatment with an Akt inhibitor, underlining a role for Akt in macrophage polarization. To elucidate whether the atheroprotective potential of macrophage Akt2 remains present under insulin resistant conditions, the current study was aimed at investigating the effects of hematopoietic Akt2 on atherosclerotic lesion development in glucose intolerant Akt2/LDLr dKO mice by reconstituting these mice with Akt2 positive bone marrow.

Material and Methods

Animals

Low-density lipoprotein receptor knockout (LDLr KO) mice and Akt2/LDLr double KO (Akt2/LDLr dKO) mice were obtained from Jackson laboratory (Bar Harbor, ME, USA). All mice used in the current study were bred and maintained at the Gorlaeus Laboratories in Leiden, the Netherlands. The studies were approved by the Dutch Ethics Committee and regulatory authority at Leiden University. The animal experiments were carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Bone marrow transplantation (BMT)

The endogenous BM of female Akt2/LDLr dKO recipient mice (13-15 weeks old) was destroyed by total-body X-ray irradiation (9-Gy) 1 day before the bone marrow transplantation (BMT). Subsequently, five-million BM cells, freshly isolated from LDLr KO mice or Akt2/LDLr dKO mice was transplanted into the recipient mice via tail vein injection. After BMT, recipient mice were maintained on antibiotic water (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate and 2.5 g/L sugar) and chow diet (RM3; Special Diet Services) for 8 weeks. Thereafter, the mice were challenged with Western-type diet (WTD, Special Diet Services, product code 824171) for 3 or 5 weeks to induce atherosclerosis development. At sacrifice, the mice were anaesthetized by an anaesthetic mixture containing xylazine (12.5 mg/kg), ketamine (100 mg/kg) and atropine (125 µg/kg) and subjected to total body PBS perfusion before organ collection.

Oral glucose tolerance test (OGTT)

The oral glucose tolerance test was performed in the recipient mice at 4 weeks on WTD after a 16-hours fasting period. Glucose levels of tail blood were determined by using a manual ACCU-CHEK Compact glucose monitor (Roche Diagnostics, Almere, the Netherlands).

Blood glucose levels before glucose administration were determined as baseline (t=0). Immediately, the mice were administered 2 gr/kg glucose by oral gavage using a 10% Beta-D (+)-glucose (Sigma-Aldrich, catalog number: G8270) solution in PBS. Additional blood samples were taken at 15, 30, 45, 60, 90 and 120 minutes after glucose administration.

Serum Cholesterol assay

Plasma total cholesterol and free cholesterol levels were determined using a standard enzymatic colorimetric assay (chemicals from Sigma Aldrich, USA or Roche Diagnostics, Mannheim, Germany) as previously described.[25]

Haematology Analysis

An automated haematology analyzer (XT-2000iV, Sysmex Corporation, Japan) was used to analyse leukocytes and quantify macrophage foam cell formation in the peritoneum of the Akt2/LDLr KO BMT recipients as described previously.[26]

Histological Analysis of Atherosclerosis

Sections of the aortic root were made by a Leica CM3050S cryostat. Subsequently, the sections were stained with Oil red-O for neutral lipids, with Masson's Trichrome (HT15-1, 4, Sigma Aldrich) for collagen and with MOMA-2 (Research Diagnostics Inc) for macrophages as described previously.[27] The atherosclerotic plaque size and composition were determined using Leica Qwin Imaging software (Leica Ltd, Cambridge, UK).

Cell culture

Bone marrow-derived macrophages (BMDMs) from LDLr KO and Akt2/LDLr dKO mice were generated by culturing bone marrow cells with 20 ng/mL M-CSF (eBioscience, catalog number: 14-8983-80) for 7 days. To induce foam cell formation, BMDMs were incubated with 20 µg/mL copper-oxidized LDL (oxLDL) for 24 hours.[28] To prime M1 or M2 macrophages, BMDMs were incubated for 24 hours with 100 ng/mL LPS (Escherichia coli0111:B4; Sigma Aldrich, catalog number: L2630-10MG) or 20ng/mL IL-4 (Peprotech, catalog:214-14). BMDMs that were treated with PBS instead of oxLDL, LPS or IL-4 served as control. Macrophage culture supernatants were collected and stored at -20°C until further analysis.

Analysis of gene expression by quantitative PCR (qPCR)

Total RNA was isolated using the guanidinium thiocyanate method.[29] cDNA was synthesized using a RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada). The mRNA expression levels were measured on a 7500 Fast Real-Time PCR system (ABI PRISM 7500; Applied Biosystems, Foster City, CA) using SYBR green technology (Applied Biosystems). The average cycle threshold (CT) of *36B4* (acidic ribosomal phosphoprotein P0) and *RPL27* (Ribosomal Protein L27) were used as housekeeping gene expression control values.

Cytokine analysis and nitric oxide assay

The supernatants of the BMDM cell cultures were thawed and the content of the cytokines TNF-α, IL-6, and IL-10 were determined according to the manufacturer's' instructions (all ELISA kits were purchased from BD Biosciences, United States). The nitric oxide (NO) concentration in the supernatants was determined using the Griess method according to the manufacturer's protocol (Sigma-Aldrich).

Statistical analysis

All the values are expressed as means ± SEM. The statistical significant differences between the groups were tested using the unpaired Student's t-test or two-way ANOVA with GraphPad Prism software (GraphPad Software Inc., San Diego, California, USA). A Welch correction was applied to the t-test in case of unequal variances in the dataset. A two-sided P value lower than 0.05 was defined as statistically significant.

Results

Bone marrow Akt2 restoration does not affect blood glucose levels and glucose tolerance of Akt2/LDLr dKO mice

Total body Akt2 deficiency in LDLr KO mice leads to impaired glucose tolerance.[18] To establish the contribution of bone marrow Akt2 to the regulation of glucose metabolism in Akt2/LDLr KO mice, blood glucose levels were determined and a glucose tolerance test was performed at 12 weeks after BMT, when mice were fed a WTD for 4 weeks. Restoration of bone marrow Akt2 did not impact on fasting blood glucose levels after either 2 or 4 weeks of WTD feeding ($p>0.05$; figure 1A-B). Upon oral glucose administration both Akt2/LDLr dKO mice transplanted with Akt2/LDLr dKO BM or LDLr KO BM showed a rapid increase in blood glucose levels. In both groups the peak glucose value was reached at 30 minutes after the glucose bolus injection (Akt/LDLr dKO BM vs. LDLr KO BM: 18.3 ± 0.7 mM vs 19.2 ± 0.5 mM, $p<0.05$ compared to basal glucose levels; figure 1C). It is worth to note that, 120 minutes after oral glucose administration, the plasma glucose levels in both groups failed to get back to basal levels ($p<0.01$ compared to basal glucose levels, figure 1C), supporting the glucose intolerant phenotype of the Akt2/LDLr dKO recipient mice. Conclusively, we observed no difference in glucose handling between the two experimental groups, indicating that restoration of bone marrow Akt2 in Akt2/LDLr dKO mice does not influence the glucose intolerant phenotype of the recipient mice.

Bone marrow Akt2 restoration does not affect atherosclerosis development in Akt2/LDLr dKO mice

To assess the effect of Akt2 restoration in bone marrow-derived cells on atherosclerosis susceptibility of Akt2/LDLr dKO mice, the atherosclerotic plaque size in the aortic root was determined after 3 and 5 weeks of WTD feeding. At both time points the lesions were primarily composed of macrophage foam cells with no deposited collagen (Figure 2A). Surprisingly, Akt2/LDLr dKO recipients transplanted with LDLr KO BM containing functional Akt2 developed atherosclerotic lesions of a similar size as Akt2/LDLr dKO transplanted controls, both after 3 weeks WTD challenge (Akt2/LDLr dKO BM vs. LDLr KO BM: $119 \times 10^3 \pm 14 \times 10^3$ μm^2 vs. $125 \times 10^3 \pm 21 \times 10^3$ μm^2, $p>0.05$; figure 2B and 2C) and after 5 weeks WTD feeding (Akt2/LDLr dKO BM vs. LDLr KO BM: $154 \times 10^3 \pm 14 \times 10^3$ μm^2 vs. $169 \times 10^3 \pm 24 \times 10^3$ μm^2, $p>0.05$; figure 2B and 2C). Thus, in contrast

to the previously published results showing that deletion of Akt2 in bone marrow-derived cells of LDLr KO mice is athero-protective,[17,23] restoration of Akt2 in glucose intolerant Akt2/LDLr dKO mice did not increase atherosclerotic lesion development.

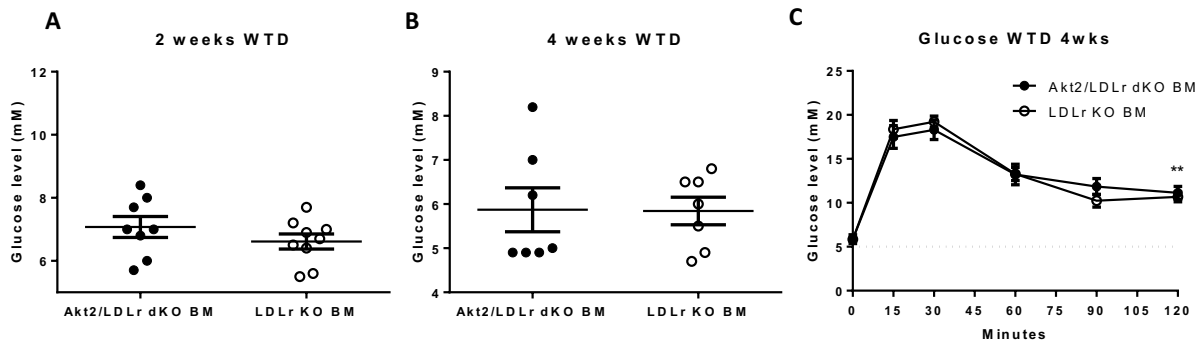

Figure 1 Restoration of Akt2 in bone marrow of Akt2/LDLr double KO mice does not alter recipient's blood glucose levels and glucose tolerance.

Akt2/LDLr dKO mice received LDLr KO bone marrow (BM) or Akt2/LDLr dKO BM. The fasting blood glucose levels were measured after A) 2 weeks Western-type diet (WTD) or B) 4 weeks WTD challenge. C) Glucose tolerance test by oral gavage of 2 g/kg glucose in fasted recipient mice is shown at 4 weeks WTD. **p<0.01, compared to the basal glucose concentration. (n=7-9).

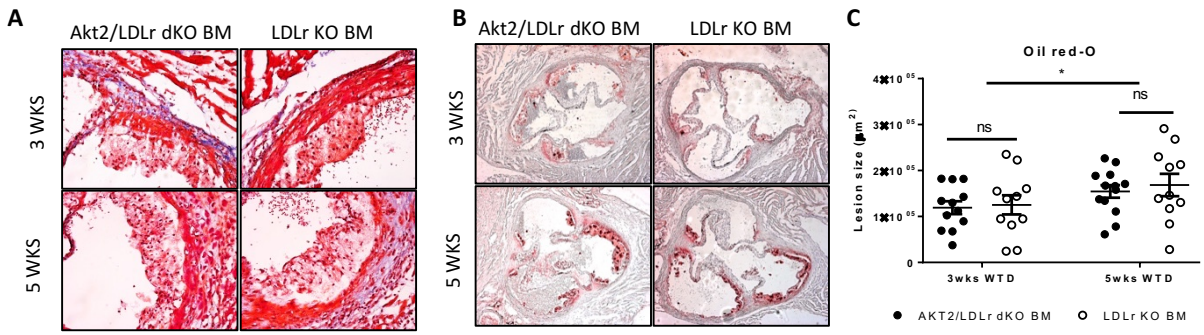

Figure 2 Restoration of bone marrow Akt2 does not affect atherosclerosis development in the aortic root of Akt2/LDLr dKO mice

A) Masson's Trichrome stained atherosclerotic lesions in the aortic root of Akt2/LDLr dKO mice transplanted with Akt2/LDLr dKO or LDLr KO bone marrow (BM) after 3 and 5 weeks Western-type diet. B) Representative pictures of Oil red-O stained atherosclerotic lesions in the aortic root and C) quantification of the atherosclerotic lesion size. ns, p>0.05; *p<0.05; (n=11-12).

Bone marrow Akt2 restoration leads to increased foam cell formation in the peritoneal cavity of Akt2/LDLr dKO mice

Akt2 has been shown to influence macrophage migration.[30-32] Therefore, next we investigated the leukocyte populations in the peritoneal cavity of Akt2/LDLr dKO recipients after 3 weeks and 5 weeks WTD challenge. The total number of peritoneal leukocytes and macrophages in Akt2/LDLr dKO mice transplanted with LDLr KO bone marrow containing functional Akt2 were similar as in Akt2/LDLr dKO BM transplanted controls (figure 3A and B). This indicates that bone marrow Akt2 restoration likely had no impact on overall leukocyte and macrophage recruitment into the peritoneal cavity of Akt2/LDLr dKO mice. Interestingly, the counts of macrophage foam cells were increased upon restoration of bone marrow Akt2 in Akt2/LDLr dKO mice (3 weeks WTD, +88.0 %, p=0.04; 5 weeks WTD, +59.5 %, p=0.07; figure 3C). This increase in foam cell number could not be attributed to changes in plasma cholesterol levels, as mice transplanted with reconstituted BM

Akt2 showed similar plasma cholesterol levels as compared to control mice, both on chow diet and after a WTD challenge (table 1). Moreover, the plasma lipoprotein distribution profile was also not altered upon restoration of Akt2 in bone marrow-derived cells of Akt2/LDLr dKO mice (figure 4).

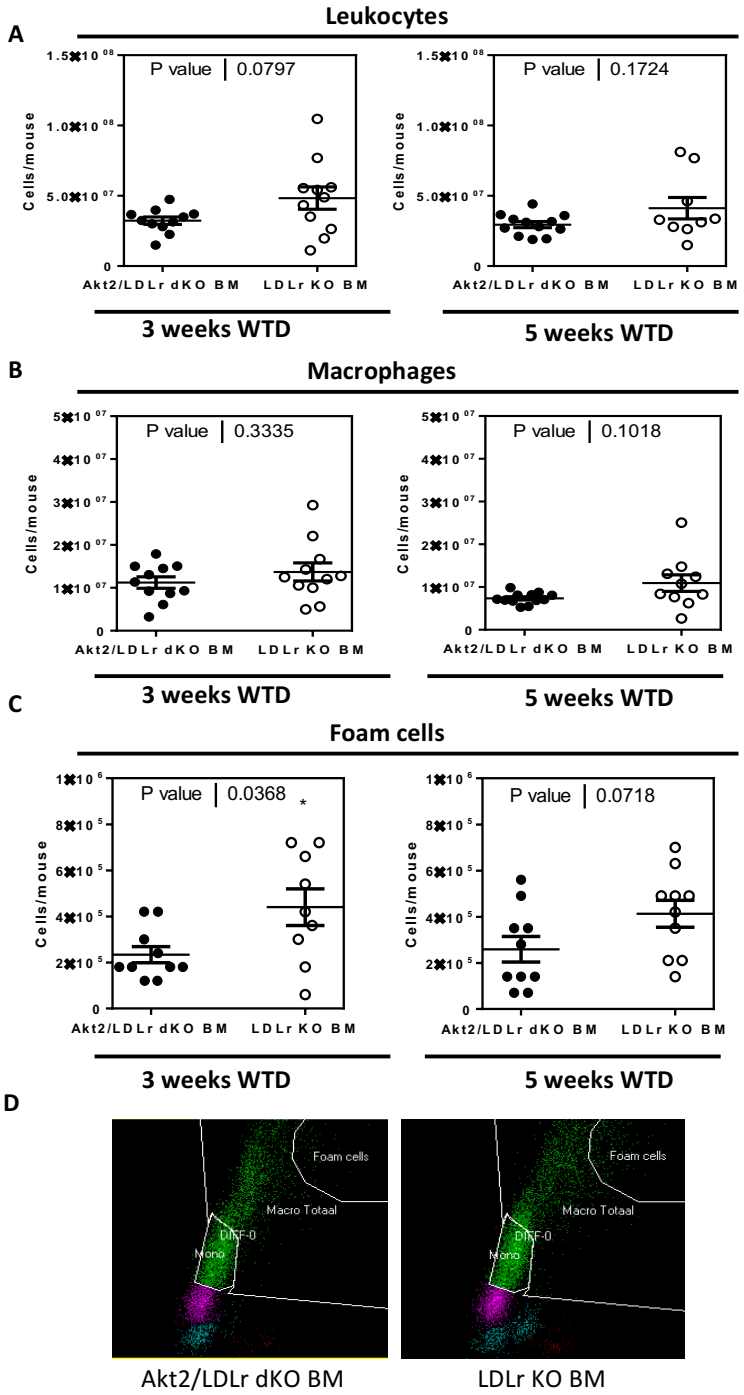

Figure 3 Restoration of bone marrow Akt2 in Akt2/LDLr dKO mice induces foam cell formation in the peritoneal cavity.

Peritoneal leukocytes were harvested from Akt2/LDLr dKO recipients transplanted with LDLr KO BM and Akt2/LDLr dKO BM after 3 weeks or 5 weeks Western-type diet (WTD) feeding. A) total leukocyte counts, B) macrophage counts and C) foam cell counts were determined by using a Sysmex hematology analyzer. D) Example picture of the hematology analysis (from the 3 weeks WTD challenge group). *p<0.05. n=11-12.

Table 1 Restoration of bone marrow Akt2 does not impact plasma cholesterol levels in Akt2/LDLr dKO mice.

	Chow diet		WTD		
mg/mL	FC	TC		FC	TC
Akt2/LDLr dKO BM	0.75 ± 0.05	2.39 ± 0.12	3 weeks	3.26 ± 0.19	9.43 ± 1.10
			5 weeks	2.65 ± 0.16	7.35 ± 0.68
LDLr KO BM	0.77 ± 0.03	2.45 ± 0.10	3 weeks	2.68 ± 0.44	7.32 ± 1.89
			5 weeks	2.51 ± 0.24	7.23 ± 0.60

Plasma was collected by tail bleeding of Akt2/LDLr dKO recipients that received LDLr KO BM and Akt2/LDLr dKO BM while on chow diet and at sacrifice after 3 and 5 weeks Western-type diet (WTD) feeding. The free cholesterol and total cholesterol levels were determined. (n=6-12).

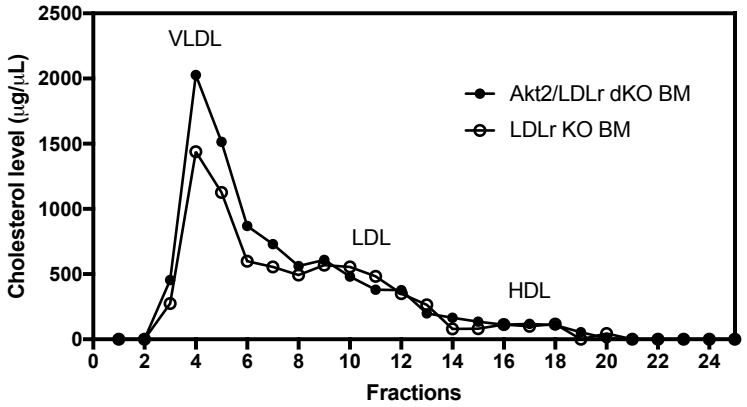

Figure 4 Restoration of bone marrow Akt2 does not impact on plasma cholesterol distribution among the different types of lipoproteins in Akt2/LDLr dKO mice.

Plasma was collected by tail-bleeding of Akt2/LDLr dKO recipients that received LDLr KO BM or Akt2/LDLr dKO BM at sacrifice after 3 weeks Western-type diet (WTD) feeding. Cholesterol lipoprotein distribution was determined by FPLC. (n=6-12 of pooled plasma).

Akt2 promotes foam cell formation in LDLr deficient macrophages by regulating the expression of cholesterol influx and efflux genes

To gain further insight into the mechanisms underlying the increased foam cell formation of LDLr KO macrophages with functional Akt2 *in vivo*, an *in vitro* foam cell formation assay was performed using LDLr KO and LDLr/Akt2 dKO BMDMs. After 24 hours of oxLDL incubation, LDLr KO macrophages showed significantly more cellular lipid accumulation as evidenced by more extensive Oil red-O staining compared to Akt2/LDLr dKO macrophages (figure 5A). The mRNA expression of *SR-A* and *CD36*, the main receptors for oxLDL uptake,[33] was significantly higher in LDLr KO macrophages compared to Akt2/LDLr dKO macrophages (*SR-A*, +60.7%, p<0.001; figure 5B; *CD36*, +24.8%, p<0.001, figure 5C) after oxLDL stimulation. The expression of low-density lipoprotein receptor-related protein 1 (*LRP1*, 1.16 ± 0.05 vs 0.87 ± 0.13, p>0.05, figure 5D) was not affected and the expression of *VLDLr*, responsible for the uptake of unmodified apoE-containing lipoproteins,[34,35] was decreased (-59.1%, p<0.05, figure 5E) in LDLr KO macrophages compared to Akt2/LDLr dKO macrophages. In addition, the expression of the cholesterol efflux genes *ABCA1* and *ABCG1* was decreased in LDLr KO macrophages compared to Akt2/LDLr dKO controls (*ABCA1*, -39.0%, p<0.05, figure 5F; *ABCG1*, -40.5%, p<0.01, figure 5G). In summary, these results suggest that LDLr KO macrophages with functional Akt2 are more susceptible to oxLDL-induced foam cell formation due to augmented uptake of oxLDL and decreased cellular cholesterol efflux.

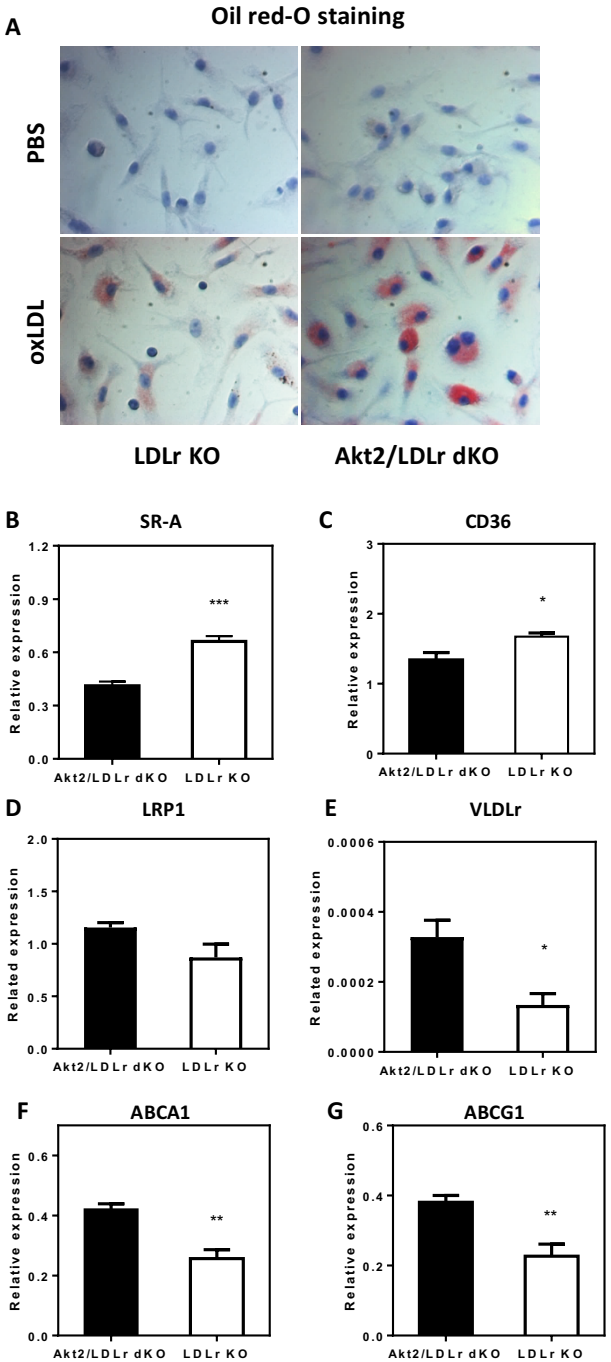

Figure 5 Akt2 presence increases susceptibility of LDLr KO macrophages to oxLDL-induced foam cell formation.

Macrophages generated from Akt2/LDLr dKO BM and LDLr KO BM were treated with 20µg/mL oxLDL for 24 hours. A) Oil red-O visualized foam cell formation, B-G) mRNA expression of genes involved cholesterol metabolism were determined using qPCR. *p<0.05; **p<0.01; ***p<0.001. (n=5).

Akt2 suppresses LPS-induced M1 macrophage polarization in LDLr KO macrophages

M2 macrophages are known to be more susceptible to foam cell formation as compared to M1 macrophages.[36] To investigate if the increased foam cell formation of LDLr KO macrophages as compared to Akt2/LDLr dKO macrophages is linked to the macrophage phenotype, a polarization assay was performed using BMDMs.

Upon LPS exposure, LDLr KO macrophages with functional Akt2 showed a decreased mRNA expression ratio of the M1 marker *iNOS* over the M2 marker *Arg1* (-77.8%, p<0.001, figure 6A), indicating a less M1-like macrophage phenotype. In support, the nitric oxide (NO) production by

LDLr KO macrophages was also significantly lower, compared to Akt2/LDLr dKO macrophages (-32.6%, p<0.001, figure 6B). Furthermore, the production of pro-inflammatory cytokines was evaluated as a reflection of macrophage function. In line with the observed reduction of the M1-like phenotype, LDLr KO macrophages displayed a less pronounced pro-inflammatory cytokine profile upon LPS stimulation, as the production of pro-inflammatory cytokines TNF-α and IL-6 was reduced (TNF-α, -20.9%, figure 6C; IL-6, -24.0%, figure 6D), whereas the production of anti-inflammatory cytokine IL-10 was increased (+89.1%, figure 6E) in LDLr KO macrophages as compared to Akt2/LDLr dKO macrophages. In support of the more M2-like phenotype, IL-4 stimulation induced a significant increase in Arg1 expression (+297%, p<0.001, figure 6F). These data clearly showed that Akt2 presence in LDLr KO macrophages leads to a less M1, and more M2-like macrophage phenotype, which might be linked to the increased susceptibility to foam cell formation that these macrophages show.

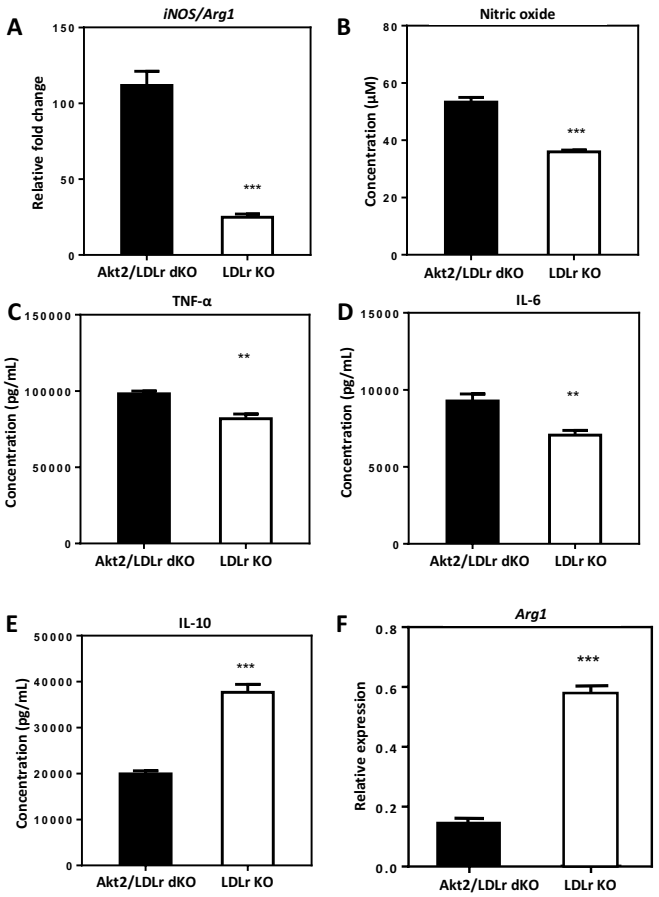

Figure 6 The presence of Akt2 suppresses the inflammatory response in LDLr KO macrophages upon LPS stimulation.

BMDMs generated from Akt2/LDLr dKO and LDLr KO bone marrow were treated with 100ng/mL LPS for 24 hours to promote M1 polarization, A) the mRNA expression ratio of iNOS/Arg1, B) nitric oxide production, and cytokine production of C) TNF-α, D) IL-6 and E) IL-10 were determined. BMDMs generated from Akt2/LDLr dKO and LDLr KO bone marrow were treated with 20ng/mL IL-4 for 24 hours to promote M2 polarization, F) the relative mRNA expression of Arg1 was determined. **p<0.01; ***p<0.001. (n=6).

Discussion

Previous studies have shown that disruption of Akt2 function specifically in bone marrow-derived cells induces an anti-inflammatory M2 macrophage phenotype, and leads to a dramatic reduction in atherosclerotic lesion development in LDLr KO mice. [17, 18] In the current study we investigated the relative contribution of macrophage Akt2 on atherosclerosis susceptibility in Akt2/LDLr double KO (dKO) mice. We show that restoration of Akt2 in bone marrow-derived cells of Akt2/LDLr dKO mice does not impact atherosclerotic lesion development, despite an observed increase in

macrophage foam cell formation. This lack of effect on lesion development can likely be explained by the observation that Akt2 restoration in macrophages also reduced the macrophage polarization towards the pro-inflammatory M1 macrophage phenotype, and correspondingly lowered the production of pro-inflammatory cytokines.

Total body Akt2 loss is known to lead to glucose intolerance in LDLr KO mice.[17,18] We here show that restoration of bone marrow Akt2 in Akt2/LDLr dKO mice does not change the blood glucose levels nor the glucose intolerant phenotype of these mice, suggesting that macrophage Akt2 is not involved in the regulation of glucose homeostasis. In line with this, *Babaev et al.* also found that deletion of bone marrow Akt2 in LDLr KO mice did not affect glucose levels.[23] These data indicate that bone marrow Akt2 is not a decisive factor for the observed glucose intolerant phenotype of mice with a total-body Akt2 deletion. Indeed, Akt2 maintains glucose homeostasis mainly by regulating hepatic glucose production and skeletal muscle insulin resistance, cell types that are not affected by bone marrow transplantation.[37-39]

Previous studies have demonstrated that bone marrow Akt2 plays a pro-atherogenic role in non-diabetic LDLr KO mice, as evidenced by reduced atherosclerotic lesion development upon bone marrow-specific deletion of Akt2.[17,23] In our study, we did not observe any impact of bone marrow Akt2 restoration on atherosclerotic lesion size in glucose intolerant Akt2/LDLr dKO mice fed a Western-type diet. Despite the lack of effect on lesion size, Akt2 restoration in bone marrow did promote macrophage foam cell formation in these mice. This increased susceptibility to macrophage foam cell formation of mice with functional bone marrow Akt2 could not be attributed to changes in the plasma cholesterol levels or lipoprotein distribution. However, *in vitro* studies showed that the increase in macrophage foam cell formation is likely to be caused by an increased influx of oxidized LDL together with a decreased cholesterol efflux as evidenced by an increased mRNA expression of the LDL cholesterol uptake receptors *SR-A1* and *CD36* and a decreased expression of cholesterol efflux pumps *ABCA1* and *ABCG1*. In agreement with our study, Rotllan *et al.* also found that macrophage Akt2 promotes foam cell formation on a wildtype background.[17]

Macrophage susceptibility to foam cell formation is influenced by the macrophage phenotype, with M2 macrophages being more prone to become foam cells as compared to M1 macrophages.[36] Indeed, in line with the increased foam cell formation, LDLr KO macrophages with functional Akt2 showed a suppressed M1 macrophage phenotype in response to LPS stimulation compared to LDLr KO macrophages lacking Akt2. This result is in contrast to previous findings showing that Akt2 deficiency leads to a more M2-like macrophage phenotype after LPS stimulation, compared to WT macrophages.[22,23,40] Several differences between our current study and the previous studies can be distinguished, such as the duration of the LPS stimulation period, the LPS concentration used, strain variabilities (LDLr KO vs WT),[41,42] macrophage heterogeneities (bone marrow-derived macrophages vs peritoneal macrophages), and culture medium compositions. Hence, further research into this topic is warranted.

It should be noted that both the previous studies on macrophage polarization[22,23,40] and the bone marrow transplantation experiments of Rotllan[17] and Babaev *et al.*[23] applied Akt2 deficient cells with a functional LDLr. For bone marrow transplantation-based atherosclerosis studies, this is a commonly used strategy as it prevents the requirement of extensive cross-breeding to generate LDLr KO donors lacking the gene of interest. However, although previous studies have shown that the presence or absence of the LDLr in bone marrow-derived cells only minimally affects

atherosclerosis per se,[43,44] it cannot be excluded that upon interaction with the gene of interest absence or presence of the LDLr does affect macrophage function and atherosclerotic lesion development. Therefore, further analysis is warranted to confirm this possible interaction between LDLr and Akt2. Understanding the underlying mechanisms of a possible gene interaction is beyond the scope of our current study. However, the difference in the LPS-induced phenotype of Akt2 deficient macrophages in the LDLr KO background in our current study as compared to the wildtype background in earlier studies, might prove a motivation for initiating further research.

Taken together, our study showed that: 1) bone marrow Akt2 is not a decisive factor in Akt2-regulated glucose metabolism, and 2) bone marrow Akt2 promotes macrophage foam cell formation but does not affect atherosclerosis development in Akt2/LDLr dKO mice, which might be due to the counteracting anti-inflammatory effects of the Akt2-induced suppressed M1-like macrophage phenotype.

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Macrophage MKP2 deficiency is associated with an M2-driven foam cell phenotype and increases atherosclerosis susceptibility of LDL receptor knockout mice

Baoyan Ren[1], Menno Hoekstra[1], Janine J. Geerling[1], Peter Van Santbrink[1], Robin Plevin[2], Miranda Van Eck[1]

1 Division of Biopharmaceutics, Cluster BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden, The Netherlands

2 Strathclyde Institute for Pharmacy and Biomedical Sciences, University of Strathclyde, Scotland, United Kingdom

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Several studies have established a role for mitogen-activated protein kinases (MAPK) in atherogenesis. The exact role of MAPK phosphatase 2 (MKP2/DUSP4), a MAPK upstream regulator, in this process is, however, still unknown. This study therefore aimed at investigating the role of MKP2 in atherosclerosis development.

MKP2 deficiency in cultured macrophages is associated with increased JNK activation (1.5-fold, $p<0.05$) and a shift towards an M2-like macrophage phenotype as compared to wild-type macrophages. This is reflected by decreased mRNA expression of iNOS (-83.7%, $p<0.001$) and TNF-α (-41.8%, $p<0.001$) and increased Arg1 (2.7-fold, $p<0.001$) and YM-1 (2.6-fold, $p<0.05$) expression. Macrophages lacking MKP2 exhibit increased expression levels of the scavenger receptors SR-A (2.6-fold, $p<0.01$) and CD36 (2.2-fold, $p<0.001$), leading to an enhanced predisposition to become foam cells. Transplantation of MKP2 knockout bone marrow into lethally irradiated hyperlipidemic LDL receptor knockout mice confirmed the atheroprotective effect of macrophage MKP2. A 1.3-fold increase ($p<0.05$) in atherosclerotic lesion size was observed in mice reconstituted with MKP2 knockout bone marrow as compared to wild-type bone marrow recipients. The increase in lesion size coincided with a 30% decrease ($p<0.01$) in lesional collagen content, suggesting that loss of macrophage MKP2 is associated with larger lesions with a relatively unstable plaque phenotype.

In conclusion, we have shown that MKP2 deficiency (1) skews cultured macrophages to an M2 phenotype, resulting in an enhanced susceptibility to become foam cells, and (2) increases atherosclerosis susceptibility *in vivo*.

Introduction

Atherosclerosis is a hyperlipidemia-induced chronic inflammatory disease characterized by the deposition of macrophage foam cells in the arterial wall.[1,2] Inside atherosclerotic lesions macrophage populations with a different inflammatory phenotype can be distinguished, including M1 and M2 macrophages.[3] In general, pro-inflammatory M1 macrophages are regarded as pro-atherogenic, while anti-inflammatory M2 macrophages are considered to be atheroprotective.[4] Interestingly, M2 as compared to M1 macrophages are more prone to oxLDL-induced foam cell formation,[5,6] highlighting a dynamic atherosclerotic role for macrophage subpopulations.

MAPKs as serine/threonine-specific protein kinases activate various cellular signaling transduction pathways by phosphorylating downstream target genes.[7] The three main subfamilies of MAPKs, i.e. extracellular signal-regulated kinase (ERK), stress-activated protein kinase (p38)[8,9] and c-Jun N-terminal kinase (JNK),[10,11] play important roles during atherosclerosis development.[12] MAPKs activity is controlled by a family called MAPK phosphatases (MKPs). MKPs inactivate MAPKs by dephosphorylating their phosphoserine/threonine and phosphotyrosine residues.[13-15] The MKP family contains at least 10 well-characterized members, divided over 2 sub-families depending on their subcellular distribution and immediate-early or late gene regulation.[16] Two members belonging to the sub-family of immediate-early gene regulators with a nuclear localization are MKP1 and MKP2.[16] The expression of MKP1 and MKP2 is induced by MAPK activation,[17] while they in turn inactivate MAPKs. Hence, they are considered the most potent regulators of the MAPK/MKP feedback loop.[16,17]

MKP2 has a molecular weight of 42.9-kDa and is ubiquitously expressed in various tissues.[18,19] Its expression is induced in response to growth factors,[20] hormones,[21] oxidative stress,[22,23] UV light[19] and lipopolysaccharides (LPS).[24] MKP2 expression is also highly responsive to cholesterol-rich diet

and fatty acids.[25] A recent study showed that MKP2 expression is strongly regulated in activated macrophages.[26] However, so far many contradictory findings on the effect of MKP2 on macrophage function have been described. In response to LPS, bone marrow-derived macrophages lacking MKP2 acquire an M2-like macrophage phenotype, reflected by enhanced Arg1 and decreased iNOS activities.[27] This phenotype was not always supported by the cytokine production profile; Al-Mutairi *et al.* found a potentiated pro-inflammatory cytokine production, while Cornell *et al.* found attenuated pro-inflammatory cytokine production by MKP2 knockout macrophages in response to LPS[27,28] Additionally, overexpression of MKP2 in macrophages significantly decreased JNK activation and the expression of inflammatory mediators.[29]

Interestingly, a pro-atherogenic function of MKP1 was recently established in mice.[30] However, the role of MKP2 in the pathogenesis of atherosclerosis is still unclear. In the current study, we therefore investigated the role of MKP2 in macrophage polarization and foam cell formation *in vitro* and evaluated the impact of macrophage MKP2 deficiency on atherosclerosis susceptibility *in vivo*.

Materials and methods

Animals

Breeding pairs of C57BL/6 wild-type (WT) mice and MKP2 knockout (MKP2 KO) mice were obtained from the Physiology & Pharmacology laboratories in Glasgow, United Kingdom.[27] Low-density lipoprotein receptor KO (LDLr KO) mice were bought from The Jackson Laboratory (Bar Harbor, ME, USA) and expanded locally at the Gorlaeus Laboratories, Leiden, The Netherlands. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Isolation of thioglycollate-elicited peritoneal macrophages

WT and MKP2 KO mice were intraperitoneally injected with 3% Brewer's modified thioglycollate medium (Becton, Dickinson and company Sparks, MD, USA; Product number: 211716) to elicit macrophage infiltration into the peritoneal cavity. Five days later, peritoneal macrophages were harvested through peritoneal lavage with PBS. Cells were cultured overnight in complete DMEM medium (Lonza Walkersville, USA, catalog number: BE12-708F) containing 10% fetal calf serum (HyClone™ Calf Serum (U.S.), catalog number: SH30073.03). Non-adherent cells were washed away to acquire the peritoneal macrophage cultures used for further research.

Phosphorylation levels of ERK, JNK, p38 MAPK determination by ELISA assay

A total of 30.000 peritoneal macrophages were plated per well in a 96-wells culture plate. Phosphorylation levels of MAPK (P38, ERK, JNK) were analysed after 24 hours using a cell-based ELISA kit (RayBiotech, Norcross, GA, USA. catalog number: CBEL-ERK-SK) according to the manufacturer's instructions. Absorbances were read at 450 nm and 570 nm using a plate reader (model PowerWave 340, Biotek, USA).

mRNA expression determination using Real-time PCR

Total RNA isolation and cDNA synthesis was performed as described previously.[31,32] The mRNA expression of genes of interest was determined using a 7500 Fast Real-Time PCR system (Applied Biosystems, CA) using SYBR green (GC Biotech, catalog number: QT625-20 supplier) technology. The average cycle threshold (CT) of RPL27 and 36B4 was used as housekeeping control.

Lipid assays

Plasma triglycerides, total cholesterol and free cholesterol were determined using standard enzymatic colorimetric assays. The triglycerides colorimetric assay kit was obtained from Roche Diagnostics (catalog number: 11488872216). The cholesterol assay was performed as described previously.[33] Precipath control serum (Roche, catalog number:11285874122) was used as standard for the assays. The distribution of cholesterol over the different lipoprotein classes was assessed by fast protein liquid chromatography using a Superose 6 column (GE Healthcare, Uppsala, Sweden).

Cytokine protein measurements

TNF-α, IL-6, IL-10, IL-12p40, and MCP-1 protein levels were measured by enzyme-linked immunosorbent assays (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's protocols.

Hematology analysis and Flow Cytometry Analysis

Total leukocytes and leukocyte subtypes were analyzed using an automated Sysmex XT-2000iV Veterinary Haematology analyzer (Sysmex Corporation). Fluorescence-activated cell sorting (FACS) analysis was performed on a FACS Canto II machine (BD Biosciences, CA, USA) using FACS antibodies (eBioscience).

Bone Marrow Transplantation

LDLr KO recipient mice were exposed to 9 Gy X-ray irradiation[34] to destroy the endogenous bone marrow. One day after, five-million bone marrow cells, freshly isolated from WT and MKP2 KO donor mice, were transplanted into the lethally irradiated LDLr KO recipients via tail vein injection. Bone marrow recipient mice were allowed to recover for 8 weeks on a regular chow diet, after which they were switched to a Western-type diet (WTD) for 9 weeks to induce the development of atherosclerotic lesions.

Histological Analysis of the Aortic Root

At sacrifice, mice were subjected to whole body perfusion with PBS. Subsequently, hearts were isolated and fixed in 4% Formal-Fixx buffer (Thermo Scientific™ Shandon™) for 24 hours, before embedding in Tissue-Tek O. C. T. compound (Sakura Finetek, USA) overnight. Ten-micron cryosections of the aortic root were cut using a Leica CM3050s cryostat. Atherosclerotic lesion area (in μm^2) and lesional collagen and macrophage content were determined by respectively Oil red O staining, Masson's Trichrome (MTC) staining and MOMA-2 immunohistochemical staining, respectively (dilution 1:50, Research Diagnostics Inc). All quantification analysis of the sections was performed by a blinded operator using the Leica image analysis system (Leica Ltd, Cambridge, UK).

Statistical Analysis

Data are expressed as means ± SEM. Statistical significant differences between the groups were determined by two-tailed unpaired Student's t-test or 2-way ANOVA using GraphPad Prism software (GraphPad Software Inc., San Diego, California, USA). Welch correction was applied to the t-test in the case of unequal variances in the dataset. A p value of <0.05 was considered statistically significant.

Results

Loss of MKP2 in macrophages leads to enhanced JNK activation

To study the role of MKP2 in macrophage MAPK activation, the phosphorylation status of JNK, p38 and ERK1/2 (hereafter referred to as ERK) were determined. Loss of MKP2 in macrophages resulted in an enhanced activation of JNK (1.5-fold, p<0.05; Figure 1A), whereas p38 activation was decreased in MKP2 KO macrophages as compared to WT macrophages (-25.5%, p<0.001; Figure 1B). Macrophage ERK activation was not affected by MKP2 deficiency (p>0.05; Figure 1C).

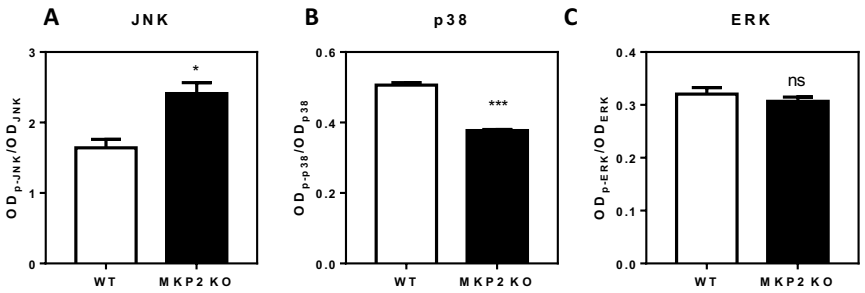

Figure 1 MKP2 deficiency alters the relative phosphorylation status of MAP-kinases in peritoneal macrophages

Phosphorylated and total JNK, p38, and ERK protein levels were measured by ELISA in thioglycollate-elicited peritoneal macrophages from WT and MKP2 KO mice. Data are expressed as optical density (OD) ratios of A) phosphorylated JNK/total JNK, B) phosphorylated P38/total P38 and C) phosphorylated ERK/total ERK. *p<0.05, ***p<0.001, ns p>0.05.

MKP2 deficiency skews macrophages to an M2-like phenotype

JNK and p38 are involved in macrophage polarization.[35-37] As MKP2 deficiency affected both JNK and p38 activation, we next investigated whether MKP2 deficiency also affected macrophage polarization. Relative mRNA expression levels of the M1 markers iNOS (-83.7%, p<0.001) and TNF-α (-41.8%, p<0.001) were significantly decreased in MKP2 KO macrophages as compared to WT macrophages (Figure 2A-B). In contrast, mRNA expression of the M2 markers Arg1 and YM-1 was significantly increased in MKP2 KO macrophages as compared to WT macrophages, by 2.7-fold (p<0.001; Figure 2C) and 2.5-fold (p<0.05; Figure 2D) respectively. Collectively, these data suggest that loss of MKP2 polarizes macrophages towards an M2 phenotype.

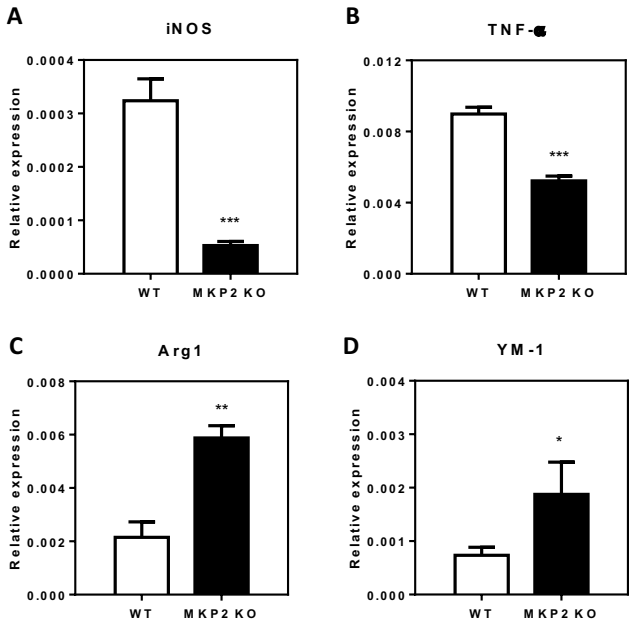

Figure 2 Thioglycollate-elicited macrophages lacking MKP2 are skewed towards an M2-like phenotype.

Relative mRNA expression levels of the M1 macrophage markers A) iNOS and B) TNF-α and the M2 macrophage markers C) Arg1 and D) YM-1 were measured by quantitative PCR. *p<0.05, **P<0.01, ***p<0.001.

MKP2 deletion upregulates the expression of lipid uptake genes and enhances macrophage foam cell formation.

Expression of SR-A and CD36, the main receptors for uptake of modified LDL, are upregulated in M2 macrophages.[6,38] As a result, M2 macrophages are more susceptible to foam cell formation.[39] Therefore, we hypothesized that MKP2 deletion in macrophages, via stimulation of M2 polarization, is likely to predispose macrophages to foam cell formation. In agreement with the more M2-like macrophage phenotype, SR-A (2.6-fold; p<0.01) and CD36 (2.2-fold; p<0.001) mRNA expression was higher in MKP2 KO macrophages, as compared to WT macrophages (Figure 3A-B). The mRNA expression of ABCA1, ABCG1 and HMG-CoA reductase were similar between the two genotypes (p>0.05; Figures 3C-E), suggesting that cholesterol efflux and endogenous cholesterol synthesis were likely not affected by the loss of MKP2. Probably, as a result of the augmented SR-A and CD36 expression, MKP2 KO macrophages already formed foam cells when maintained in 10% fetal calf serum-containing culture medium for 24 hours; an effect barely found in WT macrophages under the same culture conditions (Figure 3G). This supports the hypothesis that MKP2 loss in macrophages predisposes the cells to become foam cells. Correspondingly, we also observed more extensive foam cell formation in MKP2 KO macrophages, as compared to MKP2 KO macrophages after oxLDL treatment (Figure 3G).

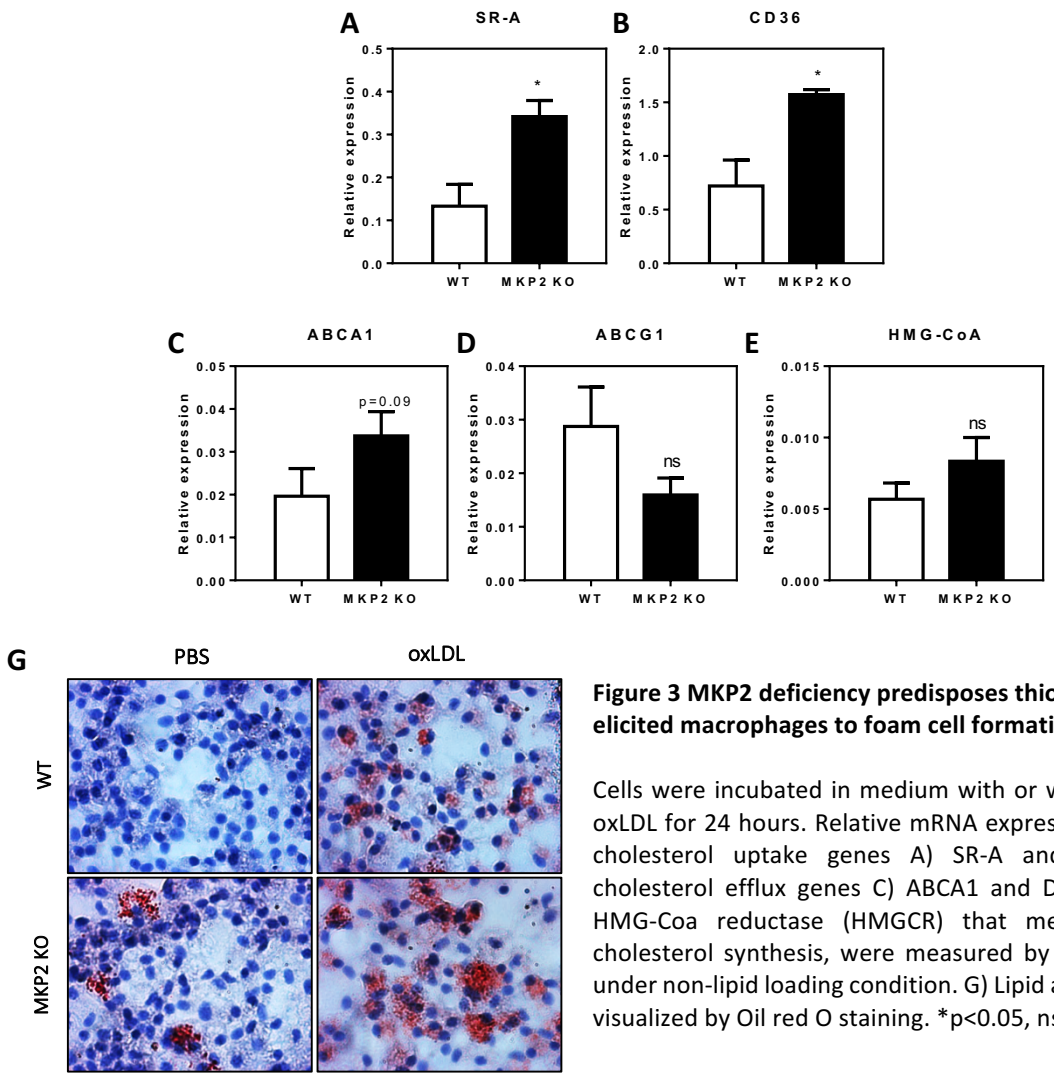

Figure 3 MKP2 deficiency predisposes thioglycollate-elicited macrophages to foam cell formation.

Cells were incubated in medium with or without 10 µg/mL oxLDL for 24 hours. Relative mRNA expression levels of the cholesterol uptake genes A) SR-A and B) CD36, the cholesterol efflux genes C) ABCA1 and D) ABCG1, and E) HMG-Coa reductase (HMGCR) that mediates de novo cholesterol synthesis, were measured by quantitative PCR under non-lipid loading condition. G) Lipid accumulation was visualized by Oil red O staining. *p<0.05, ns p>0.05.

Hematopoietic MKP2 deficiency in LDLr KO mice does not alter plasma lipid profile or white blood cell counts

To verify the potential pro-atherogenic effect of macrophage MKP2 deficiency on atherosclerosis development *in vivo*, lethally irradiated LDLr KO mice, transplanted with WT or MKP2 KO bone marrow, were challenged with WTD for 9 weeks to induce the development of atherosclerotic lesions. Hematopoietic MKP2 deficiency did not impact on plasma free cholesterol and total cholesterol levels (p>0.05, Figure 4A) or to the distribution of cholesterol over the different lipoprotein classes (Figure 4B). The total blood leukocyte count was also not different between the two groups of bone marrow recipients (p>0.05, Figure 4C). We further analysed the blood leukocyte composition profile using flow cytometry. No difference was found in the percentage of neutrophils, total monocytes, Ly6C^{low} patrolling monocytes or Ly6C^{hi} pro-inflammatory monocytes (p>0.05, Figure 4D). Representative flow cytometric plots of circulating leukocytes are shown in figure 4E.

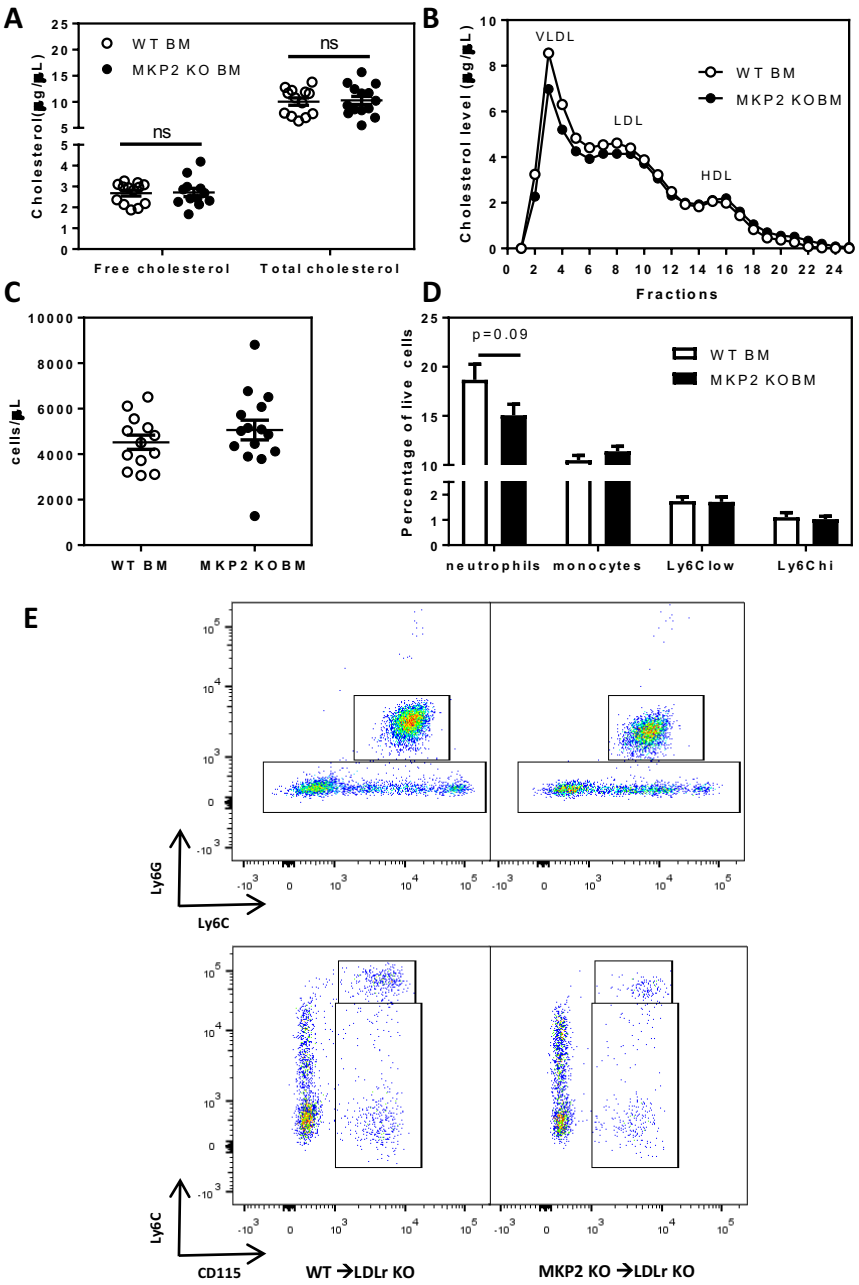

Figure 4 MKP2 deletion in hematopoietic cells enhances anti-inflammatory cytokine production but does not affect the plasma lipid profile.

LDLr KO mice transplanted with WT or MKP2 KO bone marrow were challenged with a Western-type diet for 9 weeks. A) Plasma cholesterol levels, B) the cholesterol distribution over the different lipoprotein fractions, C) total circulating leukocytes counts and D) neutrophil/monocyte percentages were determined in the LDLr KO recipients. E) Representative flow cytometric plots of circulating leukocytes. ns p>0.05; (n=8-15).

Hematopoietic MKP2 deficiency in LDLr KO mice is associated with decreased M1 peritoneal macrophage activation and an anti-inflammatory plasma cytokine profile

After 9 weeks WTD feeding, peritoneal cells of the WT and MKP2 KO bone marrow transplanted mice were harvested. No difference was found in the total number of isolated peritoneal leukocytes between the two genotypes (p>0.05, figure 5A). Flow cytometric analysis showed that the percentage of macrophages ($CD11b^+$ and $F4/80^+$) was also not different between recipients of MKP2 KO bone marrow or WT bone marrow (p>0.05, figure 5B-C), indicating that MKP2 deficiency in bone marrow of LDLr KO mice does not affect macrophage recruitment into the peritoneal cavity.

In line with the anti-inflammatory M2-like phenotype of MKP2 KO macrophages *in vitro*, the mean fluorescent intensity (MFI) of the M1 macrophage activation markers CD86 and MHC-II was significantly lower in peritoneal cells of MKP2 KO bone marrow recipients, compared to WT recipients (CD86: -18.8%, p<0.001; MHC-II: -59.0%, p<0.01; figures 5D-5E).

We next investigated plasma cytokine levels in the two groups of bone marrow transplanted mice after 9 weeks WTD feeding. LDLr KO mice with MKP2 KO bone marrow showed a striking 6.9-fold higher plasma level of the anti-inflammatory cytokine IL-10 as compared to LDLr KO mice reconstituted with WT bone marrow (817.3±106.1 pg/mL for MKP2 KO versus 118.4±35.68 pg/mL for WT; p<0.001; Figure 5F). In further support of a more anti-inflammatory phenotype, MKP2 KO bone marrow transplanted mice exhibited decreased plasma levels of the pro-inflammatory cytokine IL-12p40 as compared to their WT bone marrow recipient control mice (8.5±1.6 pg/mL for MKP2 KO versus 30.3±5.3 pg/mL for WT; p<0.01; Figure 5G).

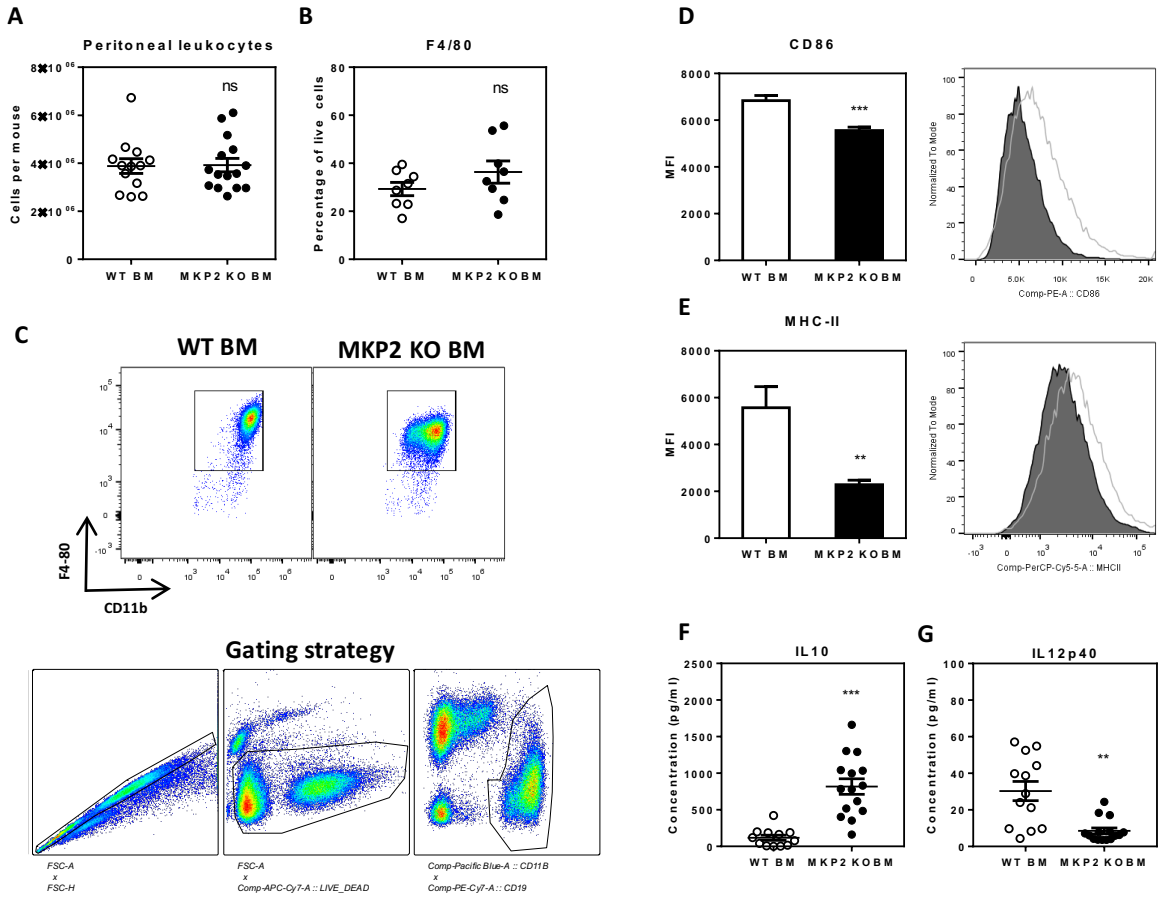

Figure 5 Hematopoietic MKP2 deficiency in LDLr KO mice is associated with decreased M1 macrophage activation in the peritoneal cavity.

LDLr KO mice reconstituted with WT or MKP2 KO bone marrow were challenged with a Western-type diet for 9 weeks. Peritoneal leukocyte populations were analyzed by flow cytometry. A) Total peritoneal leukocytes. B) Peritoneal macrophages as percentage of total leukocytes. C) Representative flow cytometric plots of $CD11b^{+}$ / $F4/80^{+}$ macrophages and gating strategy used in the analysis. D) Median fluorescence intensity of CD86 and E) MHC-II in WT (open bars) and MKP2 KO peritoneal macrophages (closed bars). Plasma IL-10 (F) and IL-12p40 (G) levels were determined by ELISA. **p<0.01, ***p<0.001, ns p>0.05; (n=8-15).

Hematopoietic MKP2 deficiency in LDLr KO mice is associated with increased atherosclerotic lesion development

Atherosclerotic lesion size was analysed in Oil red-O stained sections of the aortic root after 9 weeks WTD feeding. Bone marrow-specific MKP2 deficiency was associated with an increased atherosclerotic lesion size ($2.1\pm0.2\times10^5$ μm^2 for MKP2 KO recipients versus $1.6\pm0.1\times10^5$ μm^2 for WT recipients; $p<0.05$; Figure 6A). No difference in lesional macrophage content was observed between both groups (Figure 6B). However, compared to WT bone marrow recipient mice, MKP2 KO bone marrow transplanted mice did show a lower lesional collagen content (27±2% for MKP2 KO versus 38±3% for WT; $p<0.01$; Figure 6C), suggesting that loss of MKP2 in bone marrow-derived cells is associated with larger lesions with a less stable phenotype.

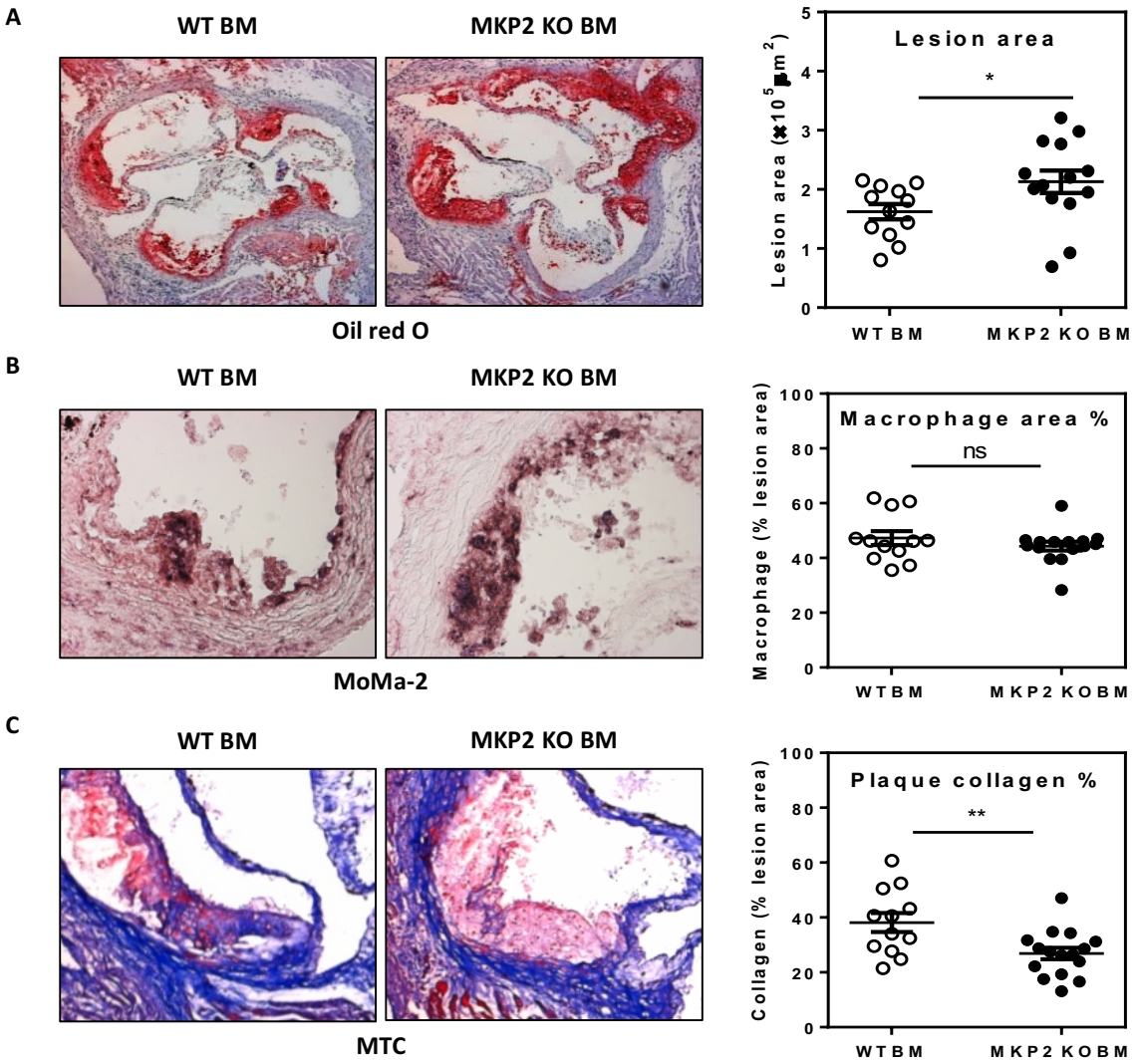

Figure 6 Hematopoietic MKP2 deletion in LDLr KO mice promotes atherosclerosis.

LDLr KO mice reconstituted with WT or MKP2 KO bone marrow were challenged with a Western-type diet for 9 weeks. A) Oil red O-positive atherosclerotic lesion area within the aortic root. B) Lesional macrophage content. C) Plaque collagen content. *$p<0.05$, **$p<0.01$, ns $p>0.05$.

Peritoneal macrophage M1 markers are negatively correlated to aortic lesion size in LDLr KO recipients

To gain insight in the possible mechanism underlying the increased atherosclerosis susceptibility of LDLr KO mice transplanted with MKP2 KO bone marrow, atherosclerotic lesion size was plotted against the cytokine concentrations in plasma, or the expression of the M1 macrophage markers on peritoneal cells. The concentration of anti-inflammatory cytokines IL-10 and IL-12 in the plasma of the recipient mice did not correlate with the atherosclerotic lesion size in the aortic root ($p>0.05$, figure 7A and 7B). However, the MFI of the M1 macrophage markers CD86 and MHCII were found to negatively correlate with atherosclerotic lesion size in the LDLr KO recipients ($r = -0.584$, $p<0.05$ for CD86; $r = -0.6963$, $p<0.01$ for MHCII).

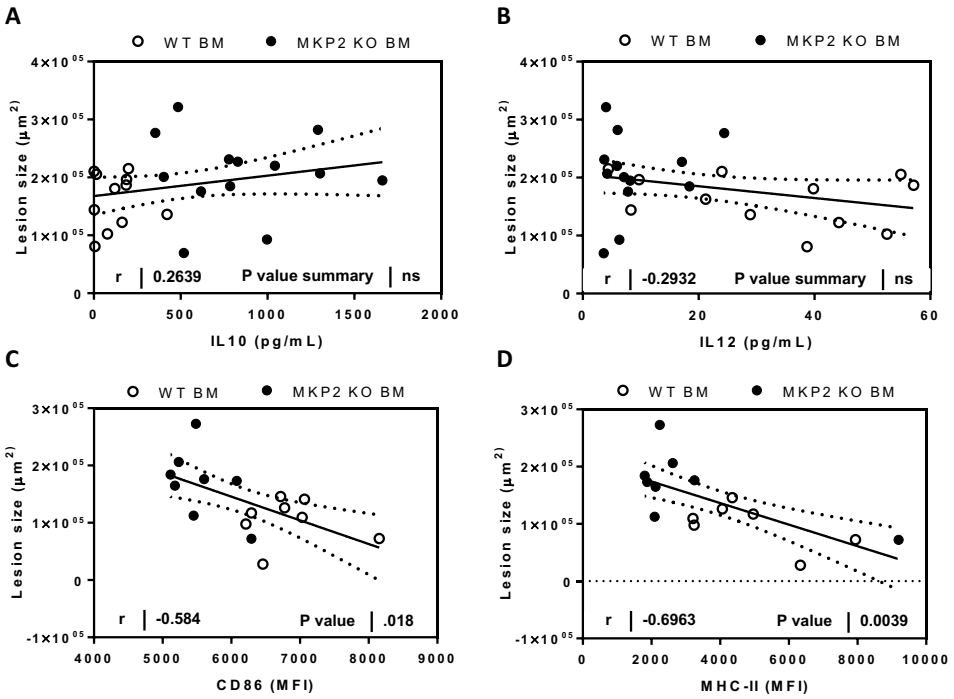

Figure 7 Expression of peritoneal macrophage M1 markers are negatively correlated to aortic lesion size in LDLr KO recipients

Correlation analysis on the plasma concentration of IL-10 (A) and IL-12 (B) and peritoneal macrophage expression of CD86 (C) and MHC-II (D) with aortic root atherosclerotic lesion size. White dots represent individual WT bone marrow transplanted mice, while black dots represent individual MKP2 KO bone marrow recipient mice. Solid lines represent regression lines. Dotted lines represent the 95% confidence interval for individual predictions. (n=16-25).

Discussion

In the current study, we investigated the role of MKP2 in macrophage polarization, foam cell formation and atherosclerosis. Deletion of MKP2 in macrophages is associated with hyperactivation of the MAPK family member JNK, enhanced polarization towards an M2-like phenotype and aggravated foam cell formation *in vitro*. Correspondingly, bone marrow-specific MKP2 deficient mice showed accelerated atherosclerotic lesion development *in vivo*.

MKP2 is a member of the subclass of nuclear inducible MKPs, which also contains MKP1. Hematopoietic deletion of MKP1 induces atherosclerosis susceptibility of LDLr KO mice to a similar

extend as what we show for hematopoietic MKP2 deletion.[40] The effects of MKP1 deletion were primarily attributed to effects on monocyte migration, in response to MCP1. In the current study, we showed that hematopoietic MKP2 deletion did not affect blood monocyte counts and phenotype nor macrophage migration, indicating that hematopoietic MKP1 and MKP2, despite their similar MAPK inactivating function, differentially influence atherosclerosis.

MKP2 is highly homologous with MKP1 at the C-terminal catalytic domain,[41] but their N-terminal domains are less closely related.[19] The unique sequence in the N-terminal domain of MKPs determine their MAPK substrate preference.[42] MKP1 is able to dephosphorylate all three MAPKs, but its effects on atherogenesis are likely through deactivation of JNK and p38.[43,44] MKP2 appears to have only ERK2 and JNK as preferred substrates but not p38.[19] Notably, our current findings, together with previously published studies, indicate that the substrate preference of MKP2 is cell-type dependent.

Here we show that deletion of MKP2 in thioglycollate-elicited macrophages stimulated JNK activation. In line, a recently published study by Mashael *et al.* showed that, upon LPS stimulation, JNK activation was largely increased in bone marrow-derived macrophages lacking MKP2.[45] However, the JNK activation status was not changed under basal conditions in this type of macrophages.[45] This is probably due to a phenotypic difference, as naïve bone marrow-derived macrophages are monocyte-like, while peritoneal macrophages are activated in response to thioglycollate.[46] In agreement with the Mashael *et al.* study,[27] ERK activation in peritoneal macrophages was not affected in the absence of MKP2. We did also observe a decrease in p38 phosphorylation on the MKP2 KO macrophages. This is, however, most likely not a direct consequence of the MPK2 loss, since MKPs deactivate MAPKs. JNK and p38 negatively regulate each other's activation in many cell types,[47,48] including thioglycollate-elicited macrophages.[49] As such, the decreased phosphorylation of p38 in MKP2 KO macrophages can perhaps be attributed to the enhanced JNK activation. Taken together, our study suggests that JNK is the preferred substrate of MKP2 in murine macrophages.

Macrophage polarization is extensively regulated by phosphorylation and subsequent dephosphorylation of proteins involved in cell signal transduction pathways. Associated with the augmented JNK activation in peritoneal macrophages lacking MKP2, the expression of M1 signature genes was downregulated and M2 signature genes upregulated, suggesting an M2-like phenotype. Similar to our study, Stuart *et al*., also found enhanced expression and activity of the M2 markers Arg1 in naive MKP2 deficient bone marrow-derived macrophages. However, other *in vitro* studies showed that JNK activation is enhanced and prolonged during LPS-induced M1 macrophage polarization[50,51] Correspondingly, JNK deficient macrophages display a lower expression of M1-associated pro-inflammatory cytokines and chemokines upon LPS stimulation.[29,35] These results are in contrast to our findings, probably because in the latter studies the macrophages were stimulated with LPS, an extremely potent stimulator, that skews M2 macrophages towards an M1 phenotype.[52] LPS binding to toll-like receptor 2 stimulates the JNK activation-induced production of pro-inflammatory cytokines.[53] In agreement, Mashael *et al.* showed a pro-inflammatory cytokine production profile in the "M2-like" MKP2 deficient bone marrow-derived macrophages upon stimulation with LPS.[27] This indicates that loss of MKP2 might lead to a macrophage phenotype that is hyper-responsive to LPS stimulation. More research is needed to confirm this hypothesis and to address the exact mechanism of how MKP2 regulates macrophage polarization.

Macrophage phenotype is an important factor determining atherosclerosis susceptibility.[54] M2 macrophages were considered as promising therapeutic targets for the treatment of atherosclerosis due to their anti-inflammatory properties.[55] However, recent evidence suggests that M2 macrophages are more susceptible to foam cell formation.[39,56] In the current study, for the first time a striking effect of MKP2 deficiency on macrophage foam cell formation was shown. M2 macrophages are more susceptible to oxLDL-induced foam cell formation[57] due to an increased expression of the scavenger receptors CD36 and SR-A.[38,58] SR-A and CD36 are responsible for up to 90% of the oxLDL uptake by macrophages *in vitro*.[59] In line with the M2-like phenotype of MKP2 KO macrophages, the expression of CD36 and SR-A, as well as the associated foam cell formation, were induced in the absence of macrophage MKP2. JNK activation stimulates oxLDL-induced foam cell formation by inducing the CD36 / JNK / SR-A pathway.[60] Hence, we speculate that the MKP2 deficiency-induced macrophage foam cell formation is JNK-dependent and acts likely through the CD36 / JNK / SR-A pathway (Figure 8, left panel).

In line with the observation that MKP2 KO macrophages were more prone to develop into foam cells, bone marrow-specific deletion of MKP2 increased atherosclerotic lesion development in the aortic root of LDLr KO mice. Interestingly, although the atherosclerotic lesions were larger upon MKP2 deletion in bone marrow-derived cells, the collagen content was lower. This can be explained by a previous finding showing that M2 macrophages are responsible for collagen degradation.[61] In line with the anti-inflammatory properties of M2 macrophages, plasma IL-10 levels were highly elevated, while plasma IL-12p40 levels were decreased in the MKP2 KO bone marrow recipients as compared to WT bone marrow recipients. IL-10 is a prototypic anti-inflammatory cytokine, and although it has been shown to stimulate oxLDL-induced foam cell formation, it is in general considered to be athero-protective.[62,63] Therefore, the increased IL-10 production in the current study is unlikely to have contributed to the increased lesion sizes observed in MKP2 KO bone marrow transplanted mice. This is supported by the correlation analysis, which showed that the M1 macrophage marker expression, rather than plasma IL-10/12 cytokine levels, is significantly associated with atherosclerotic lesion size in the aortic root. As such, we anticipate that the M2 polarization and enhanced foam cell formation, rather than the reduced inflammation, underlies the augmented atherosclerosis development due to MKP2 loss (Figure 8, right panel).

In addition to macrophages, MKP2 is also expressed in other bone marrow-derived cells, including B cells, T cells, and dendritic cells.[64-66] Although in our bone marrow transplantation model MKP2 deficiency did not influence B cell and T cell counts, we cannot rule out a potential contribution of MKP2 in these cells to the protection against atherosclerosis.

In conclusion, we have shown that (1) MKP2 deficiency predisposes *in vitro* cultured macrophages to acquire an M2 phenotype, resulting in an enhanced susceptibility to become foam cells, and, (2) MKP2 deficiency in bone marrow-derived cells enhances the susceptibility to atherosclerotic lesion development *in vivo* (Figure 8).

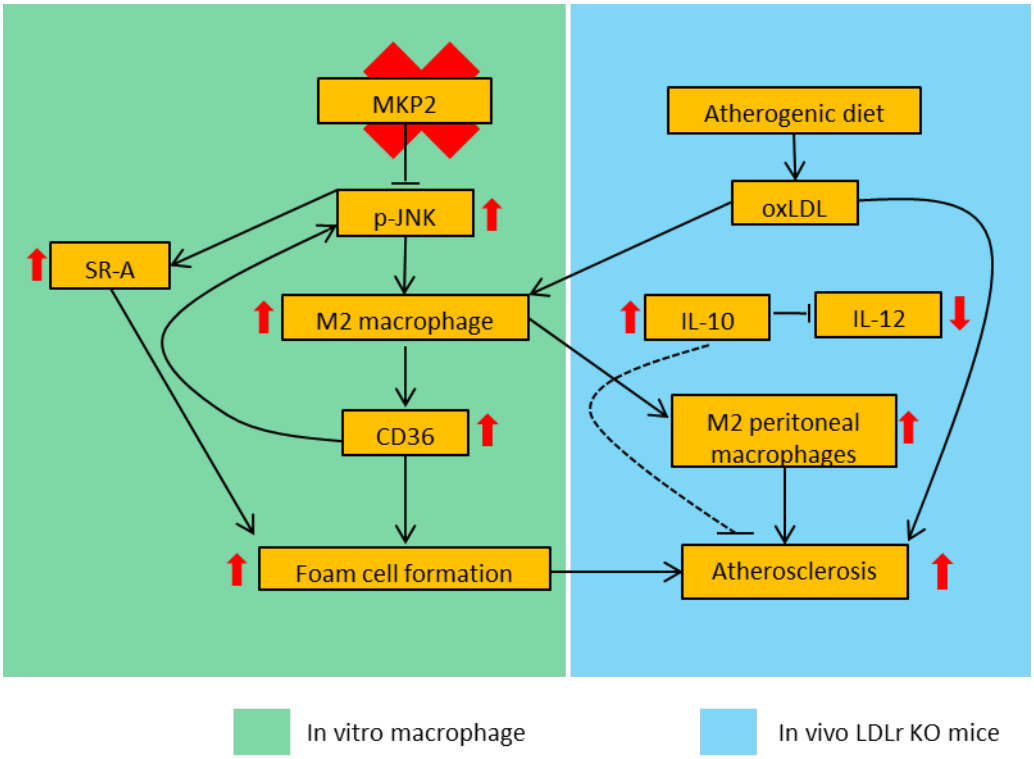

Figure 8 Diagram of MKP2 effects deletion on macrophage activation and atherosclerosis.

MKP2 deficiency in macrophages stimulates JNK activation, leading to M2 polarization. CD36 expression is upregulated in M2 macrophages which in turn stimulates JNK phosphorylation and subsequent SR-A upregulation. Under oxLDL conditions, stimulation of the CD36/JNK/SR-A pathway promotes foam cell formation. MKP2 induced M2 macrophage and foam cell formation overweigh the athero-protective role of elevated anti-inflammatory cytokine production, promoting atherosclerosis development *in vivo*.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Enhanced atherosclerotic lesion development in LDL receptor knockout mice lacking Upstream Stimulating Factor 1 (Usf1) in bone marrow-derived cells

Baoyan Ren[1], Pirkka-Pekka Laurila[2,3,9], Reeni B. Hildebrand[1], Jarkko Soronen[3,4], Vanessa Frodermann[1], Zhuang Li[5], Mariëtte R. Boon[5,6], Janine J. Geerling[1], Patrick C.N. Rensen[5,6], Christian Ehnholm[3], Petri T. Kovanen[7], Matti Jauhiainen[3,8], Menno Hoekstra[1], Miranda Van Eck[1]

1 Division of Biopharmaceutics, Cluster BioTherapeutics, Leiden Academic Centre for Drug Research, Gorlaeus Laboratories, Leiden, The Netherlands
2 Department of Medical Genetics, University of Helsinki, Helsinki, Finland
3 Genomics and Biomarkers Unit, National Institute for Health and Welfare, Biomedicum 1, Helsinki, Finland
4 Pharmaceuticals Division Bayer Oy BOF-PH-MRA-MA, Medical Affairs PO, Espoo, Finland
5 Department of Medicine, Division of Endocrinology, Leiden University Medical Center, Leiden, The Netherlands
6 Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands
7 Wihuri Research Institute, Biomedicum, Helsinki, Finland
8 Minerva Foundation Institute for Medical Research, Biomedicum, Helsinki, Finland
9 Institute for Molecular Medicine Finland, FIMM, Helsinki, Finland

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Upstream Stimulatory Factor 1 (Usf1), a ubiquitous transcription factor associated with familial combined hyperlipidemia, regulates the expression of genes involved in lipid metabolism. Previous studies showed that mice lacking Usf1 developed a beneficial cardiometabolic profile. In this study, we investigated the atherogenic effect of hematopoietic Usf1 in low-density lipoprotein receptor (LDLr) knockout (KO) mice.

Bone marrow from Usf1 KO mice and wild-type mice was transplanted into male LDLr KO mice. After 8 weeks recovery on a regular chow diet, the mice were challenged with a pro-atherogenic Western-type diet for 20 weeks. Specific deletion of hematopoietic Usf1 also protected against diet-induced obesity. However, opposed to total-body Usf1 deficiency, deletion of hematopoietic Usf1 in LDLr KO mice led to a significant increase in atherosclerotic lesion size (130%, $p<0.05$). The increased atherosclerosis susceptibility coincided with increased neutrophil counts in the circulation (200%, $p<0.01$) and elevated VLDL cholesterol levels (162%, $p<0.05$). Interestingly, the phenotype induced by hematopoietic Usf1 deficiency in LDLr KO mice is likely attributed to an impaired clearance of VLDL by white adipose tissue (WAT). The mRNA expression of Peroxisome proliferator activated receptor gamma, Lipoprotein lipase and VLDL receptor, key players in regulation of VLDL clearance, were downregulated in WAT of Usf1 KO bone marrow recipients (-41.7%, $p<0.05$; -30.1%, $p<0.05$; and -49.4%, $p<0.005$; respectively) and associated with a decreased lipid content in WAT.

Taken together, these results suggest that hematopoietic ablation of Usf1 does not account for the beneficial effects of global Usf1 deletion.

Introduction

Upstream Stimulatory Factor 1 (Usf1) is a ubiquitously expressed transcription factor that plays an important role in lipid metabolism.[1] Usf1 regulates the expression of many genes involved in lipid metabolism, including apolipoprotein A2 (*apoA2*),[2] *apoA5*,[3] *apoC3*,[4] *apoE*,[5] hepatic lipase (*HL*),[6] ATP-binding cassette transporter 1 (*ABCA1*),[7,8] and fatty acid synthase (*FASN*).[9-11] Two forms of the Usf protein have been identified, which are referred to as Usf1 and Usf2, respectively.[12] The Usf proteins form hetero- and (less common) homo-dimers and bind to the E-box motif. Although the *Usf* genes are ubiquitously expressed in mammalian cells, the relative abundance of the *Usf1* and *Usf2* gene products varies among cell types.[13] Importantly, the function of Usfs is modulated in a cell-specific manner.[14] Variants of Usf1 have been associated with familial combined hyperlipidemia (FCHL), characterized by increased serum total cholesterol, triglycerides or both.[11,15] Whole-body or liver-specific over-expression of human *Usf1* significantly decreased total plasma cholesterol levels in C57BL/6J mice, while triglycerides tended to be slightly higher.[16] Conversely, Laurila *et al.* showed that deletion of Usf1 in C57Bl/6J mice led to elevated plasma total cholesterol, primarily in high density lipoprotein (HDL) particles, and decreased very low density lipoprotein (VLDL) triglycerides.[17]

In addition to its role in lipid metabolism, there are indications that Usf1 might also modulate the immune response. A correlation was found between *Usf1* and IL-6 on transcriptional level in the liver of transgenic mice overexpressing human *Usf1*.[13,16]Moreover, downregulation of *Usf1* in the RAW 264.7 macrophage cell line upregulates mitochondrial uncoupling protein 2 (*UCP2*) which suppresses the production of pro-inflammatory mitochondria-derived reactive oxygen species (mtROS).[18] In agreement, global Usf1 deficiency also led to lower circulating inflammatory cytokines in mice.[17] Collectively, these data imply that Usf1 might play an important role in lipid

metabolism and the immune response. However, only limited research on the link between Usf1 and atherosclerosis has been described.[17,19-21] Recent studies by Laurila and colleagues showed that low-density lipoprotein (LDL) receptor (r) knockout (KO) mice lacking Usf1 display remarkably decreased susceptibility to atherosclerotic lesion development.[17] However, the mechanisms underlying the reduction of atherosclerosis induced by Usf1 deficiency are poorly defined. For instance, it is not known whether Usf1 merely affects blood lipid levels or whether it also modulates atherosclerosis susceptibility by impacting the immune system. Bone marrow-transplantation (BMT) allows to specifically delete Usf1 in BM-derived leukocytes. The aim of the current study was, therefore, to specifically assess the role of Usf1 in immune cells and the consequences for atherosclerosis development. Hereto, BM from Usf1 KO mice was transplanted into male LDLr KO mice and atherosclerosis susceptibility was determined after 20 weeks challenge with a pro-atherogenic Western-Type Diet (WTD).

Material and methods

Animals and bone marrow transplantation

LDLr KO mice (C57Bl/6J background) purchased from the Jackson Laboratories, were maintained and bred under standard laboratory conditions at the Gorlaeus Laboratories in Leiden, the Netherlands. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Usf1 KO and wild-type (WT) littermates (both C57BL/6J background) were bred at the National Institute for Health and Welfare, and University of Helsinki, in compliance with the Finnish government guidelines. Experiments were conducted in conformity with the Finnish regulations and the European parliament Directive 2010/63/EU. Other details regarding Usf1 KO mice are available in a recent report.[17]

Bones were harvested from Usf1 KO and WT mice and transported to Leiden in Dulbecco's modified Eagle medium (DMEM). Within 36 hours of collection of the bones, bone marrow (BM) was isolated. LDLr KO mice recipients (male, approx. 12 weeks old) were transplanted with either Usf1 KO BM or WT BM. BMT was performed by intravenous tail vein injection of 5×10^6 cells into the recipients, one day after lethal irradiation (Röntgen, 8 Gy). The recipients were allowed to recover for 8 weeks on a chow diet (RM3; Special Diet Services). Subsequently, the mice were fed a pro-atherogenic WTD, containing 0.25% cholesterol, 15% cocoa butter and 1% corn oil (SDS, Sussex, UK). After 20 weeks of WTD feeding, the mice were sacrificed. In short, the mice were anaesthetized using a mix of xylazine, ketamine and atropine. Blood was collected by retro-orbital bleeding (for flow cytometric analysis and testing on a veterinary haematology analyzer (Sysmex)) or by tail cut (for lipid analysis). Subsequently, the animals were perfused with PBS, and the heart and other organs were collected for further research. Erythrocytes in the blood were lysed with erythrocyte lysis buffer (0.15 mol/L NH_4Cl, 10 mmol/L $NaHCO_3$, 0.1 mmol/L EDTA, pH 7.3) and subsequently the white blood cells were used for flow cytometric analysis.

Plasma lipid determination

After 4 hours fasting, blood was collected via tail sampling in potassium-EDTA microvette CB 300 tubes (Sarstedt, Nümbrecht, Germany), and centrifuged with 2,000 rpm at 4°C for 5 minutes to separate out the plasma. Free cholesterol and total cholesterol levels were determined in plasma as previously described.[22] Furthermore, plasma was used for lipoprotein profile analysis using fast protein liquid chromatography (FPLC) using a high-resolution size-exclusion chromatography Superose 6 HR column (3.2 × 30 mm; Smart-System, Pharmacia, Uppsala, Sweden).

Hepatic lipid extraction

Total lipids were extracted from liver samples using the Bligh & Dyer method that was described previously,[23] and dissolved in 2% Triton X-100. The cholesterol and triglyceride content in the homogenate were measured and divided by the protein content as determined using a BCA assay,[24] and expressed as "µg lipid/mg protein".

Adipose tissue lipid content quantification

Paraffin embedded sections (5 µm) from gonadal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) of the transplanted LDLr KO recipients were prepared and stained by hematoxylin and eosin. The lipid–droplet-positive area as percentage of the total WAT and BAT area was qualified using Image J software (version 1.47).[25]

Sysmex haematology analyser and flow cytometry

Blood leukocyte counts were analysed using an automated Sysmex XT-2000iV Veterinary Haematology analyser (Sysmex Corporation). Fluorescent activated cell sorting (FACS) analysis was performed on a FACS Canto II apparatus (BD Biosciences, Mountain View, CA) to detect cell surface markers on blood cells. The antibodies anti-Ly6C, anti-Ly6G and anti-CD11b were all from eBioscience, Ltd. Nile red (Sigma-Aldrich, USA) and used to detect lipid-rich leukocytes. Data were analysed using FlowJo Software v10 (TreeStar Inc).

Atherosclerotic lesion analysis in aortic root

Hearts were fixed in 4% Shandon Zinc Formal-Fixx (Thermo Fisher Scientific, 9990245) for 24 hours, and subsequently embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, USA) until further processing. Cryosections (7 µm) at the level of the aortic sinus were obtained using a Leica CM3050s cryostat. Lipid-rich atherosclerotic plaques were stained with Oil Red O. Plaque area (in μm^2) quantitation was performed using a Leica image analysis system (Leica Ltd, Cambridge, UK).

mRNA expression analysis by real time PCR

Total RNA was isolated from liver, gonadal white adipose tissue and interscapular brown adipose tissue samples obtained at sacrifice after 20 weeks WTD challenge. cDNA were synthesized using RevertAid M-MuLV reverse transcriptase (Thermo Scientific, USA) according to the manufacturer's protocol (Thermo Scientific, USA). Quantitative gene expression was measured on a 7500 Fast Real-Time PCR system (Applied Biosystems, CA) using SensiMix SYBR green (GC biotech B.V., The Netherlands) technology. The average expression of the housekeeping genes *β-actin*, *RPL27* and

36B4 was used as a reference for calculation of the relative expression of the genes of interest. The primer sequences are shown in Table 1.

Table 1 qPCR primer sequences.

Primer sequences used for qPCR		
Gene	Orientation of primers	Sequences
36B4	Forward primer	CTGAGTACACCTTCCCACTTACTGA
	Reverse primer	CGACTCTTCCTTTGCTTCAGCTTT
ABCA1	Forward primer	AGAGCAAAAAGCGACTCCACATAGAA
	Reverse primer	CGGCCACATCCACAACTGTCT
ApoA	Forward primer	ATGTGTCCCAGTTTGAATCCTCCT
	Reverse primer	TTTCTCCAGGTTATCCCAGAAGTCC
β-actin	Forward primer	AACCGTGAAAAGATGACCCAGAT
	Reverse primer	CACAGCCTGGATGGCTACGTA
CD68	Forward primer	TGCCTGACAAGGGACACTTCGGG
	Reverse primer	GCGGGTGATGCAGAAGGCGATG
FASN	Forward primer	GGCGGCACCTATGGCGAGG
	Reverse primer	CTCCAGCAGTGTGCGGTGGTC
HMG-coA	Forward primer	CGAGCCACGACCTAATGAAGAATG
	Reverse primer	TGCATCACTAAGGAACTTTGCACC
LPL	Forward primer	CCCCTAGACAACGTCCACCTC
	Reverse primer	TGGGGGCTTCTGCATACTCAAA
LRP	Forward primer	CTTCTGGTGGCTGGCGTGGTG
	Reverse primer	CATCCGCTGGTGCTGGAAGCC
MTTP	Forward primer	TCTCACAGTACCCGTTCTTGGT
	Reverse primer	GAGAGACATATCCCCTGCCTGT
PPARg	Forward primer	AAGCCCTTTGGTGACTTTATGGAGCC
	Reverse primer	TGCAGCAGGTTGTCTTGGATGTCC
RPL27	Forward primer	CGCCAAGCGATCCAAGATCAAGTCC
	Reverse primer	AGCTGGGTCCCTGAACACATCCTTG
SCD1	Forward primer	TACTACAAGCCCGGCCTCC
	Reverse primer	CAGCAGTACCAGGGCACCA
SR-B1	Forward primer	AAACAGGGAAGATCGAGCCAGTAG
	Reverse primer	CGTAGTGAAGAACCTGGGGCAT
UCP1	Forward primer	CCAAGCTGTGCGATGTCCATGTACA
	Reverse primer	AAACATGATGACGTTCCAGGACCCG
Usf1	Forward primer	AGTTGGGAGATACAAAGTCCTCCG
	Reverse primer	TGCACTGTTCCCTCTTCGGTT
VLDLr	Forward primer	TGGAGATGAAGACTGTGCGG
	Reverse primer	CGAAGTCAGACTCAGCACACG

Statistical analysis

All values are expressed as means ± SEM. Differences between the groups were statistically analysed with an unpaired Student's T-test or two-way ANOVA using GraphPad Prism software (GraphPadSoftware Inc., San Diego, California, USA). Welch correction was applied in case of unequal variances in the dataset. A two-sided P value lower than 0.05 was considered as statistically significant.

Results

Hematopoietic Usf1 deficiency lowers body weight gain in LDLr KO mice

Total body deletion of Usf1 leads to a beneficial metabolic profile in C57BL/6 mice associated with leanness, increased lipolysis and improved insulin sensitivity compared to their WT littermates.[17] To investigate whether hematopoietic Usf1 deficiency influences body weight gain of the mice, changes in body weight were monitored throughout the study. Mice lacking Usf1 in bone marrow-derived cells gained less weight compared to mice that received WT BM (Figure 1A). The 2 groups started to diverge at week 18 after BMT (10^{th} week on WTD) (Figure 1B). At sacrifice (20^{th} week on WTD), LDLr KO mice with Usf1 KO BM were 2.5 g (8.5%) lighter in weight compared to mice transplanted with WT BM (Figure 1C).

Interestingly, *Usf1* mRNA expression in the gonadal white adipose tissue of LDLr KO mice reconstituted with Usf1 KO BM was 44% lower compared to the mice that received WT BM ($p<0.05$; Figure 1D). The decrease was likely not the result of a reduced macrophage content of the adipose tissue as no significant difference was found in the expression of the macrophage marker *CD68* between the 2 groups ($p=0.11$; figure 1E). The reduced *Usf1* expression in white adipose tissue was associated with 30% lower expression of lipoprotein lipase (*LPL*, $p<0.05$; Figure 1F) and 48% lower very-low density lipoprotein receptor (*VLDLr*, $p<0.05$; Figure 1G) expression, genes responsible for VLDL-TG-derived fatty acid uptake, and 42% lower Peroxisome proliferator-activated receptor-γ (*PPARg*, $p<0.05$; Figure 1H) expression, which is an important transcriptional factor regulating *LPL* and *VLDLr* expression. The expression of intracellular lipolysis related genes, including hormone-sensitive lipase (*Hsl*), adipose triglyceride lipase (*Atgl*), and the glucose transporter type 4 (*Glut4*) and lipid droplet-associated protein (*Plin*) were not changed (data not shown). Collectively, these data indicate decreased uptake of VLDL-TG-derived fatty acids by white adipose tissue upon deletion of Usf1 in bone marrow-derived cells. Therefore, lipid content of WAT was measured in paraffin-embedded gonadal WAT. In line with the body weight and gene expression data, a decreased WAT lipid content was observed in the Usf1 BMT mice compared to the WT mice, (-7%, $p<0.05$; Figure 1I). Morphological examination also confirmed a small decrease in adipocyte cell size (Figure 1J).

Plasma cholesterol is elevated in LDLr KO mice reconstituted with Usf1 KO bone marrow

Deletion of Usf1 in bone marrow-derived cells of LDLr KO mice resulted in a significant increase in total cholesterol and free cholesterol levels (+36%, $p<0.01$ and +26%, $p<0.05$ respectively; Figure 2A-B) after 20 weeks on WTD, while this effect was not observed on chow diet (data not shown). Determination of the lipoprotein distribution pattern showed that the increase in plasma cholesterol levels on WTD could be attributed to increased VLDL cholesterol levels (+60%, $p<0.05$; Figure 2C and D). Moreover, a trend towards higher plasma triglycerides was observed in LDLr KO mice with Usf1 KO BM on WTD (+17%, $p=0.12$; Figure 2E). No effect of BM Usf1 deletion was found on plasma glucose levels in the LDLr KO recipients (data not shown).

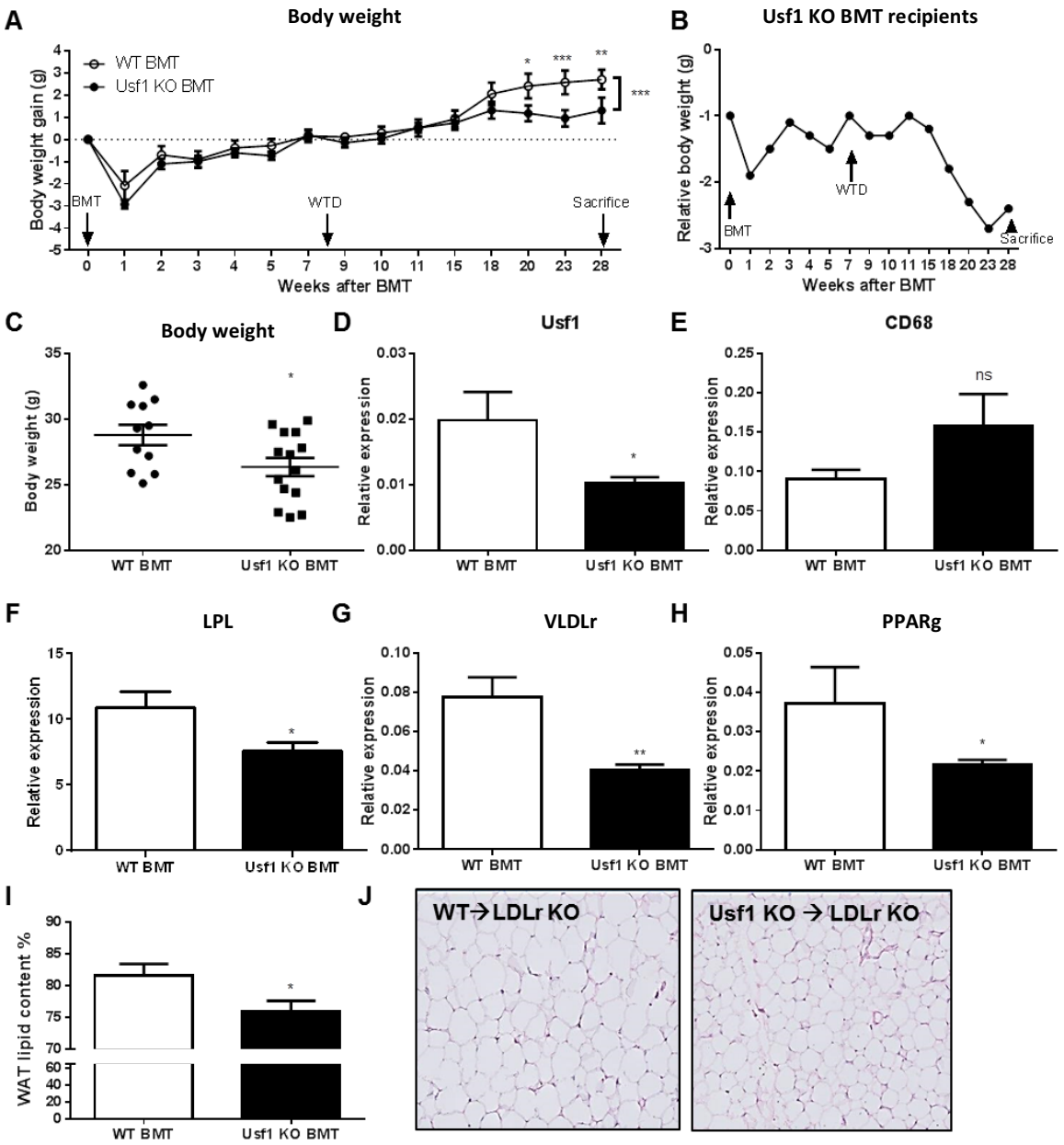

Figure 1 Deletion of hematopoietic Usf1 in LDLr KO mice attenuates body weight gain and adiposity.

LDLr KO mice received 5×10^{6} bone marrow cells intravenously from either WT or Usf1 KO mice after 8 Gy X-ray lethal irradiation. The mice were allowed to recover for 8 weeks on chow diet, and were then fed WTD diet for 20 weeks to induce atherosclerosis. A) Bodyweight gain of LDLr KO recipients reconstituted with WT bone marrow (open circles "o") or Usf1 KO bone marrow (closed circles "●") from 0-28 weeks after BMT. 2-way ANOVA was used to analyze the statistical significant difference in time. B) Relative body weight of LDLr KO recipients with Usf1 KO BM corrected by the bodyweight of recipients with WT, the distinctive bodyweight difference starts on week 15 (7 weeks WTD feeding). C) Bodyweight of LDLr KO recipients at sacrifice. D-H) Relative mRNA expression of *Usf1*, *CD68*, *LPL*, *VLDLr* and *PPARg* in inguinal adipose tissue of LDLr KO mice with WT bone marrow (open bar) or Usf1 KO bone marrow (closed bar). I) Lipid content of gonadal WAT in LDLr KO recipients with WT bone marrow (open bar) or Usf1 KO bone marrow (closed bar). J) Representative histology photographs of hematoxylin/eosin stained paraffin sections of gonadal white adipose tissue (original magnification10X).*P<0.05 **P<0.01 as compared to LDLr KO mice reconstituted with WT bone marrow (n=7-14).

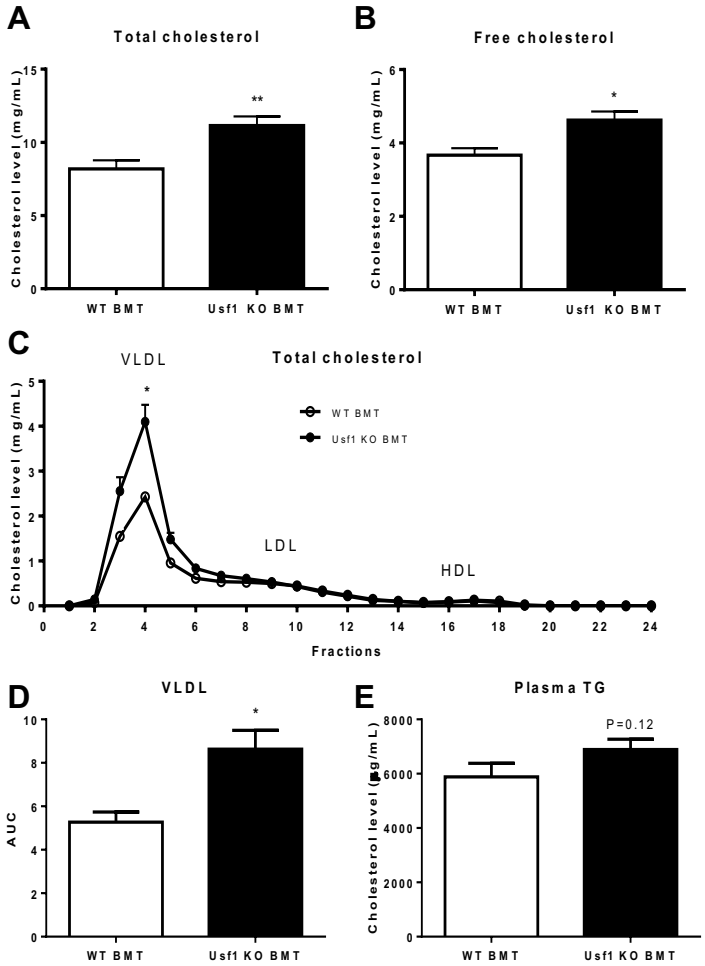

Figure 2 Deletion of hematopoietic Usf1 in LDLr KO mice induces plasma cholesterol levels.

After 20 weeks WTD feeding, plasma from LDLr KO mice reconstituted with WT bone marrow or Usf1 KO bone marrow was collected subsequently to 4 hours fasting. A) Total plasma cholesterol and B) free plasma cholesterol levels of LDLr KO recipients of WT bone marrow (open bars) or Usf1 KO bone marrow (closed bars). C) Plasma samples were fractionated with FPLC size-exclusion chromatography and cholesterol levels were measured from these fractions. Fractions 2-6 represent VLDL, fractions 7-14 LDL, fractions 15-19 HDL. Open circles (○) represent LDLr KO recipients with WT bone marrow, closed circles (●) represent LDLr KO recipients with Usf1 KO bone marrow. D) Area under the curve of cholesterol content in VLDL particles from recipients with WT bone marrow (open bar) or with Usf1 KO bone marrow (closed bar). E) Plasma triglycerides levels on of recipients. *P<0.05, **P<0.01 as compared to mice with WT bone marrow (n=4-5).

Deletion of hematopoietic Us1 in LDLr KO mice increased hepatic cholesteryl ester accumulation, but kept unaffected the mRNA expression of genes involved in hepatic lipid metabolism

In order to investigate if the increased plasma cholesterol in BM-specific Usf1 KO mice was associated with an altered hepatic lipid metabolism, the lipid content and mRNA expression levels of genes involved in lipid homeostasis were determined. BM-specific Usf1 deletion led to increased Oil red O staining for neutral lipids (Figure 3A) in the liver after 20 weeks WTD feeding, accompanied by increased cholesteryl ester accumulation evidenced by quantitative analysis after Bligh and Dyer extraction (+72%, $p<0.05$; Figure 3B), while free cholesterol and triglycerides were not affected ($p>0.05$; figure 3C). This is in contrast to the total body Usf1 knockout mice which were protected against hepatic steatosis, in line with their overall beneficial metabolic phenotype.[17]

Despite the observed increase in cholesteryl ester accumulation in the liver of LDLr KO mice lacking Usf1 in bone marrow-derived cells, the liver expression of genes involved in lipid synthesis, *i.e.* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMG-coA reductase*), microsomal triglyceride transfer protein (*MTTP*), stearoyl-CoA desaturase-1 (*SCD1*), *FASN* and the cholesterol esterification enzyme acyl-CoA:cholesterol acyltransferase (*ACAT*) were not affected (Supplementary figure 1A). Furthermore, while total body Usf1 deletion in mice leads to increased plasma HDL levels,[17] BM-specific Usf1 deletion did not affect HDL cholesterol. Not surprisingly, genes involved in HDL

metabolism, *i.e. ApoA1* and *ABCA1* were not affected in the liver of Usf1 KO BMT recipients compared to WT BMT recipients (Supplementary figure 1B). Also no effects were observed on the expression of genes involved in cholesterol clearance *i.e.* low-density lipoprotein receptor-related protein 1 (*LRP1*) and scavenger receptor class B type I (*SR-BI*) (Supplementary figure 1C). Moreover, the hepatic expression of *Usf1* was unchanged (p>0.05; Supplementary figure 1D). However, a 2.7-fold increase in expression was found of the Kupffer cell marker *CD68* in livers of LDLr KO mice reconstituted with Usf1 KO BM as compared to controls (p<0.001; Figure 3D), suggesting increased hepatic inflammation induced by the augmented cholesteryl ester accumulation in the liver.

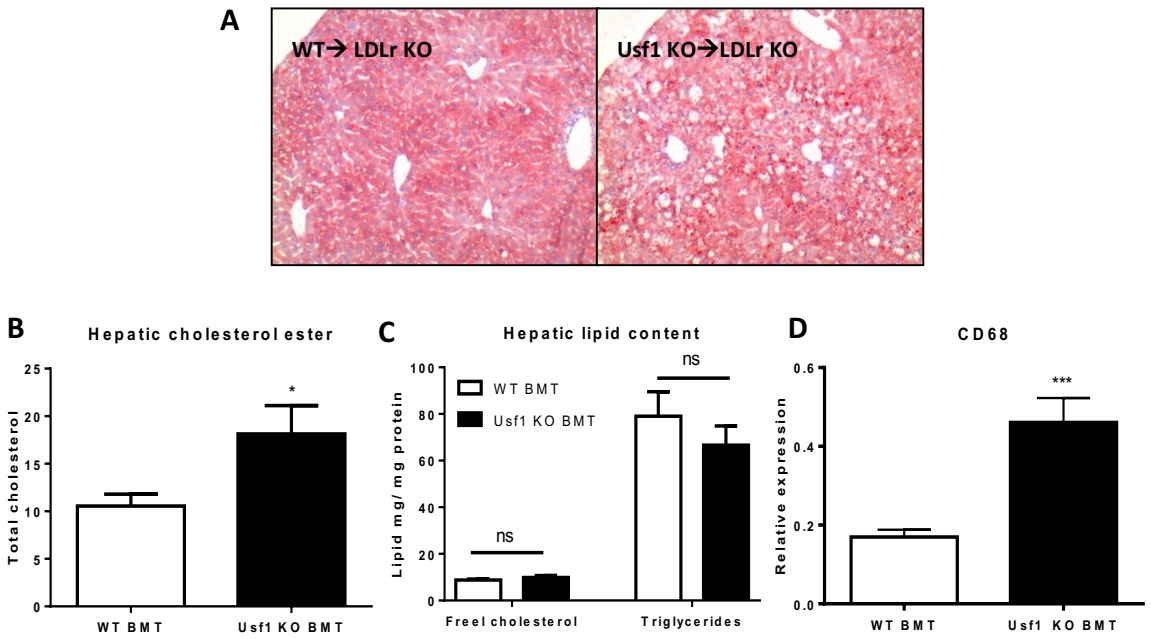

Figure 3 Effect of hematopoietic Usf1 deficiency on hepatic lipid accumulation after 20 weeks WTD feeding.

A) Representative images of Oil red O and H&E stained sections (original magnification 10x). Lipid was extracted from livers and the content of B) cholesteryl ester, C) free cholesterol and triglyceride were measured and normalized for protein level. D) Relative mRNA expression of *CD68* in liver. Open bars represent the recipients of WT bone marrow, closed bars represents the recipients of Usf1 KO bone marrow. *P<0.05; ***P<0.001 as compared to WT BMT livers (n=8).

Hematopoietic Usf1 does not affect brown adipose tissue of LDLr KO recipients

Activated brown adipose tissue (BAT) efficiently takes up fatty acids released from triglyceride-rich lipoproteins (TRL) such as chylomicrons and VLDL upon lipolysis of their core triglycerides leading to rapid clearance of the generated chylomicron and VLDL remnants by the liver.[26, 27] Mice with total-body Usf1 deletion display elevated uptake of TRL-derived fatty acids by BAT, and reduced BAT lipid content as well as smaller brown adipocyte size due to enhanced BAT thermogenesis.[17] This effect was independent of *UCP1* expression, the specific uncoupling protein of BAT, which was not changed due to the global Usf1 deletion.[17] In the current study, mRNA expression of *UCP1* in BAT of LDLr KO mice was also not altered upon deletion of Usf1 in bone marrow-derived cells. (p>0.05; Supplementary figure 2A). More importantly, in contrast to the total body Usf1 KO mice, the lipid content of BAT was not affected by BM Usf1 deletion (p>0.05, Supplementary figure 2B), suggesting a smaller contribution of BAT, if any, to the reduced bodyweight of the LDLr KO mice lacking hematopoietic Usf1.

Circulating neutrophil and monocyte counts are increased in LDLr KO mice lacking hematopoietic Usf1

High fat, high cholesterol feeding not only leads to hepatic inflammation, but also to augmented systemic inflammatory markers in mice.[28-30] Global Usf1 deficiency previously showed protection against low-grade systemic inflammation, a condition associated with metabolic disturbances.[17] To explore whether deletion of Usf1 in bone marrow-derived cells also affected the systemic inflammatory status under WTD feeding conditions, the circulating leukocyte profile was assessed using flow cytometry and haematological analysis (Sysmex). After 20 weeks of WTD feeding, a trend towards higher total white blood cell (WBC) counts was observed (+19%, p=0.09; Figure 4A). The observed trend to increased WBC counts was attributed to significantly higher amounts of circulating neutrophils (+63%, p<0.01; Figure 4B), and monocytes (+45%, p<0.05; Figure 4B), but not lymphocytes (data not shown). The results were confirmed by flow cytometric analysis. Compared to the WT controls, Usf1 KO transplanted mice showed an increase in the total amount of circulating $CD11b^+$ cells (+68%, p<0.05; Figure 5A-B); increased $CD11b^+Ly6G^+$ neutrophils (+100%, p<0.01; Figure 5C-D); and a small trend towards an increase in $CD11b^+Ly6C^{hi}$ pro-inflammatory monocytes (+23%, p=0.11; Figure 5E-F), but no difference in $CD11b^+Ly6C^{low}$ patrolling monocytes (p>0.05, Figure 5E-F).

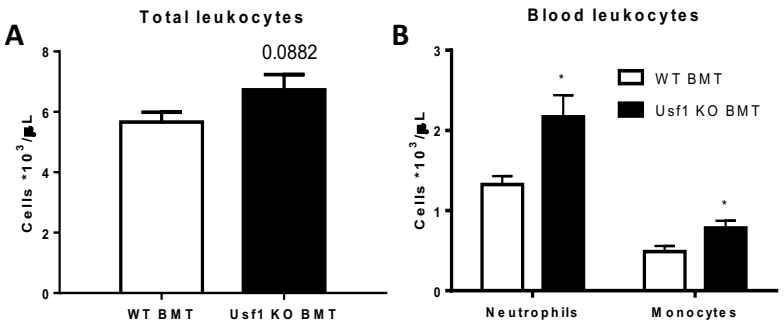

Figure 4 Effect of hematopoietic Usf1 deficiency on circulating leukocytes in LDLr KO mice.

After 20 weeks of WTD feeding, the circulating leukocyte profile was assessed using a hematological analyzer. A) Total leukocyte counts, B) Neutrophil and monocyte counts. Open bars represent LDLr KO mice reconstituted with WT bone marrow, closed bars represent mice with Usf1 KO bone marrow. *P<0.05 as compared to WT BMT mice (n=11-16).

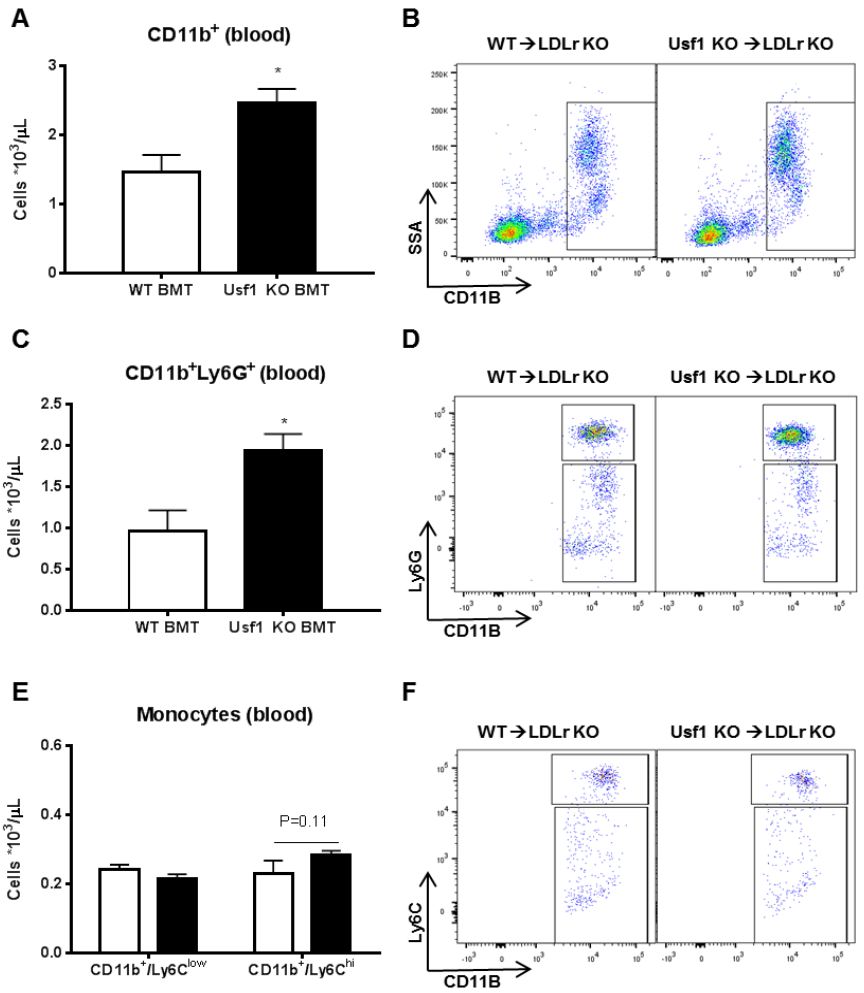

Figure 5 Flow cytometric analysis of blood cells in LDLr KO mice with hematopoietic Usf1 deficiency.

Circulating leukocytes in LDLr KO recipients were analysed by flow cytometry after 20 of weeks WTD feeding. A) Absolute numbers of $CD11b^+$ cells, C) $CD11b^+/LY6G^+$ neutrophils and E) $CD11b+/Ly6C^{low}$ anti-inflammatory monocytes and $CD11b^+/Ly6C^{high}$ pro-inflammatory monocytes were analyzed in the LDLr KO recipient mice with WT bone marrow (open bars) or Usf1 KO bone marrow (closed bars). B, D, F) Representative flow cytometric plots. *P<0.05; as compared to WT BMT mice (n=6).

Hematopoietic Usf1 deficiency in LDLr KO mice aggravates intracellular lipid accumulation in blood leukocytes

Nile red was used to quantify the cellular neutral lipid content of circulating blood cells using flow cytometry.[31, 32] In agreement with the elevated VLDL-cholesterol levels, an increased percentage of foamy leukocytes with a high nile red intensity were observed in Usf1 KO BMT mice compared to WT BMT controls on WTD (Figure 6A), indicating an induction of the amount of lipid-laden cells in the blood stream.

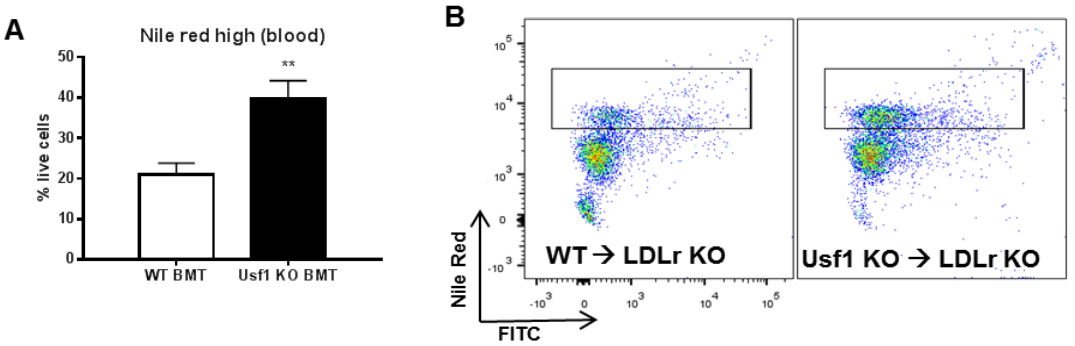

Figure 6 LDLr KO mice with hematopoietic Usf1 deficiency display aggravated intracellular lipid accumulation in blood.

After 20 weeks WTD feeding, A) Percentage of nile red positive circulating leukocytes. Open bars represent recipients of WT bone marrow, and closed bars recipients of Usf1 KO bone marrow. **P<0.01 (n=6) B) Representative flow cytometric plots of nile red staining of circulating leukocytes. (n=6).

Usf1 deficiency in bone marrow-derived cells of LDLr KO mice leads to increased atherosclerosis susceptibility

Since Usf1 deletion in bone marrow-derived cells of LDLr KO mice was associated with increased VLDL cholesterol and the appearance of more lipid-rich inflammatory cells in the circulation, we next assessed the effect of hematopoietic Usf1 deficiency on atherosclerosis susceptibility in the LDLr KO recipients. As expected, after 20 weeks WTD feeding, larger aortic root atherosclerotic lesions were found in the Usf1 KO BMT mice compared to the WT BMT controls (+31%, p<0.05; Figure 7A-B). Total cholesterol levels correlated well to atherosclerotic lesion sizes (p<0.05; figure 7C), indicating the elevated VLDL cholesterol is likely responsible for the increased susceptibility to atherosclerosis of the hematopoietic Usf1 KO mice.

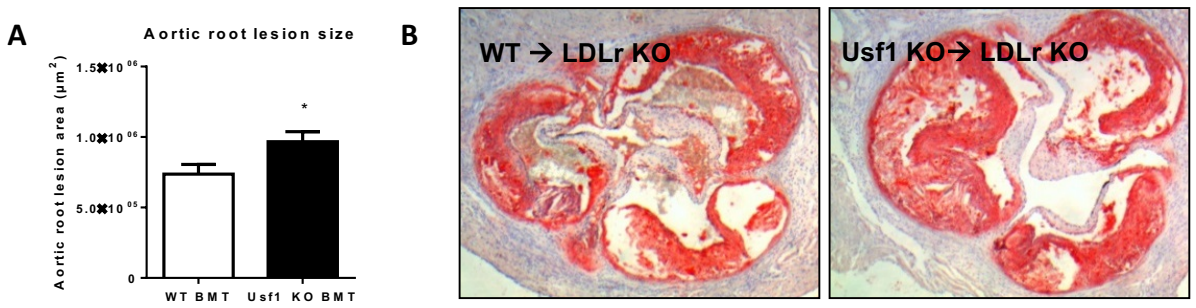

Figure 7 Bone marrow Usf1 deficiency increases atherosclerosis susceptibility of LDLr KO mice.

After 20 weeks of WTD feeding, atherosclerotic lesion development was assessed. A) Atherosclerotic lesion size in the aortic root of LDLr KO mice reconstituted with either WT bone marrow (open bars) or Usf1 KO bone marrow (closed bars). B) Representative aortic root atherosclerotic lesion stained for neutral lipids with Oil red O. *P<0.05 as compared to WT BMT mice (n=16-18).

Discussion

In the current study, we show that deletion of Usf1 in hematopoietic cells protects LDLr KO mice against diet-induced obesity, but leads to increased levels of cholesterol in circulating VLDL particles and elevated counts of lipid-laden inflammatory leukocytes in the circulation culminating into an increased susceptibility to atherosclerosis.

A recently published study by Laurila *et al.* showed that total body Usf1 KO mice are also protected against diet-induced obesity.[17] However, in contrast to our findings upon selective deletion of Usf1 in bone marrow-derived cells, total body Usf1 KO mice displayed a beneficial cardiometabolic lipid profile with decreased VLDL-triglycerides, and elevated HDL-cholesterol and were protected against atherosclerotic lesion development. In this context, it is important to note that the activity and function of Usfs is cell-type dependent.[14] The most pronounced tissue effect in the global KO mice was ascribed to BAT, although the contribution of other tissues could not be ruled out. Specific deletion of Usf1 in bone marrow-derived cells, as expected, only minimally affected the expression of *Usf1* in livers of the Usf1 KO BMT mice, while expression in BAT was not affected.

Global Usf1 deletion protects mice against the development of diet-induced obesity by increasing the activation of BAT.[17] Interestingly, in the current study we found that specific deletion of Usf1 in bone marrow-derived cells also protected LDLr KO mice from diet-induced obesity, suggesting that also bone-marrow dependent mechanisms could account for the reduced body weight of the global knockouts.[17] In contrast to total-body Usf1 KO mice, in our BM-specific Usf1 KO model, BAT lipid content was not changed nor was the expression of *UCP1* altered, suggesting that brown fat activation is not a causative factor in the lean phenotype observed in the Usf1 KO BMT mice. To fully exclude BAT activity as a causative factor in hematopoietic Usf1 deficiency-induced protection against weight gain, more comprehensive measurements of BAT activity, including measurements of BAT oxygen consumption and uptake of lipids and glucose, would have to be performed.

Adipose tissue mass can grow as a result of the expansion of the number of adipocytes (hyperplasia) or growth of the size of existing cells (hyperthrophy). Knockdown of *Usf1* in 3T3-L1 cells, a murine adipocyte model, by small interfering RNA (siRNA) represses adipogenesis,[10] indicating that Usf1 also has direct effects on adipogenesis. Notably, in our study, after challenge with WTD for 20 weeks, *Usf1* mRNA expression was largely decreased in adipose tissue of Usf1 KO BMT mice as compared to WT BMT controls. By performing a BMT with GFP-expressing BM Yuyama *et al*. previously showed that up to 16.7% of the adipocytes in mice challenged with a high fat diet for 7 weeks were derived from BM progenitors.[10] The decreased *Usf1* expression in adipose tissue of Usf1 KO transplanted LDLr KO mice is thus likely not only the consequence of deletion of Usf1 in adipose tissue macrophages (ATMs), but also in adipocytes from BM-origin. The decreased *Usf1* expression in adipose tissue coincided with lower *VLDLr* expression. VLDLr deficiency protects against obesity by lowering adipose tissue mass, which is associated with smaller adipocyte size due to a reduction in intracellular lipid droplet deposition.[33] Analysis of the lipid content of WAT in mice with BM Usf1 deletion showed a small decrease (-7%) in lipid content, but this is probably not sufficient to explain the overall 8.5% lower bodyweight. The lean body weight phenotype is thus likely also the consequence of an impaired expansion of the number of adipocytes, which is in line with the previously published role of Usf1 in adipogenesis.[10]

In contrast to total-body Usf1 KO mice that display a beneficial cardiometabolic profile,[17] in the current study we showed that selective deletion of Usf1 in bone marrow-derived cells in LDLr KO mice led to increased plasma levels of pro-atherogenic VLDL-cholesterol while HDL-cholesterol was not affected. No effects were observed on the hepatic expression of genes involved in VLDL clearance (*LRP1* and *SR-BI*) or VLDL synthesis (*HMG-coA reductase*, *MTTP*), suggesting that the observed augmented VLDL cholesterol levels are unlikely the result of an altered hepatic uptake.

Besides the liver, white adipose tissue is an important organ for cholesterol storage.[34] Adipocytes in rodents are a significant site for cholesterol synthesis and storage,[35] and, in obese subjects

adipose tissue stores up to 50% of the total body cholesterol.[34,36] Conversely, LDLr KO mice lacking adipose tissue display severe hyperlipidemia due to impaired plasma cholesterol clearance.[37] Similarly, our mice lacking Usf1 in bone marrow-derived cells remained lean and displayed increased plasma VLDL-cholesterol levels upon WTD feeding. Notably, WAT of LDLr KO mice reconstituted with Usf1 KO BM showed a decreased lipid content compared WAT of mice transplanted with WT BM, indicating reduced adipose tissue lipid deposition. Therefore, we hypothesized that an impaired VLDL-TG-derived fatty acid clearance capacity of the adipose tissue upon hematopoietic Usf1 deletion might be a causative factor in the observed elevation of circulating cholesterol levels, likely by impairing hepatic VLDL remnant clearance. LPL and VLDLr are two key proteins involved in VLDL clearance by white adipose tissue.[38] Adipocytes can remove VLDL particles directly from the circulation via the VLDLr.[39,40] or after hydrolysis of the particles' core triglycerides to free fatty acids by LPL, generating a VLDL remnant particle.[41] The expression of the *VLDLr* and *LPL* is regulated by PPAR-γ activation.[42] Yuyama *et al.* previously showed that knockdown of *Usf1* in the adipocyte cell line 3T3-L1 downregulated *PPARg* expression in this cell type.[10] Notably, *PPARg* expression was decreased in adipose tissue of LDLr KO mice transplanted with Usf1 KO BM. In support of the decreased activity of PPAR-γ in adipose tissue of LDLr KO transplanted with Usf1 KO BM, both the expression of the *VLDLr* and *LPL* were decreased. We speculate that deletion of Usf1 in bone marrow-derived cells might lead to elevated plasma VLDL levels through inhibition of VLDL clearance by adipose tissue through impairment of the PPAR-γ-VLDLr/LPL axis.

The excessive amounts of VLDL in the plasma due to impaired VLDL clearance by adipose tissue, could cycle back to the liver,[43] leading to increased hepatic cholesteryl ester accumulation. Indeed, cholesterol deposition in livers of LDLr KO recipients reconstituted with Usf1 KO BM was increased. Interestingly and in agreement with our findings, Jones and colleagues previously showed that conditional deletion of PPAR-γ in adipose tissue protects mice from high fat diet-induced obesity, and stimulates lipid accumulation in the liver.[44] The augmented cholesterol deposition in livers of LDLr KO mice transplanted with Usf1 KO BM coincided with a dramatic increase in hepatic *CD68* expression compared to WT transplanted controls, suggesting augmented hepatic inflammation.[45,46] Moreover, augmented systemic inflammation was found as evidenced by the observed increase in neutrophil counts and the trend towards increased pro-inflammatory monocyte counts in the circulation upon hematopoietic Usf1 deletion.

Besides inducing lipid accumulation in the liver, high levels of VLDL and VLDL remnant cholesterol also rapidly give rise to lipid droplet formation in monocytes, both in humans and in mice.[47,48] Consistently, BM-specific deficiency of Usf1 in LDLr KO mice led to increased counts of foamy monocytes in the circulation. Lipid-rich monocytes in blood are predictive markers for the development of atherosclerosis[49] and VLDL and remnants were reported to be the best predictor of aortic root atherosclerosis in the LDLr KO model.[50] In agreement, we observed larger atherosclerotic lesions in the aortic sinus of the Usf1 KO BMT mice after 20 weeks WTD feeding, correlating with the augmented serum cholesterol in the circulation of these animals. Interestingly, in contrast to the observed 1.3-fold increase in atherosclerotic plaque area in LDLr KO mice transplanted with Usf1 KO BM, Laurila *et al.*[17] recently reported that total body Usf1/LDLr double KO mice exhibit a 4-fold decrease in atherosclerotic plaque size after 20 weeks of WTD feeding in *en face* aortic sections, which is in line with the improved cardiometabolic lipid profile in these animals. Importantly, this observation was supported by a 45% reduction in atherosclerotic plaque area in humans being homozygous for an allele which induces 18 % decrease in *usf1* expression.[17,51] Thus, the beneficial metabolic effects caused by global Usf1 deficiency in mice and

humans are able to overcome the detrimental effects of Usf1 in bone marrow-derived cells as shown in the current study (Figure 8).

In conclusion, our study revealed a potential role of hematopoietic Usf1 in VLDL metabolism, obesity and atherosclerosis development and highlights the importance of studying tissue-specific effects of gene modifying strategies in animal models.

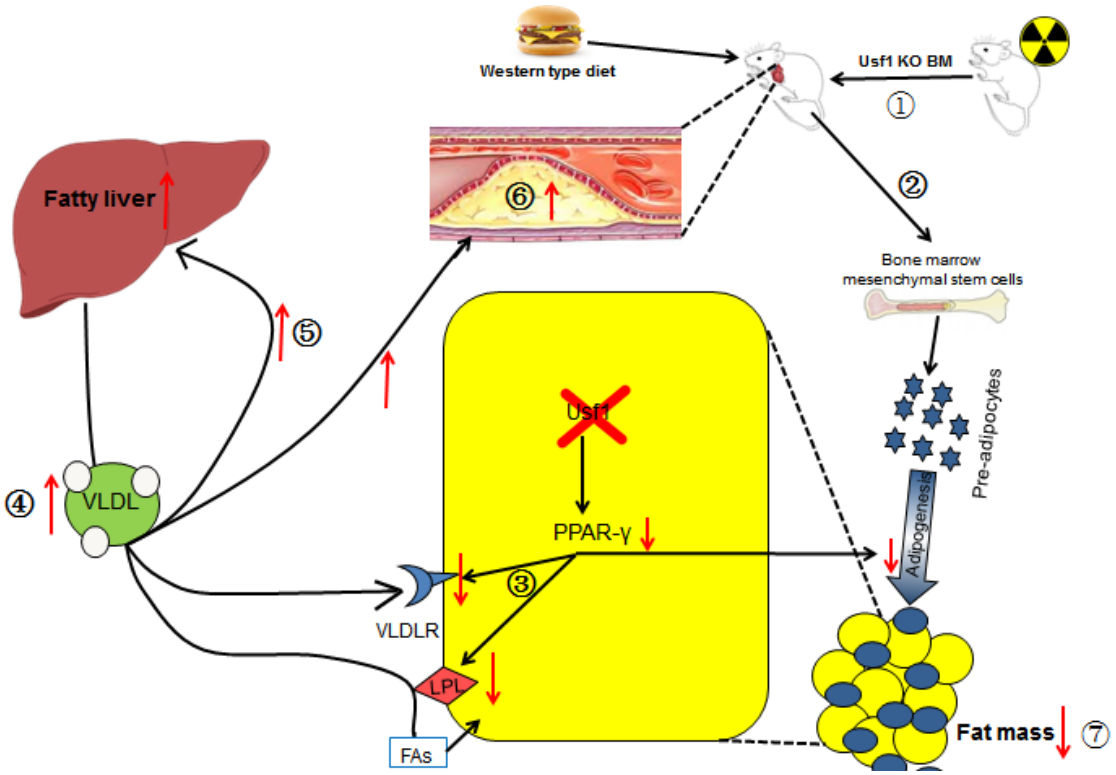

Figure 8 Effect of hematopoietic Usf1 on VLDL metabolism, adiposity, and atherosclerosis susceptibility.

1) Bone marrow transplantation deletes Usf1 in all bone marrow-derived cells of LDLr KO recipients. 2) WTD feeding induces the generation of novel adipocytes of which a fraction is derived from bone marrow progenitors lacking Usf1, contributing to decreased adipose *Usf1* expression in the Usf1 KO BMT mice. 3) Adipose Usf1 positively regulates the adipose tissue ability to take up plasma VLDL-TG-derived fatty acids through transcriptional regulation of VLDLr and LPL expression via PPAR-γ. 4) Clearance of VLDL remnant is impaired due to reduced *Usf1* expression in adipose tissue, leading to elevated plasma VLDL-cholesterol 5) Increased cycling of VLDL-cholesterol to the liver contributes to increased hepatic lipid accumulation in Usf1 BMT mice. 6) Increased plasma VLDL-cholesterol promotes atherosclerosis susceptibility in LDLr KO recipients. 7) Reduced lipid deposition and impaired adipogenesis co-lead to the decreased body weight gain and adiposity. The black lines and arrows indicate the normal pathways. The red cross means knockout and red arrows indicate the effects of hematopoietic Usf1 deficiency.

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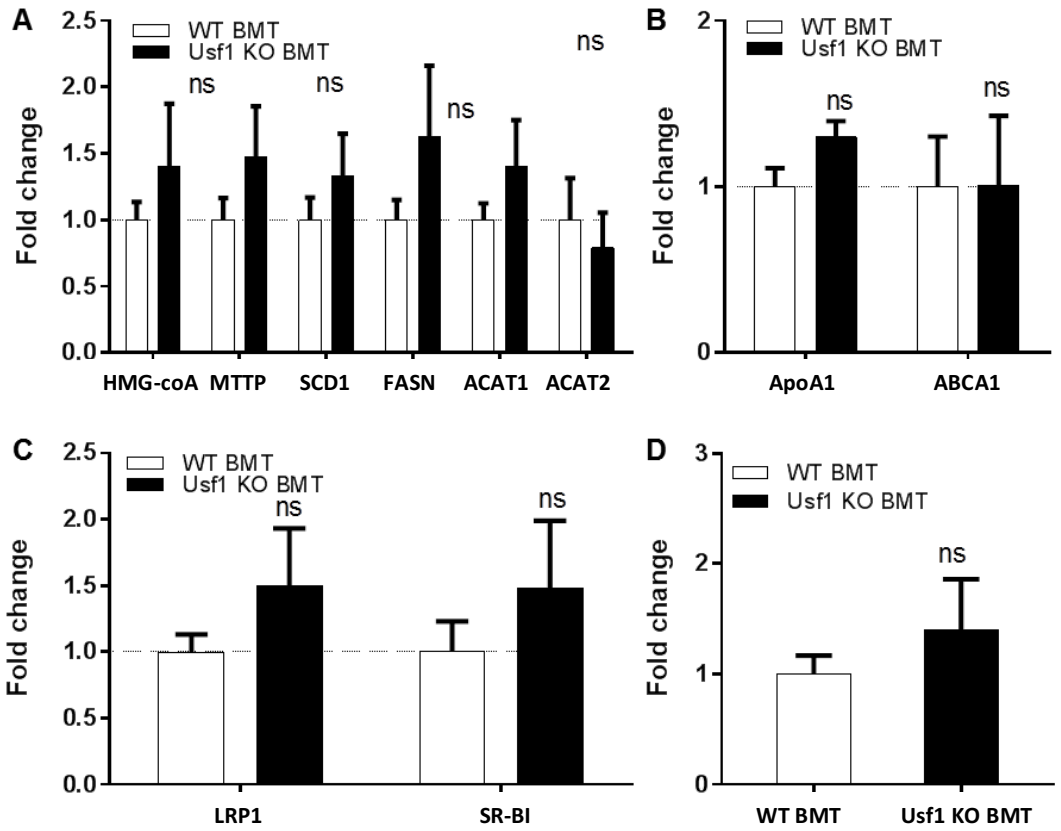
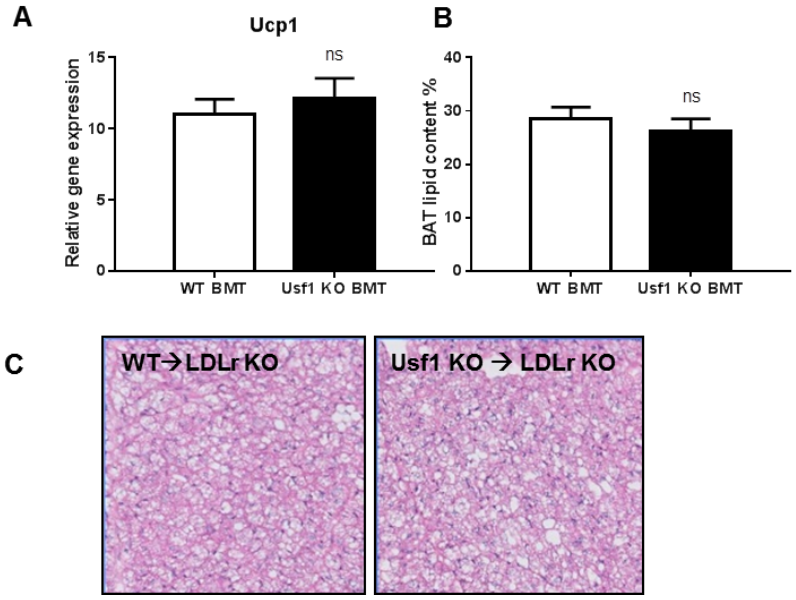
Supplementary material



Supplementary Figure 1 Hepatic lipid metabolism was not affected in LDLr KO mice lacking Usf1 in bone marrow-derived cells.

After 20 weeks WTD feeding, livers from LDLr KO mice reconstituted WT bone marrow or Usf1 KO bone marrow were collected for mRNA expression analysis. A) Relative mRNA expression of genes involved in VLDL metabolism, B) genes involved in HDL metabolism, C) genes involved in cholesterol clearance, and D) hepatic *Usf1* were analyzed. Open bars represent livers from mice reconstituted with WT bone marrow (n=8), closed bars represents mice transplanted with Usf1 KO bone marrow (n=8).



Supplementary Figure 2 Brown adipose tissue (BAT) morphology in LDLr KO mice reconstituted with either WT or Usf1 KO bone marrow after 20 weeks Western-type diet feeding.

A) mRNA expression of *UCP1*, B) lipid content in BAT was analyzed (n=9-16); ns indicates non-significant difference. C) Representative histology photographs of hematoxylin-stained paraffin sections of intercapular adipose tissue (original magnification 10×).

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6

Summary and perspectives

Table of contents

English summary

6.1.1 Introduction

Cardiovascular disease (CVD) is the number one cause of death in the Western world. In 2012, the World Health Organization reported that more than 30% of total deaths was caused by CVD, which often results from atherosclerosis.[1] Atherosclerosis is a blood vessel narrowing and hardening disease characterized by deposition of cholesterol locally in the arterial wall and leading to a low-grade chronic inflammation.[2] Strikingly, based on 2011-2014 data, 11% of United States' adults have total serum cholesterol levels that exceed the threshold of "high blood cholesterol" (>240 mg/dL) for increased CVD risk.[3] Current therapeutic strategies to prevent atherosclerosis are mainly aimed at lowering serum low-density lipoprotein (LDL) cholesterol, e.g. by using statins to inhibit *de novo* cholesterol synthesis.[4] Although an approximate 25% to 35% death rate reduction is achieved by the use of cholesterol lowering drugs in the last several tens of years,[5-7] the remaining high incidence of CVD indicates a clear necessity for exploring alternative therapeutic targets for reducing the residual risk of atherosclerosis.

6.1.2 Therapeutic targeting of M2 macrophages for atherosclerosis treatment

The development of atherosclerosis is a complex multi-factorial process.[8,9] Deposition of modified lipoproteins, rich in cholesterol, in the arterial wall causes monocytes to be attracted to the intimal area, where they differentiate into macrophages that ingest the modified lipoproteins. This process, leading to the formation of macrophage foam cells, hallmarks the development of atherosclerosis. Thus, being the major cell type of the early atherosclerotic lesion, macrophages significantly contribute to the lesional microenvironment and strongly influence atherosclerosis progression.

Increasing evidence indicates that the heterogeneity of macrophages plays an important role in shaping the size, composition, and clinical consequences of atherosclerotic lesions. Two major types of macrophages can be distinguished in lesions: pro-inflammatory M1 macrophages and anti- or less inflammatory M2 macrophages.[10] The distribution of the different types of macrophages in the lesion are both regional and stage-specific. In the early stage of atherosclerosis, the lesion is mainly comprised of M2 macrophages, whereas in the advanced stage, M1 macrophages represent the leading macrophage phenotype.[11] Furthermore, M1 macrophages dominate the rupture-prone shoulder regions of the atherosclerotic lesion,[10] whereas M2 macrophages are more often found in stable locations like peripheral regions in asymptomatic plaques.[12] These findings imply that the macrophage phenotype distribution is an important determining factor in the development of atherosclerosis.

M1 macrophages express high levels iNOS which facilitates the production of NO. High levels of NO produced by lesional macrophages, thus indicating an enhanced production of oxidative species, were shown to be pro-atherogenic.[13-15] Moreover, M1 macrophages produce many pro-inflammatory cytokines, which aggravate atherosclerosis progression. Besides modulating the inflammatory response, M1 macrophages are also negatively correlated with cap thickness of the lesion,[16] an important determinant of the susceptibility to plaque rupture. Plaque rupture in advanced atherosclerosis can lead to thrombus formation and the blockade of the arterial lumen, which represent the underlying cause of acute cardiovascular symptoms such as myocardial infarction and stroke. In contrast, M2 macrophages have long been regarded to be atheroprotective.[17] M2 macrophages produce high levels of anti-inflammatory cytokines such as

IL-10 and TGF-β, which inhibit atherosclerosis development.[18,19] Furthermore, M2 macrophages express high levels of Arg1, a major player in L-arginine metabolism. By regulating the synthesis of proline, the major precursor of collagen, M2 macrophages can contribute to plaque stability by increasing collagen production.[20] In addition, Arg1 reduces NO production by competing with iNOS for the common substrate L-arginine. Therefore, M2 macrophages protect against atherosclerosis, by inhibiting lesion progression and by increasing plaque stability. Collectively, inducing the repolarization of M1 to M2 macrophage thus seems a promising therapeutic strategy for the treatment of atherosclerosis.

We therefore first studied the role of the M2 marker Arg1 in atherosclerosis development. Subsequently the importance of Akt2 and MKP2, kinases that both have been implied in M2 macrophage polarization, in atherosclerotic lesion development was investigated. Evidence is accumulating showing that identification of disease-associated gene mutations is a successful strategy for the identification of novel drug targets. Therefore, finally, the contribution of hematopoietic Usf1, a gene associated with familial combined hyperlipidaemia, to atherosclerotic lesion formation was characterized.

6.1.3 The importance of macrophage phenotype markers in atherogenesis

Arg1 and iNOS, classic macrophage phenotype marker genes, share the same substrate L-arginine and hence negatively regulate each other's activity.[21] The role of iNOS which facilitates the production of NO, a potent signaling molecule, in atherosclerosis, has been well-studied.[17,18, 22-27] However, about the role of Arg1 in atherosclerotic lesion development still little is known. Hence, in **Chapter 2**, we aimed to unravel the role of macrophage Arg1 in atherosclerosis. LDLr KO mice received a transplantation of Arg1 KO or wild-type bone marrow, in order to specifically investigate the role of hematopoietic Arg1. We found that, deletion of Arg1 in macrophages induces a more M2-like phenotype *in vitro*. M2 macrophages have previously been reported to be more prone to foam cell formation.[28] In line, loss of Arg1 in macrophages led to an increased susceptibility to foam cell formation in response to acetylated LDL (acLDL) loading *in vitro*. In agreement with these findings, bone marrow Arg1 deficiency also resulted in enhanced foam cell formation *in vivo,* in absence of effects on circulating cholesterol levels in LDLr KO recipients fed a Western-type diet. However, the enhanced macrophage foam cell formation did not alter the susceptibility to atherosclerotic lesion development, as similar lesion sizes were found in LDLr KO recipients that received either Arg1 KO or WT bone marrow. Furthermore, the lesional collagen content was also not affected by macrophage-specific Arg1 loss. This might be explained by the fact that smooth muscle cells, the main producers of lesional collagen, are not affected by the bone marrow transplantation procedure. To further clarify the compensatory mechanisms that might counteract the enhanced foam cell formation, we investigated the leukocyte profile in the circulation and spleen of the Arg1 KO bone marrow transplanted mice. Deletion of Arg1 in bone marrow led to a decrease in B cells. B cells can protect against atherosclerosis by mediating the production of antibodies against oxLDL.[29] However, despite the observed reduction in B cells, oxLDL-specific antibodies were increased in LDLr KO mice transplanted with Arg1 KO bone marrow. Deletion of Arg1 in bone marrow-derived cells also led to significantly lower amounts of $CD11b^{+}$ cells, $CD11b^{+}/ly6C^{low+med}$ monocytes and neutrophils in the spleen of LDLr KO mice and a tendency towards decreased in pro-inflammatory $CD11b^{+}/ly6C^{high}$ cells. Considering that 30% of the total number of monocytes in atherosclerotic lesions are derived from the spleen,[30] this might provide an atheroprotective mechanism, counteracting the increased foam cell formation in the LDLr KO mice transplanted with Arg1 KO bone marrow. In conclusion, Arg1 in bone marrow-derived cells

affects foam cell formation and circulating B cells, but does not affect atherosclerosis development in LDLr KO mice, suggesting that hematopoietic Arg1 is unlikely a therapeutic target for atherosclerosis treatment.

Repolarization of pro-inflammatory M1 macrophages to less inflammatory M2 macrophages is considered a promising strategy to reduce atherosclerosis. However, simply changing one of the markers used to discriminate between the M1 and M2 macrophage phenotype, namely Arg1, is too simplistic and one should merely consider a more holistic approach to metabolically and immunologically reprogram macrophages.

Macrophages represent a continuous spectrum of cells with different markers. The current M1/M2 classification of the macrophage system is not sufficient for illustrating the full spectrum of macrophage functions and activation pathways. *In vitro*, macrophages can be skewed to an M1 phenotype by incubation with LPS/IFN-γ and to an M2 phenotype by IL-4/IL-13. *In vivo,* however within the atherosclerotic lesion, however, a wide variety of factors are present that may not induce such a clear-cut M1 or M2 phenotype as *in vitro*.[31,32] This indeed results in a bunch of macrophage phenotypes that lack or express the classic M1/M2 macrophage phenotype markers only to a limited extend.[33] This largely limits the potential value of known macrophage phenotype marker genes as diagnostic indicators and therapeutic targets.

M1 and M2 macrophages use different signaling pathways to fuel their effector functions.[34] For a more holistic approach to influence macrophage metabolic and inflammatory functions, targeting microRNAs, have been suggested as a successful strategy, as they are important posttranscriptional fine tuners of many biological and metabolic programs, have been suggested as a successful strategy. Notably, recently is was shown that inhibition of microRNA-33 skews macrophages to an M2 phenotype and reduces atherosclerosis.[34] Interestingly, this apparently works via the protein kinase AMPK. Protein kinases are pivotal for stimulus-triggered cellular processes.[35] Except AMPK, macrophage activation is also tightly regulated by the phosphoinositide-3-kinase/protein B (PI3K/Akt) pathway and mitogen-activated protein kinase (MAPK) pathway.[36-38] In the context of this thesis, we studied the role of Akt2, a member of the PI3K/Akt pathway and MKP2, a phosphatase involved in the de-activation of MAPK in atherogenesis.

6.1.4 The importance of protein kinase pathways in macrophage phenotype polarization and atherogenesis

Akt2 is a potent macrophage polarization regulator.[39-41] Macrophage Akt2 deficiency skews macrophages towards an M2 phenotype and protects against diet-induced atherosclerosis in non-diabetic LDLr KO mice.[42,43] In addition to regulating macrophage polarization, Akt2 is also a major regulator of the insulin pathway. Total body Akt2 deficiency leads to impaired glucose tolerance in mice, however, it has little to no effect on atherosclerosis development.[43,44] To better understand the role of macrophage Akt2 in atherosclerosis under glucose intolerant conditions**,** glucose intolerant Akt2/LDLr dKO mice were transplanted with either LDLr KO or Akt2/LDLr dKO bone marrow, thereby enabling investigation of macrophage-specific restoration of Akt2 expression. I**n Chapter 3** data are presented that indicating that macrophage Akt2 does not significantly contribute to the type 2 diabetic phenotype and atherosclerosis development in Akt2/LDLr dKO mice. Previous studies showed that macrophage Akt2 deficiency is associated with a decreased susceptibility to foam cell formation.[43] In agreement, we found that Akt2 restoration increased

foam cell formation. In line with the augmented foam cell formation,[43] LDLr KO macrophages with functional Akt2 signaling exhibited an M2 macrophage phenotype. Interestingly, this finding is in contrast to previous studies showing that Akt2 *deficiency* promotes M2 macrophage polarization.[42,45,46] In this view, it is worth to note that in our study, the effects of Akt2 were studied in LDLr KO macrophages, whereas previous studies investigated Akt2 function in wild-type macrophages. Overall, the results described in chapter 3 indicate that macrophage Akt2 restoration leads to more pronounced foam cell formation, but a less inflammatory M2 macrophage phenotype; ultimately, leading to unaltered atherosclerosis development in the transplanted Akt2/LDLr dKO mice. In addition to the counteracting effects of enhanced foam cell formation and reduced inflammation upon Akt2 restoration, likely also the glucose intolerant background contributes to the observed unchanged atherosclerosis susceptibility of Akt2/LDLr dKO mice transplanted with Akt2 positive/LDLr KO bone marrow. It is worth noting that the macrophages investigated in the current study were LDLr deficient, which is different as compared to the previously published studies.[42,45,46] Macrophage activation is the result of a collaborative interaction of several gene products in response to an extra cellular signaling stimulus.[47] Genetic variation could possibly disturb the cellular signaling pathways, thereby affecting macrophage activation and ultimately lead to a totally different phenotype.[48] Hence, we speculate that the presence of the LDLr on macrophages might have important consequences for M2 macrophage polarization induced by Akt2 deficiency. To confirm this further dedicated research is needed. Our study in this thesis expanded the knowledge of Akt2 in macrophage polarization and atherosclerosis. It underlined that the atherosclerotic role of Akt2 might be influenced by the genetic and metabolic background of the atherosclerosis-prone mouse model used.

MAPK phosphatases (MKP) represent a family of phosphatases that are tightly related to mitogen-activated protein kinases (MAPKs).[49] The MKP family consists of at least ten different members, each MKP family member being able to bind and de-activate their substrate MAPKs by dephosphorylating their phosphoserine/threonine and phosphotyrosine residues.[50,51] The specific functions of MKPs are different for each family member, which is at least partly due to their distinct MAPK substrate preference.[49] Interestingly, MPK2 is involved in macrophage polarization[52], and thus represents a potential target for the treatment of atherosclerosis. **Chapter 4** therefore focuses on the effect of MKP2 on macrophage function and atherosclerosis development. We demonstrated that MKP2 deficiency enhanced the activation of the MAPK member JNK in thioglycollate-elicited macrophages. In addition, macrophages lacking MKP2 express an M2-like phenotype, and in line, display an enhanced susceptibility to foam cell formation in response to oxLDL. Next, MKP2 deficient bone marrow was transplanted into LDLr KO mice. After 9 weeks WTD feeding, the lipoprotein cholesterol distribution profile of the recipient mice was not affected by bone marrow MKP2 loss. In agreement with the M2 macrophage polarization in vitro, peritoneal macrophages from MKP2 KO bone marrow recipients showed a decreased expression of the M1 markers CD86 and MHCII. Furthermore, MKP2 KO bone marrow recipients displayed increased production of the anti-inflammatory cytokine IL-10, whereas the production of the pro-inflammatory cytokine IL-12 was reduced. Strikingly, despite the observed decrease in pro-atherogenic cytokines, an increase in atherosclerotic lesion size was observed after 9 weeks of WTD diet feeding. This result indicated that the anti-inflammatory effect induced by MKP2 deletion in the end was likely overruled by the augmented foam cell susceptibility of the MKP2 knockout macrophages. Importantly, the collagen content in the lesions of the MKP2 bone marrow recipients was lower than in the lesions of the WT bone marrow recipients, indicating that bone marrow MKP2 also leads to an unstable atherosclerosis phenotype. *In vitro*, the MKP2 deficiency-

induced M2 macrophage phenotype had only a limited effect on foam cell formation in absence of oxLDL stimulation. Therefore, it is likely that high levels of circulating cholesterol magnify the effect of MKP2 deficiency on foam cell formation and ultimately overrule the beneficial anti-inflammatory effects in the LDLr KO recipients. From this view, it would be very interesting to expand the current study and investigate the role of MKP2 in atherosclerosis development under cholesterol-lowering conditions.

The protein kinase superfamily members and the proteins regulating their activity are the most intensively studied protein drug targets in current pharmacological research. Based on a 2014 report, there were more than 3000 approved and experimental kinase-based drugs in active clinical trials.[53] The majority of the trials with kinase-targeting agents focus on the treatment of cancer, but the field is now expanding to the treatment of inflammatory diseases.[53] Emerging evidence indicates that modulation of the kinase pathway could effectively alter macrophage polarization in a beneficial way.[54-59] Therefore, targeting kinases is considered a potent strategy for the development of novel pharmaceutical therapies for the treatment of atherosclerosis. In this thesis, it is indeed shown that targeting protein kinase activity, i.e. by restoration of kinase AKT2 or disruption of kinase-inactivating MKP2, is an effective way to stimulate macrophage M2 polarization and induce anti-inflammatory effects. In both studies, however, the M2 polarization also led to augmented foam cell formation and eventually had either no effect on atherosclerotic lesion development or even led to larger lesions.

6.1.5 The importance of upstream stimulatory factor 1 in atherogenesis

The hallmark of atherosclerosis is foam cell formation, a pathological process for which both macrophages and lipids are indispensable. In the first part of this thesis, we focused on genes involved in M2 macrophage polarization. In the second part of thesis, we focused on Usf1, a gene involved in lipid metabolism. Patients suffering from familial combined hyperlipidemia (FCHL) display a high risk to develop premature coronary artery disease.[60,61] Usf1 is a gene associated with familial combined hyperlipidemia (FCHL), characterized by increased LDL and triglycerides concentrations, an effect often accompanied by decreased HDL levels.[60] A previous study suggested an important role of total body Usf1 in lipid metabolism and atherogenesis.[62] In **chapter 5,** we investigated the role of hematopoietic Usf1 in atherosclerosis. In this study, we found that bone marrow restricted Usf1 deficiency induced a different phenotype in LDLr KO mice as compared to total body Usf1 deficiency. For instance, total body Usf1 deletion led to a beneficial cardiometabolic lipid profile, marked by decreased athero-promoting VLDL cholesterol and triglyceride levels, and increased levels of athero-protective HDL cholesterol. In contrast, bone marrow Usf1 deletion led to elevated VLDL cholesterol levels and increased susceptibility to atherosclerosis. This effect can likely be attributed to an impaired clearance of VLDL by white adipose tissue. Overall, both literature and our study demonstrated that Usf1 alteration significantly affect atherosclerosis development.[62] Interestingly, in contrast to the pro-atherogenic role of total body Usf1,[62] hematopoietic-specific Usf1 is athero-protective. Our study thus highlighted a tissue specific role of Usf1 in atherosclerosis development. This should be taken into account when developing novel pharmaceutics aimed at inhibiting Usf1 activity or downregulating its expression to combat atherosclerosis.

6.1.6 Concluding remarks and future perspectives

Using knockout mice and the bone marrow transplantation tool, we evaluated the effects of genetic modulation involved in macrophage polarization and lipid metabolism on the pathogenesis of atherosclerosis. First a classic M2 marker gene Arg1 was studied (**Chapter 2**), followed by key regulators of M2 macrophage activation, Akt2 (**Chapter 3**) and MKP2 (**Chapter 4**). In the end we discussed the role of Usf1 (**Chapter 5**), a gene associated with familial combined hyperlipidaemia associated gene, in atherosclerosis. Besides these selected genes, many other molecules, that are possibly also involved in macrophage activation and lipid metabolism, are still open for evaluation.

Macrophages are essential for the maintenance of tissue homeostatic functions by regulating gene expression in response to the local tissue environment. The plasticity of macrophage phenotypes is highly manipulable, which is interesting as the different types of macrophage phenotypes contribute to atherogenesis in a distinct way. These features provide a possibility to develop cell- or gene-based therapies for treating atherosclerosis by modifying macrophage phenotypes. The most successful therapy targeting macrophage function to date, is pulmonary macrophage transplantation to treat hereditary pulmonary alveolar proteinosis.[63,64] The idea of therapeutic targeting of macrophage phenotype was developed first for the treatment of tumors.[65,66] However, all clinical trials failed until now,[67-74] which is largely due to the rapid loss of the anti-tumor M1 phenotype when the pre-educated macrophages are re-exposed to the tumor microenvironment.[75-77] Therefore, establishing how to "lock" the macrophage polarization phenotype *in vivo,* and consequently modulate the microenvironment, rather than be exposed to modulation by the local microenvironment is the key point for improving the efficiency of macrophage-based therapies.

Targeting macrophage genes at the molecular level to modify macrophage polarization as described in this thesis, is an alternative. By modifying the expression of genes involved in macrophage polarization, the phenotype can be favorably altered and may lock polarization of the macrophage into a specific phenotype. For example, MKP2 loss skews macrophages from an M1 to a more M2-like phenotype. Furthermore, after bone marrow transplantation, LDLr KO mice transplanted with MKP2 deficient bone marrow showed an anti-inflammatory plasma cytokine profile. This finding suggests that genetic modulation of the macrophage phenotype and "locking" of the favorable phenotype is feasible in mice. Therapeutic strategies aimed at altering protein kinase activity, as shown in this thesis, are an effective way to stimulate macrophage M2 polarization and induce anti-inflammatory effects.

Another interesting strategy is to interfere in epigenetic regulation of monocyte/macrophage gene expression. Epigenetic modifications, such as DNA methylation, histone modification and regulation via non-coding RNAs, alter DNA accessibility and chromatin structure, thereby regulating the patterns of gene expression.[78-83] Emerging literature suggests an important role for epigenetic regulation in macrophage polarization and the development of atherosclerosis.[84-88] For instance, Jmjd3, a histone 3 Lys27 (H3K27) demethylase, facilitates expression of IRF4, a key M2-promoting transcription factor, and regulates the M2 macrophage polarization without affecting M1 macrophages.[89,90] Deleting histone deacetylase 3 (Hdac3) skews macrophage to the M2 phenotype, improves lipid handling and increases atherosclerosis plaque stability.[91] Moreover, treatment with 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation, decreases macrophage inflammation and reduces atherosclerosis development in LDLr KO mice.[92,93] Besides, the

epigenetic changes seem also transplantable. Van Kampen and colleagues found that bone marrow from mice exposed to long-term Western-type diet challenge shows hypomethylation of CpG regions in the genes encoding Pu.1 and IRF8 and that transplantation of these bone marrow cells to LDLr KO mice on a low-fat, no cholesterol chow diet leads to increased monocyte/macrophage proliferation and differentiation, and aortic root atherosclerosis plaque formation.[94] Further research to increase the understanding of epigenetic changes in macrophages during atherosclerotic lesion development and the epigenetic pathways controlling their inflammatory repertoire would greatly help the future development of novel therapies in the combat against atherosclerosis.[78]

From an inflammation point of view, the anti-inflammatory M2 macrophage phenotype is beneficial for slowing down the progression of atherosclerosis. However, M2 macrophages accelerate foam cell formation which largely reduces the therapeutic value of M2 macrophages under high cholesterol conditions. The enhanced foam cell formation results in pro-atherogenic effects and may counteract the anti-inflammatory atheroprotective effects of the M2 macrophage (**Chapter 3 and 4**). Therefore, it is important that novel therapeutic strategies aimed at skewing macrophages to an anti-inflammatory M2 phenotype are combined with plasma cholesterol lowering drugs.

Massive lowering of plasma cholesterol is a valuable strategy to induce regression of atherosclerotic lesions in mouse models of atherosclerosis. Interestingly, in regressing plaques a consistent increase in M2 over M1 macrophages is observed.[95-97] A very recent study indicated that influx of macrophages is important to induce lesion regression, likely to remove debris from regressing plaques.[98] Upon transplantation of apoE KO aorta's with plaques to a CCR2 KO mouse, no recruitment of new macrophages was seen into the existing lesions was seen and no regression of atherosclerosis could be induced. CCR2 is a chemokine receptor crucial for monocyte recruitment to atherosclerotic lesions and a marker of classical, pro-inflammatory monocytes.[99] It has been speculated that the classical $Ly6C^{high}/CCR2^{+}$ monocytes are the precursors for M1 macrophages, but this has not been firmly established. Considering that the majority of the patients enter the clinic with established atherosclerotic lesions, it will be important to investigate if induction of M2 macrophages on top of cholesterol lowering, can promote or impede atherosclerosis regression.

As described in this thesis, bone marrow transplantation is an effective way to modify gene expression in macrophages. Bone marrow transplantation is an excellent tool to uncover if, and via which mechanisms, a gene of interest influences atherosclerosis development, and to provide essential information on macrophage gene candidates as potential pharmacological targets. However, with respect to clinical application, bone marrow transplantation requires myeloablation, and hence is not suitable as a direct therapy for atherosclerosis treatment not suitable. To modulate macrophage gene expression, several strategies can be pursued. For instance, small interfering RNA (siRNA), short hairpin RNA (shRNA), and microRNA (miRNA) can be used for gene-silencing,[100-104] while lentiviral vectors and plasmid vectors are suitable for overexpression of a gene of interest.[105,106] Besides using genetic engineering methods to directly target gene expression, pharmacological inhibitors and agonist/antagonists of a gene of interest can also be specifically delivered to macrophages using macrophage-receptor-based or ligand-anchored micro-/nano-carriers.[107-109] In fact, nanoparticles loaded with pioglitazone, a potent activator of PPAR-γ, significantly skew monocyte/macrophage to an anti-inflammatory phenotype in ApoE KO mice.[110] Furthermore, nanoparticles containing contrast agents used in computed

tomography (CT) and magnetic resonance imaging (MRI), are also a potent tool to image and characterize the plaque in vivo, providing basis for the further clinical treatment strategies.[109] Overall, these strategies offer possibilities to specifically target genes and signaling pathways that skew macrophage functions and might prove interesting in further future clinical treatment.

Macrophage heterogeneity is not only pivotal for atherosclerosis, but also for various other diseases with extensive inflammation components, such as cancer, Parkinson's disease, obesity, diabetes, and arthritis.[111-117] Therefore, targeting macrophages would not only provide possibilities for the treatment of cardiovascular disease, but can also be beneficial for multiple other inflammation-based pathologies.

In conclusion, modulation of macrophage polarization combined with cholesterol lowering agents, might provide a promising strategy to treat atherosclerosis. It would be interesting to evaluate whether selective targeting of macrophage polarization is indeed an effective approach for the treatment of atherosclerosis and for the reduction of cardiovascular risk.

6.2 Nederlandse samenvatting

6.2.1 Inleiding

Hart- en vaatziekten (HVZ) is de nummer één doodsoorzaak in de Westerse wereld. In 2012 rapporteerde de internationale *World Health Organization* that meer dan 30% van het totaal aantal overlijdens in de wereld waren veroorzaakt door HVZ, dat vaak het gevolg is van slagaderverkalking ofwel atherosclerose.[1] Atherosclerose, een aandoening die leidt tot vernauwing en verstijving van bloedvaten, wordt gekarakteriseerd door een afzetting van cholesterol lokaal in de vaatwand met een chronische ontsteking van de bloedvatwand als gevolg.[2] Een belangrijke oorzaak voor deze cholesterolafzettingen is onder andere een hoog totaal cholesterolniveau in het bloed. In dit kader is het belangrijk te vermelden dat gegevens uit de jaren 2011-2014 laten zien dat een opmerkelijke hoeveelheid van 11% van de Amerikaanse volwassenen een totaal serum-cholesterolniveau heeft dat de drempelwaarde voor de definitie van 'hoog cholesterol' (>240 mg/dL) als risicofactor voor HVZ overschrijdt.[3] De huidige therapieën voor atherosclerose zijn er vooral op gericht om de hoge cholesterol niveaus getransporteerd door het zogeheten lage-dichtheidslipoproteïne (LDL) in het bloed te verlagen, bijvoorbeeld door het gebruik van statines om *de novo* cholesterolsynthese te remmen.[4] In de afgelopen decennia heeft men dankzij deze cholesterolverlagende therapieën een afname van het risico op HVZ van ongeveer 25-35% kunnen bewerkstellingen.[5-7] Echter, de resterende hoge incidentie van HVZ geeft aan dat er een duidelijke noodzaak is voor alternatieve therapeutische doelwitten teneinde het restrisico van atherosclerose te verlagen.

6.2.2 M2 macrofagen als therapeutisch doelwit voor de behandeling van atherosclerose

De ontwikkeling van atherosclerose is een complex en multifactorieel proces.[8,9] De afzetting van gemodificeerde cholesterolrijke lipoproteïnen in de intima van de arteriële vaatwand zorgt ervoor dat monocyten naar dit gebied van de vaatwand worden getrokken, waar zij differentiëren in macrofagen die de gemodificeerde lipoproteïnen opnemen. Dit proces, dat resulteert in de vorming van zogeheten macrofaag-schuimcellen, is het belangrijkste kenmerk van atherosclerose ontwikkeling. Omdat macrofagen het belangrijkste celtype vertegenwoordigen dat aanwezig is in de vroege atherosclerotische plaque, leveren zij een significante bijdrage aan het micromilieu in de plaque en hebben zij een sterke invloed op de vordering van het atherosclerotisch ziekteproces.

In toenemende mate wordt bewezen dat de heterogeniteit van macrofagen een belangrijke rol speelt in het bepalen van de grootte, compositie en klinische consequenties van atherosclerotische plaques. In deze plaques kunnen twee macrofaag-subtypen gedefinieerd worden: pro-inflammatoire M1 macrofagen en anti-, of minder, inflammatoire M2 macrofagen.[10] De verdeling van de verschillende type macrofagen in de plaque is zowel regio- als fasespecifiek. In de vroege fase van atherosclerose atherosclerose ontwikkeling bestaat de plaque vooral uit M2 macrofagen, terwijl in de meer gevorderde fase juist M1 macrofagen het voornaamste fenotype vertegenwoordigen.[11] Daarnaast zijn M1 macrofagen dominant aanwezig in de schouderregio's van de atherosclerotische plaque, een deel van de plaque dat erg gevoelig is voor een ruptuur. M2 macrofagen worden in aan de andere kant juist vaker aangetroffen in locaties waar de plaque meer stabiel is, zoals de perifere regio's in asymptomatische plaques.[12] Deze bevindingen impliceren dat de verdeling van het macrofaagfenotype een belangrijke bepalende factor is voor het ontwikkelen van atherosclerose.

M1 macrofagen brengen grote hoeveelheden iNOS tot expressie, een enzym dat de productie van NO faciliteert. Eerdere studies hebben laten zien dat het atherosclerotisch ziekte proces wordt gestimuleerd door juist die macrofagen in de atherosclerotische plaque met een hoge NO-productie.[13-15] M1 macrofagen produceren daarnaast veel pro-inflammatoire cytokinen, die de ontwikkeling van atherosclerose versnellen. Naast de modulatie van de ontstekingsreactie zijn de hoeveelheden M1 macrofagen in de plaque ook negatief gecorreleerd met de dikte van het kapsel dat de plaque afdekt.[16] Een afname in de dikte van dit kapsel is een belangrijke risicofactor voor het scheuren van de plaque, een complicatie waardoor er een bloedprop kan ontstaan die het bloedvat lokaal of verderop in de bloedstroom kan afsluiten en daarmee acute cardiovasculaire symptomen zoals een hart- of herseninfarct kan veroorzaken. In tegenstelling tot de M1 macrofagen, worden M2 macrofagen juist veelal beschouwd als beschermend ten aanzien van atherosclerose ontwikkeling.[17] M2 macrofagen produceren hoge niveaus van anti-inflammatoire cytokinen zoals IL-10 en TGF-β die erom bekend staan de ontwikkeling van atherosclerose te remmen.[18, 19] Daarnaast brengen M2 macrofagen grote hoeveelheden Arg1 tot expressie, een enzym dat een sleutelrol speel in het metabolism van L-arginine. Arg1 kan met iNOS competeren voor hun gedeelde substraat L-arginine. Op deze manier verlaagt de Arg1 expressie in M2 macrofagen de NO productie door deze cellen, waardoor M2 macrofagen de ontwikkeling van atherosclerose kunnen tegengaan. Daarnaast kunnen M2 macrofagen de stabiliteit van een plaque vergroten door de synthese van proline, de belangrijkste voorloper van collageen, te verhogen.[20] M2 macrofagen beschermen dus tegen atherosclerose door enerzijds de groei van de plaque te remmen en anderzijds de stabiliteit van de plaque te verhogen. Op basis van deze bevindingen kan de verschuiving van de macrofaagpolarisatie van een M1 naar een M2 fenotype beschouwd worden als een veelbelovende therapeutische strategie voor de behandeling van atherosclerose.

Om deze hypothese te testen, hebben we eerst onderzocht welke rol de M2 marker Arg1 speelt in de ontwikkeling van atherosclerose. Daarna hebben we de relevantie van Akt2 en MKP2, beide kinases waarvan gesuggereerd wordt dat ze betrokken zijn bij de polarisatie van macrofagen naar het M2 fenotype, in de ontwikkeling van atherosclerose bestudeerd. Tot slot, omdat er in toenemende mate bewijs wordt gevonden dat de identificatie van ziekte-geassocieerde genmutaties een succesvolle strategie is voor het ontdekken van nieuwe therapeutische doelwitten, hebben we onderzocht welke rol USf1, een gen dat geassocieerd wordt met familiare hyperlipidemie, speelt in het ontstaan van atherosclerose.

6.2.3 Het belang van macrofaagfenotype-merkers in de ontwikkeling van atherosclerose

Arg1 en iNOS, klassieke merkers voor respectievelijk het M2 en M1 macrofaagfenotype, delen het gezamenlijke substraat L-arginine en remmen hiermee elkaars activiteit.[21] De rol van iNOS, dat de productie van het potente signaleringsmolecuul NO faciliteert, is reeds uitgebreid bestudeerd.[17,18,22-27] Echter, de rol van Arg1 in de ontwikkeling van atherosclerose is nog onbekend. In **hoofdstuk 2** hebben we daarom getracht om de rol van macrofaag-specifiek Arg1 in de ontwikkeling van atherosclerose te ontrafelen. Hiertoe werd in LDLr KO muizen het eigen beenmerg vervangen door beenmerg van ofwel Arg1 KO of wildtype donoren. Na herstel van de beenmergtransplantatie werden de dieren een Westers dieet gevoed om de ontwikkeling van atherosclerose te stimuleren. Allereerst vonden we dat uitschakeling van Arg1 in macrofagen *in vitro* een meer M2-gelijkend fenotype veroorzaakte. Zo waren Arg1 KO macrofagen meer gevoelig voor het ontwikkelen van schuimcellen na blootstelling aan geacetyleerd LDL (acLDL) *in vitro*. Dit

is in overeenstemming met de literatuur, waarin eerder is laten zien dat M2 macrofagen meer gevoelig zijn voor schuimcelvorming.[28] In overeenstemming met deze *in vitro* bevindingen, leidde beenmerg-specifieke deficiëntie van Arg1 in LDLr KO muizen ook tot een verhoogde schuimcelvorming, zonder dat er een effect op de bloedcholesterolspiegels waargenomen werd. Echter, deze verhoogde schuimcelvorming had geen effect op de gevoeligheid voor atherosclerose ontwikkeling, aangezien de plaquegroottes in de Arg1 KO beenmerggetransplanteerde LDLr KO muizen vergelijkbaar waren aan die van WT beenmerggetransplanteerde LDLr KO muizen. Daarnaast was ook de hoeveelheid collageen in de plaques niet verschillend tussen de twee groepen, wat verklaard zou kunnen worden door het feit dat de gladde spiercellen, de belangrijkste producenten van collageen in de atherosclerotische plaque, niet aangedaan worden door de beenmergtransplantatieprocedure. Desalniettemin is er, ondanks de verhoogde schuimcelvorming als gevolg van beenmergspecifieke Arg1-deletie in LDLr KO muizen, geen sprake van een verhoogde atherosclerose ontwikkeling. Dit duidt op de mogelijkheid dat er een compenserend mechanisme in werking is getreden dat de effecten van de verhoogde schuimcelvorming tegengaat. Om deze mechanismen te kunnen verklaren hebben we de leukocytprofielen in zowel het bloed als in de milt van de muizen onderzocht. Hieruit bleek dat beenmergspecifieke Arg1-deletie leidde tot een significante verlaging van het aantal monocyten en neutrofiele granulocyten in de milt. Aangezien 30% van het totaal aantal monocyten in de atherosclerotische plaque vanuit de milt komen,[30] kan deze gevonden verlaging van het aantal monocyten in de milt mogelijk een verklaring vormen voor het feit dat de ontwikkeling van atherosclerose niet is verhoogd in LDLr KO muizen met Arg1 deficiënt beenmerg ondanks de verhoogde schuimcelvorming. Samenvattend beïnvloedt Arg1 in beenmergafgeleide cellen de schuimcelvorming door macrofagen, maar heeft het geen effect op de ontwikkeling van atherosclerose in LDLr KO muizen. Arg1 in beenmergafgeleide cellen is daarom waarschijnlijk geen goed therapeutisch doelwit voor de behandeling van atherosclerose.

De repolarisatie van pro-inflammatoire M1 macrofagen naar minder inflammatoire M2 macrofagen wordt beschouwd als een veelbelovende strategie om atherosclerose te verminderen. Echter, het simpelweg veranderen van een van de specifieke M1-markergenen, zoals verlaging van Arg1, is een te simplistische benadering voor deze strategie. Men zal daarom op een meer holistische wijze het metabolisme van en het afweermechanisme in macrofagen moeten herprogrammeren om daadwerkelijk een effect op atherosclerose te kunnen bewerkstelligen.

Macrofagen vertegenwoordigen een groot spectrum van cellen die elk gekenmerkt worden door verschillende merkers. De huidige M1/M2 classificatie van macrofagen is onvoldoende om het volledige spectrum van macrofaagfunctie en –activering weer te kunnen geven. Macrofagen kunnen *in vitro* door incubatie met LPS/IFN-γ gestimuleerd worden tot verandering in macrofagen met een M1 fenotype, terwijl incubatie met IL-4/IL-13 er juist voor zorgt dat macrofagen verandering in cellen met een M2 fenotype. In de atherosclerotische plaque *in vivo* is er echter een grote variëteit aan cytokinen en andere signaleringsmoleculen aanwezig, waardoor de aanwezige macrofagen mogelijk ook minder duidelijke fenotypen kunnen aannemen.[31,32] In atherosclerotische plaques worden inderdaad veel macrofaagfenotypen gevonden waarin de expressie van de klassieke M1/M2 markers slechts beperkt of zelfs geheel niet aanwezig is.[33] Dit zorgt ervoor dat het gebruik van macrofaagfenotype-merkergenen als diagnostisch hulpmiddel of therapeutische doelwit voor de behandeling van atherosclerose lastig is.

M1 en M2 macrofagen maken gebruik van verschillende signaleringspaden om hun effectorfunctie tot uiting te kunnen brengen.[34] Om op een meer holistische wijze het metabolisme van en het

afweermechanisme in macrofagen te reprogrammeren, kan het lonen om de strategie te richten op microRNA's aangezien deze veel biologische en metabole programma's op posttranscriptioneel niveau reguleren. In dit opzicht is het belangrijk om te melden dat recent is aangetoond dat remming van microRNA-33 macrofagen naar een M2 fenotype kan drijven en atherosclerose kan verminderen.[34] Dit proces lijkt gemedieerd te worden door de eiwitkinase AMPK. Eiwitkinases zijn essentieel voor stimulus-geïnduceerde cellulaire processen.[35] Macrofaagactivatie wordt, naast de AMPK-signaleringsroute, ook gereguleerd door de fosfoinositide-3-kinase/eiwit B (KI3K/Akt) en mitogen-geactiveerde eiwitkinase (MAPK) signaleringsroutes.[36-38] Binnen de context van dit proefschrift hebben wij zowel de rol van Akt2 als lid van de KI3K/Akt-route, als de rol van het fosfatase MKP2 die de MAPK-route deactiveert, in de ontwikkeling van atherosclerose bestudeerd. Deze signaleringsroutes worden hieronder in meer detail beschreven.

6.2.4 Het belang van eiwitkinase-signaleringsroutes in macrofaagpolarisatie en atherosclerose ontwikkeling

Akt1 is een sterke regulator van macrofaagpolarisatie.[39-41] Akt2-deficiëntie in macrofagen leidt tot een sterker M2 macrofaag fenotype en beschermd tegen dieet-geïnduceerde atherosclerose in niet-diabetische LDLr KO muizen.[42, 43] Naast het effect op macrofaagpolarisatie is Akt2 ook een belangrijke speler in de signaleringsroute van insuline. Wanneer Akt2 in het gehele lichaam afwezig is, resulteert dit in een verlaagde glucosetolerantie in muizen terwijl er weinig tot geen effect zichtbaar was op de ontwikkeling van atherosclerose.[43, 44] Om de rol van macrofaag-specifiek Akt2 in atherosclerose onder glucoseintolerante condities beter te begrijpen, hebben wij in **hoofdstuk 3** glucoseintolerante Akt2/LDLr dKO muizen getransplanteerd met LDLr KO of Akt2/LDLr dKO beenmerg, om zodoende het effect van macrofaag-specifiek herstel van Akt2-expressie te kunnen bestuderen. De resultaten laten zien dat Akt2 in macrofagen geen significante bijdrage levert aan het type 2 diabetes fenotype, noch aan de ontwikkeling van atherosclerose in Akt2/LDLr dKO muizen. Eerdere studies hebben aangetoond dat macrofaag-specifieke Akt2-deficiëntie geassocieerd is met een verlaagde vatbaarheid voor macrofaag-schuimcelvorming.[43] Onze studie ondersteunt deze bevindingen, omdat in onze opzet het herstel van Akt2 expressie in macrofagen de schuimcelvorming van deze cellen juist verhoogde. In tegenstelling tot de eerdere bevindingen dat Akt2-deficientie leidt tot een M2 macrofaag fenotype, laten onze studies in de LDLr KO achtergrond zien dat Akt2 expressie in macrofagen leidde tot een M2 macrofaag fenotype. Samengevat laten wij in hoofdstuk 3 zien dat het herstel van Akt2 specifiek in macrofagen resulteerde in een sterke schuimcelvorming, maar het M2 macrofaag fenotype versterkt waardoor naar verwachting de macrofagen minder inflammatoir zijn. Gezamenlijk leidde dit uiteindelijk tot een onveranderde atherosclerose ontwikkeling in de getransplanteerde Akt2/LDLr dKO muizen. Naast de tegenhangende effecten van een verhoogde schuimcelvorming en verlaagde inflammatoire repons na Akt2-herstel, speelt de glucoseintolerante achtergrond van de muizen waarschijnlijk ook een rol in de onveranderde gevoeligheid voor de ontwikkeling atherosclerose in onze muizen. In dit kader is het belangrijk om te vermelden dat de macrofagen die wij in ons onderzoek hebben bestudeerd een LDLr-deficiënte achtergrond hadden, terwijl eerdere studies vaak op een wildtype achtergrond zijn uitgevoerd.[42, 45, 46] Macrofaagactivering is het gevolg van een samenwerkende interactie tussen verschillende genproducten, in reactie op een extracellulaire signaleringsstimulus.[47] Genetische variatie, zoals een LDLr KO *versus* wildtype achtergrond, kan mogelijk de cellulaire signaleringsroutes beïnvloeden. De activatie van macrofagen in verschillende genetische achtergronden zou daarmee uiteindelijk kunnen leiden tot heel verschillende effceten op macrofaagfenotype en functie.[48] We speculeren daarom dat de aanwezigheid van de LDLr op macrofagen mogelijk belangrijke consequenties heeft voor de

polarisatie van macrofagen naar een M2 fenotype onder Akt2-deficiënte omstandigheden. Om deze hypothese te kunnen bevestigen is echter verder onderzoek nodig. Desalniettemin heeft onze studie, zoals beschreven in hoofdstuk 3 van dit proefschrift, de kennis over de rol van Akt2 in macrofaagpolarisatie en atherosclerose vergroot, en onderstreept deze de notie dat de genetische en metabole achtergrond van het muismodel dat wordt gebruikt de rol van Akt2 in atherosclerose ontwikkeling kan beïnvloeden.

MAPK fosfatases (MKP's) vertegenwoordigen een familie van minstens tien verschillende fosfatases die elk sterk gerelateerd zijn aan de MAPK's.[49] MKP's hebben elk een verschillende voorkeur voor specifieke MAPK's, die ze kunnen deactiveren via binding en daaropvolgende defosforylatie van de fosfoserine/treonine- en fosfotyrosine residuen.[50,51] Elk MKP-familielid heeft een andere functie, wat deels veroorzaakt wordt door diens specifieke voorkeur voor het MAPK-substraat.[49] In dit kader is het interessant om te melden dat MKP2 betrokken is bij macrofaagpolarisatie[52], en daarmee dus een potentieel therapeutisch doelwit is voor de behandeling van atherosclerose. **Hoofdstuk 4** richt zich daarom op het effect van MKP2 op macrofaagfunctie en atherosclerose ontwikkeling. We hebben laten zien dat MKP2-deficiëntie de activatie van het MAPK-familielid JNK verhoogt in thioglycollaat-opgewekte macrofagen. Daarnaast laten we zien dat macrofagen die geen MKP2 tot expressie brengen een M2-macrofaagfenotype laten zien en, in lijn daarmee, meer vatbaar zijn voor schuimcelvorming na blootstelling aan geoxideerd LDL. Om de rol van MKP2 in atherosclerose ontwikkeling verder te ontrafelen, hebben we MKP2-deficiënt beenmerg getransplanteerd naar LDLr KO muizen. Deze muizen lieten, na 9 weken een Westers dieet gevoerd te zijn, geen verandering zien in het distributieprofiel van cholesterol over de lipoproteïne-fracties in het bloed. In lijn met de *in vitro* aangetoonde versterkte M2-macrofaagpolarisatie, observeerden we minder M1 markers op peritoneale cellen in de macrofaag-specifieke MKP2-deficiënte muizen, en zagen we dat deze muizen meer anti-inflammatoire en juist minder pro-inflammatoire cytokinen produceerden. Echter, ondanks de verlaging in pro-atherogene cytokineproductie, zagen we dat transplantatie met MKP2-deficiënt beenmerg leidde tot een verhoogde atherosclerose ontwikkeling na 9 weken van Westerse dieetvoeding. Belangrijk om te vermelden is dat de hoeveelheid collageen in de plaques van dieren die met MKP2-deficiënt beenmerg getransplanteerd waren lager was dan in de plaques van de controledieren. Samengevat laten deze resultaten zien dat de anti-inflammatoire effecten van MKP2-deficiëntie uiteindelijk tenietgedaan werden door de verhoogde vatbaarheid voor schuimcelvorming van de MKP2-deficiënte macrofagen. Het is denkbaar dat de hoge cholesterolniveaus in het bloed van de macrofaagspecifieke MKP2-deficiënte LDLr KO muizen op een Westers dieet het effect van MKP2-deficiëntie op schuimcelvorming dusdanig vergroot heeft, dat de gunstige anti-inflammatoire effecten van MKP2 overstemd werden. In dit kader zou het interessant zijn om de huidige studie verder uit te breiden en de rol van MKP2 in atherosclerose ontwikkeling te bestuderen onder condities waarin de cholesterol-bloedspiegels actief verlaagd worden.

De eiwitkinase-superfamilie, alsmede de regulatoren van hun activiteit, zijn de meest intensief onderzochte geneesmiddel-doelwitten in het huidige farmacologische onderzoek. Op basis van een rapport uit 2014, bestaan er meer dan 3000 goedgekeurde en experimentele geneesmiddelen in klinische trials die gebaseerd zijn op kinases.[53] Hoewel de meerderheid van de klinische trials die gebruikmaken van kinase-gerichte geneesmiddelen zich richten op de behandeling van kanker, breid dit veld zich inmiddels ook uit naar de behandeling van ontstekingsziekten.[53] Er komt steeds meer bewijs dat laat zien dat de modulatie van de kinase-signaleringsroute op een effectieve en gunstige manier macrofaagpolarisatie kan beïnvloeden.[54-59] Vanuit dit oogpunt wordt het gebruik

van kinase-gerichte middelen beschouwd als een potente strategie om nieuwe therapieën voor de behandeling van atherosclerose te ontwikkelen. In dit proefschrift laten wij inderdaad zien dat het moduleren van eiwitkinase-activiteit, bijvoorbeeld door het herstellen van de expressie van de kinase Akt2 (**hoofdstuk 3**) of het verstoren van expressie van de kinase-inactiverende fosfatase MKP2 (**hoofdstuk 4**), een effectieve manier is om macrofaagpolarisatie richting het M2-fenotype te drijven en om anti-inflammatoire effecten te bewerkstellingen. Beide studies laten echter zien dat de verhoogde M2-polarisatie ook leidde tot een verhoogde schuimcelvorming, waardoor de atherosclerose ontwikkeling uiteindelijk niet aangedaan of zelfs versterkt werd.

6.2.5 Het belang van upstream stimulatory factor 1 in atherosclerose ontwikkeling

Het belangrijkste kenmerk van atherosclerose is schuimcelvorming, een pathologisch proces waarvoor zowel macrofagen als lipiden onmisbaar zijn. In het eerste deel van dit proefschrift hebben we ons gericht op genen die betrokken zijn bij de polarisatie van macrofagen richting een M2 fenotype. In het tweede deel van dit proefschrift richten we ons op een gen dat betrokken is bij het lipidenmetabolisme. Patiënten die lijden aan zogeheten familiaire gecombineerde hyperlipidemie (FCHL) hebben een hoog risico op het ontwikkelen van vroegtijdige coronaire vaatziekten.[60, 61] FCHL wordt gekarakteriseerd door verhoogde LDL- en triglyceride niveaus die vaak gepaard gaan met een verlaging in HDL-niveaus.[60] Usf1 is een gen dat geassocieerd wordt met FCHL,[60] en dat een belangrijke rol lijkt te spelen in het lipidenmetabolisme en de ontwikkeling van atherosclerose.[62] In **hoofdstuk 5**, hebben we daarom de macrofaag-specifieke rol van Usf1 in atherosclerose onderzocht. In deze studie hebben we laten zien dat beenmerg-specifieke Usf1-deficiëntie een ander fenotype liet zien in LDLr KO muizen dan Usf1 wordt uitgeschakeld in alle weefsels van het lichaam. Wanneer Usf1 in het gehele lichaam afwezig was, leidde dit tot een gunstig cardiometabool lipidenprofiel, wat gekenmerkt werd door een verlaging in de niveaus van 'slecht' VLDL-cholesterol en triglyceriden, terwijl het 'goede' HDL-cholesterolniveau juist verlaagd was. Echter, beenmerg-specifieke Usf1-deficiëntie leidde tot een verhoging in VLDL-cholesterolniveaus en een verhoogde gevoeligheid voor de ontwikkeling van atherosclerose. Dit effect kan waarschijnlijk toegeschreven worden aan een verlaagde VLDL-klaring door het witte vetweefsel. Op basis van onze eigen studies en eerdere studies uit de literatuur kan in het algemeen gesteld wordt dat modulatie van Usf1 de ontwikkeling van atherosclerose beïnvloed.[62] Een interessante bevinding in onze studies is dat, in tegenstelling tot de pro-atherogene rol van algehele USf1 expressie[62], beenmergspecifieke Usf1-expressie juist een beschermende rol heeft in het kader van atherosclerose ontwikkeling. Onze studie onderstreept dus een weefselafhankelijke rol van Usf1 in atherosclerose, waarmee rekening gehouden dient te worden bij het ontwikkelen van nieuwe geneesmiddelen gericht op het remmen van Usf1-activiteit of de downregulatie van diens expressie om atherosclerose tegen te gaan.

6.2.6 Concluderende opmerkingen en toekomstperspectieven

In dit proefschrift is, middels het gebruik van knockout-muizen en de beenmergtransplantatietechniek, onderzocht welk effect de modulatie van genen betrokken bij macrofaagpolarisatie (**hoofdstuk 2-4**) en het lipidenmetabolisme (**hoofdstuk 5**) heeft op de pathogenese van atherosclerose. Naast de in dit proefschrift bestudeerde genen, zijn er vele andere moleculen die een rol spelen in macrofaagpolarisatie en het lipidenmetabolisme, en derhalve een mogelijk interessant doelwit zijn voor de behandeling van atherosclerose.

Macrofagen en de plasticiteit van het fenotype van de macrofagen zijn essentieel voor het onderhoud van homeostatische functies van weefsels. De doen ze door hun genexpressie patronen te veranderen in reactie op de lokale omgeving van het weefsel. Omdat de verschillende typen macrofagen elk op een verschillende manier bijdragen aan de ontwikkeling van atherosclerose, is het mogelijk om cel- of gengebaseerde therapieën te ontwikkelen waarmee macrofaagfenotypen gemoduleerd kunnen worden als behandeling tegen atherosclerose. Tot nu toe is de meeste bekende macrofaag-gerichte therapie de macrofaagtransplantatie die wordt gebruikt om een erfelijk longaandoening, pulmonaire alveolaire proteinose, te behandelen.[63,64] Het idee om therapieën te ontwikkelen die specifiek gericht zijn op het moduleren van macrofaagfenotype, stamt uit het kankeronderzoeksveld, waar het ingezet werd voor de behandeling van tumoren.[65,66] Echter, tot nu toe zijn alle klinische trials op dit gebied gefaald,[67-74] voornamelijk omdat de voorbehandelde macrofagen na herhaalde blootstelling aan de micro-omgeving van de tumor hun anti-tumor M1-fenotype snel verliezen.[75-77] Het is daarom van groot belang om uit te zoeken hoe het macrofaagfenotype 'vastgezet' kan worden *in vivo* zodat zij de micro-omgeving van de tumor kunnen moduleren, in plaats van dat deze de macrofagen moduleert.

Een alternatief hiervoor zijn therapieën die zich richten op het moduleren van macrofagen op het moleculaire niveau, zoals beschreven in dit proefschrift. Door de expressie van genen die betrokken zijn bij macrofaagpolarisatie te moduleren, kan het fenotype van de macrofaag ten gunste worden veranderd en 'vastgezet' worden. Zo worden macrofagen door uitschakeling van MKP2 veranderd van macrofagen met een M1-fenotype in cellen met een meer M2-fenotype. In aanvulling hierop laten LDLr KO muizen die MKP2-deficiënt beenmerg hebben ontvangen een meer anti-inflammatoir cytokinenprofiel in hun bloed zien. Deze bevinding suggereert dat genetische modulatie van het macrofaagfenotype en het 'vastzetten' van dit fenotype haalbaar is in muizen. Daarnaast hebben wij in dit proefschrift ook laten zien dat therapeutische strategieën gericht op het moduleren van eiwitkinase-activiteit ook een effectieve manier is om M2-macrofaagpolarisatie te bewerkstelligen en anti-inflammatoire effecten te induceren.

Een andere interessante strategie om genexpressie in monocyten en/of macrofagen te moduleren is door te interfereren in de epigenetische regulatie van deze genen. Epigenetische modificaties kunnen worden bewerkstelligd door DNA-methylatie, histonmodificatie, en door regulatie van niet-coderende RNA's. Deze modificaties zorgen ervoor dat de toegankelijkheid van het DNA en de chromatinestructuur verandert, waardoor genexpressiepatronen gewijzigd worden.[78-83] In de literatuur zijn er steeds meer studies die laten zien dat epigenetische regulatie een belangrijke rol speelt in zowel macrofaagpolarisatie als atherosclerose ontwikkeling.[84-88] Zo faciliteert Jmjd3, een histon 3 Lys27 (H3K27) demethylase, de expressie van IRF4, een belangrijke M2-stimulerende transcriptiefactor die M2-macrofaagpolarisatie reguleert zonder een effect te hebben op M1-macrofagen.[89,90] Daarnaast hebben eerdere studies laten zien dat uitschakeling van histon deacetylase 3 (Hdac3) macrofagen richting een M2-fenotype drijft, een gunstig effect heeft op de verwerking van lipiden, en de stabiliteit van atherosclerotische plaques vergroot.[91] Tot slot verlaagt behandeling met een DNA-methylatieremmer, 5-aza-2'-deoxcycytidine, macrofaag ontstekingsprocessen en verlaagt het de ontwikkeling van atherosclerose in LDLr KO muizen.[92,93] Interessant genoeg lijken epigenetische veranderingen ook transplanteerbaar te zijn. Van Kampen en collega's vonden dat beenmerg van muizen die gedurende lange tijd gevoed zijn met een Westers dieet een hypomethylatie van CpG-regionen lieten zien in de genen die coderen voor Pu.1 en IRF8.[94] Transplantatie van deze beenmergcellen naar LDLr KO muizen die gevoed werden met een controledieet dat laag in vet en cholesterol is, leidde tot een verhoging van de monocyt-

/macrofaagproliferatie en differentiatie en een verhoging van de atherosclerose ontwikkeling. Het ontrafelen van epigenetische veranderingen in macrofagen tijdens atherosclerose ontwikkeling, en van de epigenetische signaleringsroutes die de inflammatoire respons van macrofagen beïnvloeden, zal de toekomstige ontwikkeling van nieuwe therapieën in de strijd tegen atherosclerose sterk kunnen ondersteunen.[78]

Vanuit het oogpunt van de ontstekingsreactie, lijkt het drijven van macrofaagpolarisatie richting het anti-inflammatoire M2-fenotype een gunstige strategie voor het afremmen van de atherosclerose ontwikkeling. Echter, M2-macrofagen versnellen de schuimcelvorming, waardoor de therapeutische waarde van M2-macrofagen sterkt afneemt wanneer er sprake is van hoge cholesterolniveaus in de circulatie. De versterkte schuimcelvorming heeft een pro-atherogene werking en kan zelfs het anti-inflammatoire, beschermende effect van M2-macrofagen tenietdoen (**hoofdstuk 3 en 4**). Het is daarom van groot belang dat nieuwe therapeutische strategieën gericht op het stimuleren van een M2-macrofaag fenotype gecombineerd worden met cholesterolverlagende medicijnen.

Sterke verlaging van het cholesterolniveau in het bloed is een waardevolle strategie voor het induceren van regressie van atherosclerotische plaques in muismodellen. In dit kader is in eerdere studies een constante verhoging van de M2-over-M1-ratio van macrofagen in de plaques onder regressie condities aangetoond.[95-97] Het is belangrijk te vermelden dat een recente studie heeft laten zien dat de influx van gezonde macrofagen in de atherosclerotische plaque belangrijk is om uiteindelijk regressie van de plaque te kunnen induceren, waarschijnlijk omdat macrofagen nodig zijn voor het opruimen van de dode cellen en het necrotische weefsel in de kleiner wordende plaques.[98] Deze studie liet zien dat wanneer plaque-bevattende apoE KO aorta's naar muizen getransplanteerd werden die deficiënt waren voor de chemokinereceptor CCR2, er geen rekrutering van nieuwe macrofagen plaatsvindt in de reeds bestaande plaques en dat er geen regressie van atherosclerose bewerkstelligd kon worden. CCR2 is een chemokinereceptor die essentieel is voor de rekrutering van monocyten naar atherosclerotische plaques.[99] Gezien het feit dat de meerderheid van de patiënten die zich melden met HVZ klachten reeds vergevorderde atherosclerotische laesies heeft, is het belangrijk om te onderzoeken of de inductie van M2-macrofaagpolarisatie bovenop cholesterolverlaging regressie van atherosclerose ontwikkeling kan versterken of dat regressie hierdoor juist tegengegaan wordt.

Beenmergtransplantatie is een effectieve manier om genexpressie in macrofagen te moduleren, zoals ook in dit proefschrift beschreven is. Om deze reden is beenmergtransplantatie een zeer geschikt middel om te ontrafelen of en zo ja, via welke mechanismen, een gen van interesse de ontwikkeling van atherosclerose kan beïnvloeden. Op deze manier biedt de beenmergtransplantatietechniek ook essentiële informatie over potentiële 'drugable' macrofaag-kandidaatgenen. In de kliniek is de techniek echter niet bruikbaar, omdat voor beenmergtransplantatie een myeloablatie, de vernietiging van endogeen beenmerg, benodigd is. Om in mensen alsnog de expressie van macrofaaggenen te kunnen moduleren, kunnen meerdere strategieën toegepast worden. Zo kan small interfering RNA (siRNA), short hairpin RNA (shRNA) en microRNA (miRNA) gebruikt worden om de genexpressie teniet te doen,[100-104] terwijl lentivirale en plasmavectoren juist gebruikt kunnen worden om een gen van interesse tot overexpressie te brengen.[105, 106] Naast het gebruik van genetische methoden om op directe wijze de expressie van genen te moduleren, kan men ook farmacologische remmers en (ant)agonisten van het gen specifiek afleveren aan macrofagen middels het gebruik van macrofaagreceptor-gebaseerde of ligand-geankerde micro- of nanodeeltjes.[107-109] Zo heeft een eerdere studie laten zien dat

behandeling van ApoE KO muizen met nanodeeltjes geladen met pioglitazon, een potente activator van PPAR-γ, monocyt-/macrofaagpolarisatie succesvol naar een anti-inflammatoir fenotype kan drijven.[110] Daarnaast is het gebruik van nanodeeltjes geladen met contrastvloeistof, in combinatie met de visualisatietechnieken CT en MRI, een succesvolle methode gebleken om atherosclerotische laesies *in vivo* te visualiseren en te karakteriseren, wat een belangrijke basis biedt voor verdere klinische behandelstrategieën.[109] Samengevat bieden deze verschillende technieken de mogelijkheid om te interfereren in specifieke genexpressiepatronen en signaleringsroutes en macrofaagfuncties te moduleren, en bieden als zodanig een basis voor toekomstige klinische behandelstrategieën.

Tot slot, macrofaagheterogeniteit is niet alleen essentieel voor atherosclerose, maar ook voor veel andere ziekten die een sterke ontstekings component kennen, zoals kanker, de ziekte van Parkinson, obesitas, diabetes en arthritis.[111-117] De ontwikkeling van macrofaag-gerichte therapieën biedt dus niet alleen nieuwe behandelmogelijkheden op het gebied van hart- en vaatziekten, maar zal ten gunste komen aan een grote variëteit van ontstekings-gebaseerde aandoeningen.

Concluderend kan gesteld worden dat modulatie van macrofaagpolarisatie, in combinatie met cholesterolverlaging, een veelbelovende strategie is voor de behandeling van atherosclerose. Vervolgonderzoek dient aan te wijzen of macrofaagpolarisatie-gerichte therapieën inderdaad een effectieve benadering voor de behandeling van atherosclerose zijn, en of zij de beoogde verlaging in cardiovasculair risico kunnen bewerkstelligen.

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Abbreviations

ABC	ATP-binding cassette
acLDL	acetylated-low density lipoprotein
Akt	protein kinase B
Apo	apolipoprotein
Arg1	arginase 1
Atgl	adipose triglyceride lipase
BAT	brown adipose tissue
BMDMs	Bone marrow-derived macrophages
BMT	bone marrow transplantation
CAD	coronary artery disease
CETP	cholesteryl ester transfer protein
dKO	double knockout
DUSPs	dual-specificity phosphatases
ERK	extracellular regulated protein kinase
FACS	fluorescent activated cell sorting
FASN	fatty acid synthase
FCHL	familial combined hyperlipidemia
FPLC	fast protein liquid chromatography
Glut4	glucose transporter type 4
HDL	high-density lipoprotein
HL	hepatic lipase
HSL	hormone-sensitive lipase
IDL	intermediate-density lipoprotein
IL-1β	interleukin 1β
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LDL	low-density lipoprotein
LDLr KO	LDL receptor knockout
LPS	lipopolysaccharides
LRP1	low-density lipoprotein receptor-related protein 1
M1	classically activated macrophages
M2	alternatively activated macrophages
MAPK	mitogen-activated protein kinases
MKPs	MAP kinase phosphatases
MTC	Masson's Trichrome
mtROS	mitochondria-derived reactive oxygen species
MTTP	microsomal triglyceride transfer protein
nNOS	endothelial NOS (eNOS), neuronal NOS
NO	nitric oxide
OAT	ornithine amino-transferase
OGTT	oral glucose tolerance test
oxLDL	oxidized LDL

p38	stress-activated protein kinase
Plin	lipid droplet-associated protein
RCT	reverse cholesterol transport
ROS	reactive oxygen species
SMCs	smooth muscle cells
SR-BI	scavenger receptor class B type I
TG	triglycerides
TNF-α	tumor necrosis factor α
TRL	triglyceride-rich lipoproteins
UCP2	uncoupling protein 2
Usfs	upstream stimulatory factors
VCAM-1	vascular cell adhesion molecule-1
VLDL	very-low-density lipoprotein
VSMC	vascular smooth muscle cells
WAT	white adipose tissue
WBC	white blood cell
WT	wild-type
WTD	Western-Type Diet

List of publications

Baoyan Ren, Erik Van Kampen, Theo JC Van Berkel, Sheena M. Cruickshank, Miranda Van Eck. Hematopoietic arginase 1 deficiency results in decreased leukocytosis and increased foam cell formation but does not affect atherosclerosis. *Atherosclerosis* 256 (2017): 35-46.

Baoyan Ren, Pirkka-Pekka Laurila, Reeni B. Hildebrand, Jarkko Soronen, Vanessa Frodermann, Zhuang Li, Mariëtte R. Boon, Janine J. Geerling, Patrick C.N. Rensen, Christian Ehnholm, Petri T. Kovanen, Matti Jauhiainen, Menno Hoekstra, Miranda Van Eck. Enhanced atherosclerotic lesion development in LDL receptor knockout mice lacking Upstream Stimulating Factor 1 (Usf1) in bone marrow-derived cells. (submitted for publication).

Baoyan Ren, Menno Hoekstra, Janine J. Geerling, Peter Van Santbrink, Robin Plevin, Miranda Van Eck. Macrophage MKP2 deficiency is associated with an M2-driven foam cell phenotype and increases atherosclerosis susceptibility of LDL receptor knockout mice. (submitted for publication).

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Olga S.C Snip, **Boayan Ren**, D. Huyen Tran, Janine J. Geerling, Miranda Van Eck. Leukocyte ABCA1 Impedes Progression of Established Atherosclerotic Lesions after Dietary Cholesterol Lowering in LDLr-/- Mice. (Manuscript in preparation)

Rick van der Geest, Janine J. Geerling, Menno Hoekstra, **Baoyan Ren**, Lidewij R. de Leeuw, Richard Verbeek, Johannes M. van Noort, Miranda Van Eck. Heat shock protein alpha B-crystallin promotes macrophage foam cell formation and aggravates early atherosclerosis in LDL receptor-deficient mice. (Manuscript in preparation)

Jianhua Li, Qianzhong Han, Pengtao Gong, Tuo Yang, **Baoyan Ren**, Shijie Li, Xichen Zhang. Toxoplasma gondii rhomboid protein 1 (TgROM1) is a potential vaccine candidate against toxoplasmosis. *Veterinary parasitology* 184, 2 (2012): 154-160.

Guilian Yang, Jianhua Li, Xichen Zhang, Quan Zhao, Pengtao Gong, **Baoyan Ren**, Guocai Zhang. Eimeria tenella: Cloning and characterization of telomerase reverse transcriptase gene. *Experimental parasitology* 124, 4 (2010): 380-385.

Curriculum Vitae

Baoyan Ren was born on February 3rd 1985 in Dingzhou, P. R. China. She grew up there and graduated from Experimental High School of Dingzhou in 2004. Afterwards, she went to Jilin University and received five-years education in Veterinary Medicine. In 2009, she obtained her bachelor degree and continued her three-years postgraduate study in the key laboratory of Zoonosis Research under the supervision of Prof. Dr. Xichen Zhang in the same university with a full scholarship funded by Jilin University. During her master studies, she focused on parasite antigens as possible targets for cancer immunotherapy. She finished her master studies and received her master degree in 2012. In Leiden, the Netherlands, she started her PhD program with a full scholarship funded by China Scholarship Council (CSC) in that same year. There she worked at the division of Biopharmaceutics of the Leiden Academic Centre for Drug Research (LACDR) at Leiden University under supervision of Prof. Dr. Miranda van Eck. During her PhD studies, her research focused on bone marrow transplantation in mice as a tool to investigate M2 macrophage activation pathways in atherogenesis, as described in this thesis.

PhD Portfolio

Courses and Workshops

2015	Effective communication
2015	Cardiovascular PhD-training course
2014	Time management, self-management
2014	Communication in science
2014	Introduction to teaching & supervision for LACDR PhD students
2014	LACDR PhD Introductory Course on Drug Research
2014	On being a scientist
2014	Health Physics expert level 5B
2013	ULLA Summer school
2013	Proefdierkunde (Laboratory Animal Science Course)
2012	LACDR course on Atherosclerosis

(Inter)National Poster Presentations

2016	LACDR Spring Symposium, Leiden, The Netherlands
2015	17th International Symposium on Atherosclerosis (ISA2015), Amsterdam, The Netherlands,
2015	LACDR Spring Symposium, Leiden, The Netherlands
2015	Cardiovascular PhD-training course, Arnhem, The Netherlands
2014	LACDR Spring Symposium, Leiden, The Netherlands
2014	20th Annual Scandinavian Atherosclerosis Conference, Humlebæk, Denmark
2014	The 5th Rembrandt Symposium, Noordwijkerhout, The Netherlands
2013	ULLA summer school, London, UK
2013	The 4th Rembrandt Symposium, Noordwijkerhout, The Netherlands
2013	LACDR Spring Symposium, Leiden, The Netherlands

Teaching

2016	9-month research project MSc student Bio-Pharmaceutical Sciences (BPS)
2015	International BPS Summer School
2015	Therapeutic Modulation of Atherosclerosis BSc BPS Laboratory course
2014	Drug Administration and Distribution BSc BPS Laboratory course
2014	10-week research projects BSc students BPS
2014	4-month research project Erasmus MSc student Chemistry and Pharmaceutical Technology (Parma, Italy)
2013	Therapeutic Modulation of Atherosclerosis BSc BPS Laboratory course
2013	Pharmaceutical administration and distribution BSc BPS Laboratory course
2013	10-week research projects BSc students BPS

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