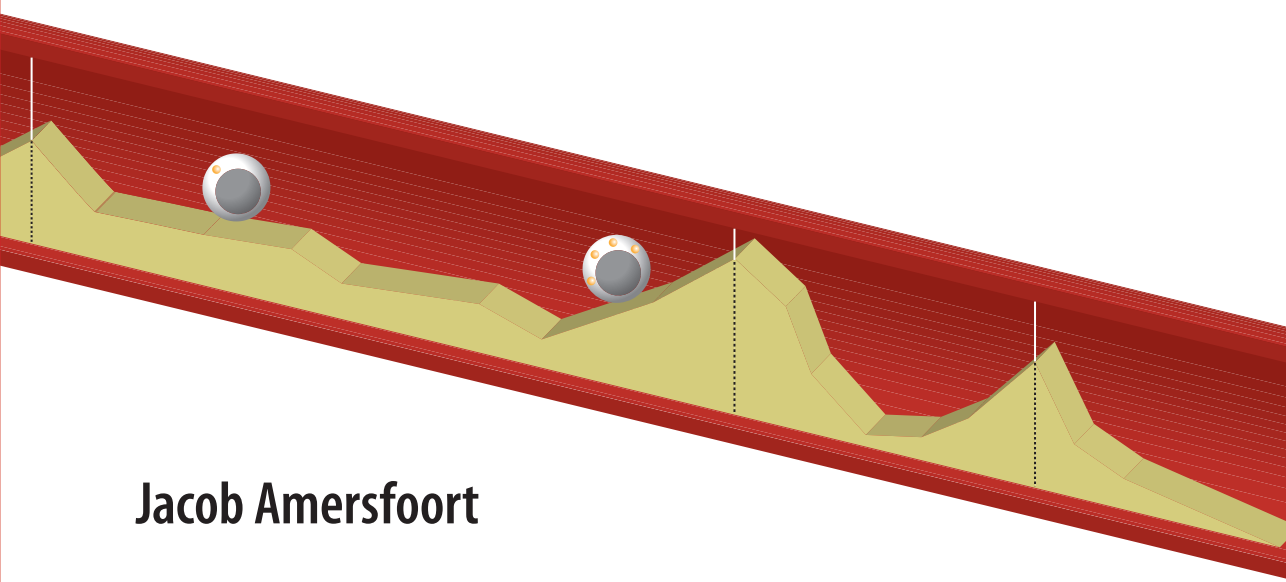
Dyslipidemia, metabolism and autophagy : antigen-independent modulation of T cells in atherosclerosis

Amersfoort, J.

Dyslipidemia, metabolism and autophagy

Antigen-independent modulation of T cells in atherosclerosis



Jacob Amersfoort

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CHAPTER 1

General introduction

1. CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) comprises all diseases which affect the heart and/or blood vessels. CVD in the form of ischemic heart disease and stroke is the leading cause of death worldwide accounting for more than 15 million deaths annually [1]. Ischemic heart disease occurs when stenosis in coronary arteries induces a regional reduction in blood flow. This reduction creates an imbalance between the supply and demand of oxygen and nutrients (ischemia) in the downstream myocardial tissue. Ischemic stroke is a medical condition in which ischemia of brain tissue is caused through similar mechanisms as ischemic heart disease. Alternatively, a hemorrhagic stroke creates ischemia as a result of the rupture of a blood vessel. Major risk factors for CVD include dyslipidemia, hypertension, a sedentary life-style, stress and smoking [2]. Years of research and campaigning by health organizations have created awareness in the Western world of the link between the aforementioned risk factors and the development of CVD. In the past years, the number of CVD deaths has declined in the United States [2] and Europe [3], which is largely due to improved prevention and treatment [2,4]. Nevertheless, CVD remains the most prominent health issue, even accounting for 45% of total annual deaths in Europe [3]. The high prevalence of CVD is stimulated by the fact that the incidence of diseases associated with CVD, such as obesity and diabetes, has increased over the past decades [2]. Moreover, familial hypercholesterolemia, an inherited disorder characterized by dyslipidemia and premature coronary artery disease, is among the most common inherited diseases with a prevalence of 1 in 500 worldwide [5,6] . The classic risk factors for CVD like dyslipidemia and smoking promote the development of the main underlying pathology of CVD: atherosclerosis.

Atherosclerosis is a lipid-driven autoimmune-like disease of the medium and large-sized arteries, characterized by progressive growth of (multiple) stenotic lesions. In the advanced stage, these lesions contain large amounts of lipids and (dead) immune cells, hence the 'athero' part of atherosclerosis, which refers to the gruel-like, pasty materials in atherosclerotic lesions. Additionally, matrix proteins such as collagen and calcifications contribute to the 'sclerosis' part of atherosclerosis as it refers to the stiffened aspect of advanced lesions. In humans, the development and growth of atherosclerotic lesions, also called plaques, which progressively narrow the arterial lumen, can start in the first decades of a human life and progress during a lifetime [7].

When atherosclerosis-based perfusion defects are present in myocardial tissue and ischemia progresses, coronary artery atherosclerosis becomes symptomatic and electrocardiographic changes and angina, i.e. chest pain, are present. In patients with stable angina, these symptoms arise under (exercise induced) stress and resolve during rest. Whether coronary artery atherosclerosis progresses into a potentially lethal disease depends on lesion composition rather than on the severity of the stenosis. This

seemingly counterintuitive notion is explained by the life-threatening complication of atherosclerosis called myocardial infarction. Myocardial infarction (MI) is caused by myocardial cell death caused by prolonged (>20 min) and acute myocardial ischemia, which occurs after plaque rupture or erosion causes a thrombus to occlude a coronary artery [8]. Plaque rupture is an underlying pathological event in MI in which the rupture of a plaque exposes tissue factor present in the necrotic core to coagulation factors in the blood, which initiates the coagulation cascade [9]. Alternatively, plaque erosion causes a thrombotic event through dysfunction of endothelial cells, which gradually exposes tissue factor in the underlying basal layer [8]. Another complication of such a thrombotic event is the dissociation of the thrombus after which it circulates in the blood and occludes an artery elsewhere, for example in the brain (causing stroke).

Initially, atherosclerosis was considered to be a primarily cholesterol-driven disease [10] and the inflammatory cell changes associated with atherosclerotic plaques were considered to be a secondary effect of the pathological process [11]. In the 19th century, the German pathologist Rudolf Virchow proposed that it is actually the cells which drive the pathological process [11]. After this, research has focused on the role of the immune system in the pathophysiology of atherosclerosis and has uncovered it to be a complex multifactorial process. This has resulted in the generally accepted theory in which atherosclerosis is the result of a plethora of immune cells responding to abnormal amounts of (modified) lipoproteins which accumulate in the vessel wall and progressively induce fundamental architectural and morphological changes, as described below.

2. ATHEROSCLEROSIS

2.1 Early atherosclerosis

Even though lipids and immune cells circulate throughout the vascular system atherosclerosis only develops at specific sites of the vasculature. Presumably, this is because early atherogenesis is tightly linked to local disturbances in blood flow. The innermost layer (intima) of arteries and veins is lined with a monolayer of cells called endothelial cells (EC). ECs can regulate the vascular tone by inhibiting and stimulating smooth muscle cells (SMC) in the medial layer, regulate nutrient permeability through their intimal integrity and facilitate immune cell transmigration to surrounding tissues [12]. Dysfunction of endothelial cells occurs at arterial segments where shear stress is low or oscillatory, e.g. in the curvature of coronary arteries or in bifurcations [13]. Endothelial dysfunction in the presence of pro-atherogenic factors such as elevated circulating lipid levels (dyslipidemia) can initiate early atherogenesis through two crucial processes. First, the expression of adhesion molecules associated with EC dysfunction, such as vascular cell adhesion molecule-1 and E- and P-selectin molecules, is increased on the

EC membrane [14,15]. These adhesion molecules bind to cognate ligands (such as integrin α4β1) on the cell membranes of circulating immune cells, thus binding immune cells to ECs [12].

Second, disturbances in the EC tight junctions, e.g. caused by alterations in VE-cadherin expression [16], decrease the intimal integrity and increase its permeability to lipoproteins [17]. Lipoproteins are particles consisting of various classes of lipids and core proteins through which hydrophobic lipids can circulate in the body. In atherosclerosis, the most relevant lipoproteins are chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). VLDL and, particularly, the cholesterol-rich LDL are considered the most pathological lipoproteins in atherosclerosis. LDL particles present in the subendothelial space can be modified (e.g. through oxidation), which promotes their retention, the latter by interactions with certain proteoglycans [18–20]. Alternatively, LDL circulates the body in its oxidized form (oxLDL) and then infiltrates the subendothelial space [21]. Simultaneously, ECs secrete chemokines, such as monocyte chemoattractant protein 1 (MCP-1, also called CCL2), which recruit circulating immune cells such as monocytes [22,23]. Next, intracellular adhesion molecule-1 and VCAM-1 facilitate firm adhesion and a full arrest of bound monocytes [15,24], after which they spread and migrate between or through the EC layer in the process of diapadesis.

Under influence of local growth factors and cytokines, infiltrated monocytes then differentiate into specialized immune cells called macrophages [25,26]. Macrophages are phagocytes, meaning that these cells engulf extracellular foreign or toxic materials to minimize tissue damage [27]. In the subendothelial space macrophages engulf (modified) lipoproteins in an unregulated fashion via scavenger receptors such as CD36 and SR-A1 [28,29]. When this process persists, intracellular lipid storage organelles termed lipid droplets expand in number and size, which induces a morphologically and functionally distinct type of macrophage called foam cell [30,31]. Foam cells secrete inflammatory factors such as cytokines and chemokines, especially in response to cholesterol crystals [32], which in turn can result in additional recruitment of monocytes and other innate and adaptive immune cells, including neutrophils [33] and T cells [34]. This inflammatory environment in early developing lesions renders the ECs to remain 'leaky' and activated, thereby further promoting the recruitment of more inflammatory cells. Therefore, this inflammatory response is not beneficial but pathological as a vicious cycle involving lipids and immune cells causes the ongoing inflammation to remain unresolved. In this stage, an atherosclerotic lesion is classified as a fatty streak (fig. 1A), which is asymptomatic and can disappear after normalization of serum cholesterol levels, i.e. by counteracting dyslipidemia [35]. However, when serum lipids are not normalized and no therapeutic intervention is performed to inhibit inflammation, the vicious inflammatory cycle causes the early atherosclerotic lesion to progress.

2.2 Advanced atherosclerosis

Over decades, ongoing and recurring pathogenic processes, as described below, remodel the plaque into complex lesions which cannot be resolved and have distinct histological characteristics [35,36]. Advanced atherosclerotic lesions are characterized by a fibrous cap, increased SMC content and intraplaque necrotic areas. One of the main pathological mechanisms causing the necrotic areas in the plaque is the induction of apoptosis of foam cells (and other immune cells) due to continuous lipid overload as a result of lipotoxicity [37–39]. In line, endoplasmic reticulum (a crucial organelle in cellular cholesterol metabolism) stress induced by atherogenic lipoproteins induces programmed cell death known as apoptosis [40]. As lesions progress, foam cells switch from secondary necrosis as a result of apoptosis, which usually results in cellular debris to be engulfed by phagocytes, to non-programmed cell death (i.e. primary necrosis) [41,42] which results in large amounts of debris inside the lesions and progresses inflammation [43]. Progressive cell death leads to the formation of necrotic core regions which increase lesion burden [44,45].

Additionally, under the influence of growth factors and inflammatory cytokines, which are largely secreted by T cells, SMCs are activated and: **a**) proliferate, **b**) migrate into the intima, **c**) acquire a foam cell-like phenotype [46], and **d**) secrete extracellular matrix proteins such as collagen [47–51] (fig. 1B). Eventually this results in the formation of a fibrous cap. Therefore, SMCs are predominantly atheroprotective as the extracellular matrix proteins, such as collagen, which they secrete, stabilize the lesion and encapsulate the plaque content at the luminal side [48,49]. Through these remodeling processes, an early fatty streak develops into an atheroma, a stenotic plaque characterized by intraplaque lipid accumulation and necrotic core expansion. Eventually, an atheroma progresses into a lesion classified as a fibroatheroma in which the SMC-derived matrix proteins have formed a fibrous cap [52]. Further intraplaque remodeling can occur over the next decades. Hypoxia in the plaque induces neovascularization [53], further facilitating inflammatory cell influx [54]. Furthermore, apoptosis and necrosis cause intraplaque calcium depositions, which contribute to the calcification of lesions [55], which can be a characteristic of unstable lesions prone to rupture [56].

Advanced atherosclerotic lesions are clinically relevant when the degree of stenosis is severe enough to cause symptoms (e.g. stable angina) or when the lesion is at risk of rupturing or eroding and cause a thrombus (fig. 1C). Degradation of the matrix proteins in the fibrous cap and the lesion is caused by matrix degrading proteins, such as matrix metalloproteinases (MMP) [57]. MMPs can be secreted by dedifferentiated SMCs [58], neutrophils [59], mast cells [60] and macrophages [34]. Through degradation of the extracellular matrix, MMPs such as MMP-9 contribute to the instability of atherosclerotic lesions and render them more prone to cause a major adverse cardiac event such as an MI.

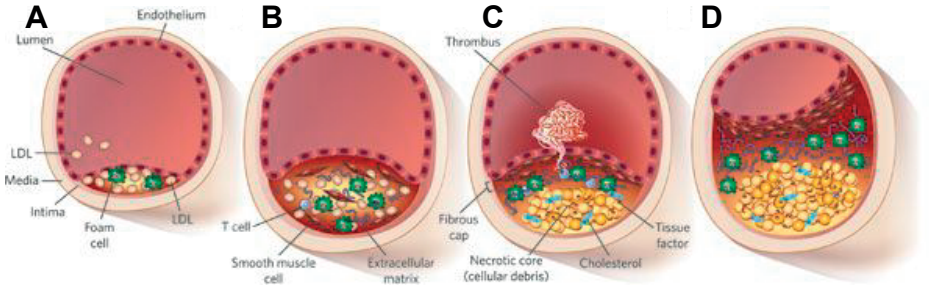

Figure 1 Development of atherosclerotic lesion. (A) Atherosclerosis is initiated after endothelial dysfunction causes monocyte recruitment and lipoproteins such as low-density lipoprotein (LDL) to infiltrate the subendothelial space (intima). Monocytes differentiate locally to macrophages which engulf the infiltrated lipoproteins. When lipoprotein accumulation persists, macrophages turn into foam cells. (B) When fatty streaks are not resolved, inflammation persists and other immune cells such as T cells are recruited towards the atherosclerotic lesion. In response to cytokines and growth factors secreted by foam cells and other immune cells, smooth muscle cells migrate and proliferate and secrete extracellular matrix proteins (such as collagen). (C) Lipotoxicity causes cell death, thereby inducing the formation of a necrotic core and cholesterol crystals to be deposited inside the lesion. Further smooth muscle cell activity leads to the formation of a fibrous cap which encapsulates a lipid and necrosis rich core and occludes the arterial lumen (stenosis). When the fibrous cap is degraded through rupture or erosion, tissue factor comes into contact with coagulation factors in the blood thereby initiating the coagulation cascade and thrombus formation. These thrombi can cause ischemic heart disease or stroke. (D) Alternatively, the fibrous cap remains intact and lesion growth progresses, thereby further occluding the lumen. This stage of atherosclerosis can cause stable angina. *Reproduced with permission from Nature Publishing Group (Springer Nature). D.J. Rader and A. Daugherty, Translating molecular discoveries into new therapies for atherosclerosis, Nature, 2008, 21;451(7181):904-913.*

At that stage, atherosclerosis has caused an acute and possibly life-threatening condition. This acute clinical stage requires immediate therapeutic intervention in the form of balloon angioplasty with or without additional stent placement to restore normal blood flow [61,62]. Alternatively, in the non-acute stage where a lesion causes severe stenosis (fig. 1D) and is at risk of causing a major adverse cardiac event or stroke, endarterectomy surgery can be performed to remove the plaque. Of note, endarterectomy surgery can also be performed in addition to thrombolytic therapy in the acute stage of stroke. Furthermore, vascular bypass surgery can be performed to circumvent a stenotic or occluded vessel using a vein graft. Unfortunately, these surgical interventions are invasive and can cause complications, such as restenosis of the transplanted vessel. Pharmacologically, the main strategy of treatment of atherosclerosis and CVD is lipid lowering, mainly by the use of statins [63–65]. Statins inhibit the rate-limiting enzyme HMGCoA reductase in the cholesterol synthesis pathway and thereby lower LDL-cholesterol levels in patients with an elevated risk of a cardiac event such as familial hypercholesterolemia patients [63] or patients with a history of ischemic heart disease. The use of statins has been shown to be clinically successful in lowering LDL-cholesterol by 25-40% [66] and reducing the number of deaths from ischemic heart disease [67]. Nevertheless, some patient groups

have only limited benefit from statins as statins sometimes fail to lower LDL-cholesterol levels [66] in so-called non-responders. Therefore, experimental and clinical researchers have sought to develop additional treatment methods to prevent CVD. Recently, the therapeutic use of monoclonal antibodies targeting PCSK9 has shown significant added therapeutic value to statins to lower LDL-cholesterol levels and the incidence of cardiac events [68,69]. These therapeutic approaches primarily target systemic lipid metabolism, although statins have been identified to have cell-specific anti-inflammatory properties as well [70,71]. Recently, dampening inflammation through antibody-mediated inhibition of a potent inflammatory cytokine, called interleukin-1β (IL-1β), has been shown to successfully decrease the incidence of CVD [72]. The results of these trials underline the significance of biomedical research to unravel and examine novel therapeutic targets and approaches to treat atherosclerosis and prevent CVD.

2.3 Experimental animal models of atherosclerosis

Atherosclerosis was first induced in experimental animals by Alexander Ignatowski in the beginning of the 20th century. Ignatowski induced aortic atherosclerotic lesions in rabbits by feeding them a cholesterol- and protein-rich diet [73]. Since then, experimental atherosclerosis has been described in swines [74], rats [75], non-human primates [76] and mice. As for many disease models, the mouse is usually the model of choice as many genetically modified models are available, they are easy to house and breed and are relatively cheap to purchase and keep. The most commonly used wild-type laboratory mouse is the C57/BL6 mouse. C57/BL6 mice develop fatty streak-like lesions when fed an atherogenic diet [77], but this is time-consuming and does not reflect a clinically relevant stage of atherosclerosis. Two genetically modified mouse strains have been extensively used in atherosclerosis research as they can develop lesions which more closely resemble the clinical stage of atherosclerosis and allow for the examination of therapeutic intervention in different stages of atherosclerosis. The LDL receptor deficient mouse (*Ldlr*$^{-/-}$) and the apolipoprotein E (apoE) deficient mouse (*apoE*$^{-/-}$) are the most common experimental models for atherosclerosis. In *Ldlr*$^{-/-}$ mice, the lack of LDL receptor-mediated uptake of VLDL and LDL from the circulation by the liver increases the amount of circulating cholesterol-rich lipoproteins [78]. Without dietary intervention, *Ldlr*$^{-/-}$ mice develop early lesions over the course of months. Therefore, a high fat diet is required to induce dyslipidemia and atherosclerosis in a timely fashion and if required in an advanced stage. The apoE protein is present in chylomicron remnants and VLDL. It binds to the LDL receptor which facilitates their uptake by the liver. *apoE*$^{-/-}$ mice have elevated serum cholesterol levels when fed a normal chow diet and slowly develop atherosclerosis without any additional dietary intervention [79]. Of note, apoE is involved in antigen presentation by antigen presenting cells (APC) [80], and other inflammatory processes [81] suggesting that *apoE*$^{-/-}$ mice are a less suitable model than *Ldlr*$^{-/-}$ mice to study specific inflammatory pro-

cesses in atherosclerosis. Recently, several reports have shown that injecting mice with viral vectors encoding a gain-of-function form of PCSK9 is suitable to efficiently induce atherosclerosis without the need of germline mutations [82,83]. Gain-of-function mutations in PCSK9 increase its targeting of the LDL receptor for lysosomal degradation, thereby inducing an *Ldlr*$^{-/-}$-like phenotype. Of note, PCSK9 has also been shown to target CD36 for lysosomal degradation, thereby affecting triglyceride metabolism [84], suggesting that viral vector-induced PCSK9 overexpression in mice might have LDL-independent effects on lipid metabolism. Nevertheless, the described mouse models are suitable to study atherosclerosis due to aberrations in their systemic lipid metabolism.

3. SYSTEMIC AND CELLULAR LIPID METABOLISM

Increases in dietary cholesterol intake or *de novo* cholesterol synthesis can drive atherosclerosis by elevating the abundance of circulating atherogenic lipoprotein particles. Adequate systemic lipid metabolism processes dietary lipids and synthesizes lipids, thus producing lipoprotein particles which provide tissues with the essential amounts of cholesterol and specific fatty acids. Thereby, metabolism is required to provide tissues and cells with cholesterol which is an essential building block for cell membranes, regulates membrane fluidity and lipid raft formation [85], is involved in steroid hormone synthesis [86] and is required for bile acid synthesis [87]. Disturbed lipid metabolism, however, can cause dyslipidemia, in the form of elevated levels of circulating cholesterol (hypercholesterolemia) or triglycerides (hypertriglyceridemia), and thereby contributes to atherosclerosis and CVD.

Systemic lipid metabolism can be divided into an exogenous and endogenous pathway. The liver is a key organ in lipoprotein metabolism as it is a major organ in both the exogenous and the endogenous pathway.

In the exogenous pathway, the uptake of dietary lipids primarily takes place in the small intestine [88] where digested lipids form micelles which are partly degraded and taken up by intestinal mucosal cells and transported to the interstitial space as chylomicron particles [89]. Alternatively, free fatty acids (FFA) are directly transported from the small intestine to the liver via the portal vein [90]. The chylomicrons travel through the interstitial space, eventually enter the lymphatic system and then enter the blood circulation via the thoracic duct [91]. Triglycerides in the chylomicron particles are hydrolyzed in the capillaries of skeletal muscle and white adipose tissue by lipoprotein lipases secreted by ECs, thus releasing FFA in the circulation to be taken up by peripheral tissues [89]. Chylomicron remnants are subsequently taken up by liver cells.

The endogenous pathway starts with the processing of the contents of chylomicron remnant particles and proceeds with the *de novo* synthesis of cholesterol and FFA. Sub-

sequently, the (newly synthesized) lipids are esterified, packaged and secreted as VLDL particles containing apoE and ApoB100. VLDL particles are particularly triglyceride-rich but also contain cholesteryl esters and thus supply peripheral tissues with FFA and cholesterol [89]. When lipoprotein lipase in the capillaries hydrolyze the triglycerides in VLDL, VLDL particles transition into intermediate density lipoproteins. Subsequently, intermediate density lipoproteins are degraded by hepatic lipases which hydrolyze the remaining triglycerides and remove the apoE protein, resulting in LDL particles. LDL is subsequently transported in the circulation to provide tissues with cholesteryl esters or is taken up in the liver via the LDL receptor and scavenger receptors. The liver then stores the lipids from excess LDL particles or processes the cholesterol to be excreted via the gut [92]. Another essential process in systemic lipid metabolism and atherosclerosis is reverse cholesterol transport [93] in which cholesterol is extracted from cells through interactions between circulating high-density lipoproteins (HDL) and cholesterol efflux transporters. The core protein of HDL particles is ApoA-1 which is produced in the liver and intestine. ApoA-1 binds to ATP-binding cassette (ABC) transporters located at the cell membranes which facilitates the efflux of cellular cholesterol to immature and mature HDL particles [94]. HDL particles subsequently travel to the liver where they acquire cholesterol from liver cells after which the cholesterol can be cleared via the intestines. Thus, on a systemic level, chylomicrons, VLDL and LDL function to provide peripheral tissues with lipids whereas HDL functions to extract lipids from peripheral tissues. When dyslipidemia persists, lipid accumulation occurs in liver cells (mainly driven by FFA) which can lead to hepatic steatosis and eventually hepatosteatitis.

On a cellular level, the synthesis and influx and the degradation and efflux of cholesterol and FFAs are mainly regulated by the transcriptional activities of the nuclear receptor liver-X-receptor (LXR) and sterol regulatory element binding protein (SREBP) [95,96]. Upon endocytosis of cholesterol-rich lipoproteins, the free cholesterol which is released from lysosomes into the cytoplasm is modified to different types of oxysterols which serve as a ligand for LXR [97]. Upon its activation, LXR inhibits cholesterol synthesis and promotes cholesterol efflux by increasing the expression of ABC transporters [98], thus forming a negative feedback mechanism for elevated intracellular cholesterol levels. On the other hand, SREBP1 and SREBP2 are activated by low amounts of cholesterol in the endoplasmic reticulum and their target genes function to increase the lipid content in cells by increasing the expression of the LDL receptor and genes which promote cholesterol- and FFA synthesis [99]. One of these genes encodes HMGCR, the target of statins.

Peroxisome proliferator activated receptors (PPAR) represent another class of nuclear receptor which act as transcription factors and are activated by intracellular lipids, mainly FFA and FFA-derivatives [100], and modulate lipid metabolism through their target genes. Three types of PPARs have been identified: PPARα, PPARδ (also called PPARβ) and PPARγ. These PPARs have different tissue distribution and physiological functions but share

some overlap in their activating ligands and transcriptional targets [100,101]. Many target genes of PPARs are involved in lipid metabolism but each PPAR has also been described to have immunomodulatory effects. PPARα activation has been described to negatively regulate inflammatory gene expression, which might be in part through its direct interaction with NF-kappa B [102,103]. Metabolically, PPARα regulates systemic lipid metabolism by controlling the expression of lipoprotein lipase and apolipoproteins [104,105]. Furthermore, target genes of PPARα are involved in peroxisomal and mitochondrial β-oxidation of FAs [106]. PPARδ is ubiquitously expressed, suggesting that it is fundamentally required for lipid metabolism. Its target genes are primarily involved in mitochondrial biogenesis, mitochondrial β-oxidation and, in skeletal muscle, repression of glucose metabolism [107]. The role of PPARδ in the regulation of inflammation remains debated, although the loss of hematopoietic PPARδ expression has been shown to reduce atherosclerosis [108] and PPARδ activation inhibits foam cell formation [109]. Like other PPARs, PPARδ is activated by specific subclasses of FAs and FA-derivatives, mainly polyunsaturated FAs and specific eicosanoids [100]. PPARγ activation is involved in adipogenesis, as its transcriptional targets regulate adipocyte differentiation, FA uptake and synthesis [100,110]. PPARγ activation generally has anti-inflammatory effects [102]. Thus, on a systemic level, lipid metabolism is mainly regulated by the dietary intake of lipids and hepatic lipoprotein metabolism. On a cellular level, it is mainly regulated by transcription factors which respond to perturbations in intracellular lipid abundance, by modulating the expression of their target genes.

4. IMMUNE SYSTEM

As mentioned above, while dyslipidemia raises lipoprotein levels, it is the inflammatory response induced by lipoproteins which represents the other cornerstone of the atherosclerosis pathophysiology. The immune system responds to pathogens which are recognized as 'non-self' and which could potentially be detrimental to the health of the organism and therefore require neutralization. In the acute phase of an immune response, innate immune cells quickly respond to invading pathogens in a mostly non-selective manner. If inflammation persists, adaptive immune cells are recruited to generate a specific response and induce immunological memory. Atherosclerosis is characterized by chronic inflammation in which various cell types of the innate- and adaptive immune system contribute to the disease process (fig. 2). In the innate immune system, monocytes, macrophages, dendritic cells and neutrophils are involved in atherogenesis. In the adaptive arm, T helper cells, cytotoxic T cells, natural killer T cells and B cells have been shown to contribute to atherosclerosis [111,112]. Below, the specific immune cells relevant for this thesis will be discussed but various additional types of immune

cells have been described to have pro- or anti-inflammatory effects in atherosclerosis, including dendritic cells [113,114], mast cells [115], natural killer cells [116], eosinophils [117], γδ-T cells [118,119] and B cells [120].

4.1 Macrophages

The contribution of macrophages to the pathogenesis of atherosclerosis is significant as they are among the first immune cells to be present at the site of a developing lesion. As previously described, macrophages differentiate from monocytes which are recruited from the circulation and have entered the subendothelial space [121,122].

Monocytes are innate immune cells which mature in the bone marrow and, after entering the circulation, patrol the blood stream in search of sites of inflammation [123]. In atherosclerosis, they respond locally to chemokines which can be secreted by, for example, ECs and SMCs [26]. As previously mentioned, MCP-1 is a crucial chemokine for the recruitment of monocytes during the early stage of atherosclerosis. Therefore, mice deficient for C-C chemokine receptor type 2 (CCR2), the receptor for MCP-1, have strongly reduced atherosclerosis development [124]. CX3C chemokine receptor 1 (whose ligand is the chemokine CX3CL1) represents an additional receptor which has been shown to mediate monocyte homing to atherosclerotic lesions [125–128]. Monocytes are roughly divided into two categories based on the proteins present on their cell membrane, their gene expression profiles and inflammatory potential. In humans, classical monocytes have pro-inflammatory properties and are defined as $CD14^{+}CD16^{-}$ monocytes. In mice, pro-inflammatory monocytes express high levels of the membrane protein Ly6C (thus termed $Ly6C^{hi}$ monocytes) and have the highest potential to differentiate into inflammatory macrophages in tissues [129,130]. Additionally, inflammatory monocytes highly express CCR2, thus enhancing their capacity to respond to MCP-1 [131]. Non-classical patrolling monocytes are defined in humans as $CD14^{dim}CD16^{+}$ monocytes and in mice as $Ly6C^{lo}$ monocytes [130]. Non-classical monocytes are more likely to differentiate into anti-inflammatory macrophages [132]. During atherosclerosis, hypercholesterolemia induces monocytosis (increased circulating monocyte numbers) and an increase in the amount of $Ly6C^{hi}$ monocytes which differentiate into macrophages inside atherosclerotic lesions [133]. Recently, lipid accumulation in classical monocytes has been shown to be associated with increased CCR2 expression and transmigration, suggesting that elevated cholesterol levels during hypercholesterolemia can cause intrinsic changes in monocytes which directly affect their inflammatory function [134].

Macrophages are crucial in the development of atherosclerosis as was demonstrated in *apoE*$^{-/-}$ mice deficient for macrophage-colony stimulating factor (M-CSF) which have an 86% decrease in atherosclerosis as compared to *apoE*$^{-/-}$ mice [135]. As mentioned, macrophages accumulate lipids derived from oxLDL and VLDL via scavenger receptors inside the atherosclerotic lesion [29,30,136]. Additionally, Toll-like receptors (TLR), which recognize

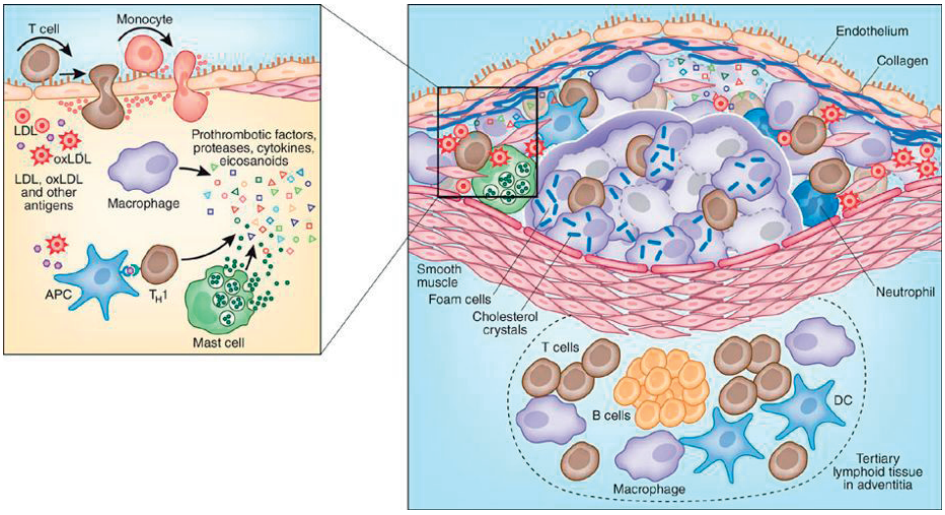

Figure 2 Immune cell types in atherosclerosis. Inflammation in the lesion is largely but not exclusively mediated by macrophages and T cells. Monocyte-derived macrophages take up (modified) lipoproteins such as oxLDL and secrete atherogenic factors such as proteases and inflammatory cytokines, thereby promoting lesion growth and instability. Macrophages can act as antigen presenting cells (APC) which present antigens derived from LDL and other proteins to T cells. Additionally, dendritic cells (DCs) are very potent APCs in atherosclerosis. The main pathogenic T cell in atherosclerosis is the T helper 1 (Th1) cell which secretes inflammatory cytokines when activated by an APC presenting its cognate antigen. The cytokines which T helper cells secrete modulate other immune cells and smooth muscle cells and endothelial cells. Innate immune cells such as neutrophils promote atherosclerosis through their granular secretion of cytokines and proteases. Also depicted: other immune cell types involved in the pathophysiology of atherosclerosis, including mast cells and B cells. *Reproduced with permission from Nature Publishing Group (Springer Nature). G.K. Hansson, A. Hermansson, The immune system in atherosclerosis, Nat. Immunol., 2011;12,204-212.*

structurally conserved molecules such as lipopolysaccharides during bacterial infection, recognize oxLDL and contribute to foam cell activation [137,138]. Notably, TLR4 activation by oxLDL increases the secretion of pro-inflammatory cytokines such as IL-1β and IL-6 [34], thereby partly explaining the inflammatory effects of oxLDL.

A crucial process which connects the innate immune system to the adaptive immune system is the process of antigen presentation in which pathogen-derived peptide fragments are processed and presented on major histocompatibility complex (MHC) molecules. In mice, MHC-I and MHC-II molecules are loaded with peptides which can activate $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells, respectively. Macrophages are potent APCs and can present peptides on MHC molecules as well as lipid antigens on CD1d (an MHC-like molecule) which subsequently specifically activate natural killer (NK) T cells [139,140]. Another mechanism through which macrophages can drive atherosclerosis is through the secretion of MMPs, thereby contributing to plaque instability [141]. However, MMP expression by macrophages heavily depends on their differentiation status

and macrophages can also express tissue inhibitor of metalloproteinases, which inhibit MMP activity [142].

In vitro, a clear dichotomy in the inflammatory phenotype of macrophages has been described. Monocytes are differentiated into M0 macrophages by M-CSF. After this, they can be differentiated to classical M1 macrophages which are pro-inflammatory or non-classical M2 macrophages which dampen inflammation and tissue damage. M1 macrophage differentiation is induced by TLR ligands and interferon gamma (IFNγ). M1 macrophages secrete inflammatory cytokines such as TNFα, IL-1β, IL-6 and MMPs and have poor phagocytic capacity [143,144]. Macrophages are polarized towards the M2 phenotype by IL-4 [145]. M2 macrophages secrete less inflammatory cytokines than M1 macrophages, but more anti-inflammatory cytokines such as IL-10. Moreover, their capacity to phagocytose apoptotic debris is enhanced [146] as compared to M1 macrophages.

The macrophage population in atherosclerotic lesions is too heterogeneous to be divided in just M1 and M2 macrophages. Nevertheless, pro-inflammatory and anti-inflammatory macrophages have both been described in atherosclerosis. As atherosclerotic lesions contain high levels of IFNγ and other inflammatory cytokines, newly differentiated M0 macrophages are likely to differentiate into M1-like macrophages. Hence, M1-like macrophages have been described in both human [147] and murine atherosclerotic lesions [148]. M2-like macrophages have also been described in human and murine atherosclerotic lesions [147,149]. Of note, macrophages display great plasticity in their polarization as M1 and M2 macrophages can switch phenotype under the right environmental circumstances.

Many other types of macrophages have been suggested to contribute to atherogenesis, including Mox macrophages which are generated by oxidized lipids and have a gene expression profile distinct from M1/M2 macrophages [148]. Therefore, research examining macrophage populations inside atherosclerotic plaques is currently limited by the inevitable oversimplification of the dynamics in macrophage heterogeneity over time *in vivo*. Moreover, a single cell atlas of macrophages derived from murine atherosclerotic lesions revealed great heterogeneity in the macrophage phenotypes [150]. This suggests that the modulation of macrophage-mediated immunity as a therapy might be difficult to translate to the human situation where the macrophage population is also heterogeneous and might be quite distinct from murine models of atherosclerosis. Nevertheless, given their abundance in all stages of atherosclerosis, the modulation of the atherosclerotic macrophage population towards anti-atherogenic phenotypes remains a promising therapeutic approach.

4.2 Neutrophils

Neutrophils are another type of innate immune cells which contribute to atherosclerosis [151]. Neutrophils are short-lived granulocytes [152], which reside in the bone marrow and upon their egression circulate in the blood until they respond to inflammatory signals

and migrate into tissues [153]. Neutrophils are among the first cells to respond in many inflammatory processes, including the one in atherosclerosis [154]. Upon activation, neutrophils release granules filled with inflammatory mediators such as lipocalin-2, MMPs and antimicrobial agents such as myeloperoxidase and reactive oxygen species [155,156]. Neutrophils have been observed in early murine lesions [154], advanced murine lesions [157] and human atherosclerotic lesions [158], albeit in relatively low numbers. This may be due to their short life-span and the fact that they undergo apoptosis upon activation [33]. Nevertheless, experimental and observational evidence has shown that neutrophils affect early and advanced atherosclerosis. In early atherosclerosis, hypercholesterolemia induces neutrophilia by driving maturation and egression of neutrophils from the bone marrow, and depletion of neutrophils with the 1A8 antibody reduces lesion size by ~50% [154]. Mechanistically, neutrophils promote atherogenesis amongst others through the secretion of myeloperoxidase [155] and reactive oxygen species [156]. Given their potential to secrete MMPs, neutrophil activation might contribute to plaque destabilization during advanced stages of atherosclerosis [59]. Interestingly, intraplaque neutrophils show a positive association with acute coronary events [159]. A distinct mechanism through which neutrophils may contribute to CVD is through the formation of neutrophil extracellular traps (NET) [160,161]. In the process of NETosis, neutrophils spill out condensed chromatin in a web-like structure, thereby 'trapping' pathogens and inflammatory factors [162]. Accordingly, NET formation has been shown to be pro-atherogenic [163]. NET formation also promotes thrombotic events which are associated with plaque erosion [164], suggesting that neutrophils contribute to different stages of atherosclerosis through distinct mechanisms in both murine and human atherosclerotic lesions.

4.3 T cells

A crucial process in adaptive immunity is the presentation of antigens to T cells by APCs. Many types of APCs are known to be involved in atherosclerosis, including macrophages, dendritic cells, B cells and mast cells. Of these, the dendritic cells are professional APCs which have the highest capacity to activate T cells [165]. T cells are a type of lymphocyte which is involved in targeted immunity and can form immunological memory. T cell precursors originate from the bone marrow from which they migrate towards the thymus for their maturation. In the thymus, T cell precursors mature, partly via DNA recombination events, into double positive cells expressing a unique combination of TCRα and TCRβ subunits, which comprise the T cell receptor (TCR). The TCR recognizes antigens presented by MHC-I and MHC-II molecules (in humans termed human leukocyte antigen molecules) in part through interactions with the co-receptors CD8 and CD4, respectively, which are present in the TCR complex. During positive selection, only T cells which have medium or high affinity for the binding of various peptides by APCs are selected [166]. In this process, T cells also go through a 'commitment' phase in the thymus in which they

commit to MHC-I or MHC-II peptide recognition and lose either CD4 or CD8 expression. T cells which do not bind to the presented molecules go into apoptosis. Subsequently, during negative selection, T cells which are autoreactive (i.e. their cognate antigens are derived from self-molecules) are either instructed to die by apoptosis or to differentiate into regulatory T (Treg) cells [167] which act in the periphery to maintain tolerance to self-molecules. Through these selection mechanisms, naïve T cells which migrate out of the thymus into the circulation comprise a unique and diverse TCR repertoire, capable of maintaining self-tolerance and, equally important, respond to pathogen-derived antigens in peptide-MHC complexes. In the periphery, full naïve T cell activation occurs by three signals during an APC-T cell interaction. The first signal represents the MHC-antigen complex which binds to the TCR and induces intracellular signaling events through the TCR complex which instruct the T cell to proliferate. The second signal is a costimulatory signal which can be induced by the interaction between CD28 on the cell membrane of a T cell which binds to CD80 or CD86 on the APC. Other costimulatory signals exist and have either activating or inhibitory effects on T cells. A third signal is the release of cytokines by the APC which skews differentiation of the activated T cell [168]. These three signals together instruct a T cell to clonally expand and differentiate into specialized subsets of T cells, capable of destroying the pathogen from which the antigen was derived or induce tolerance to self-antigens. In atherosclerosis, APCs engulf lipoproteins such as LDL and oxLDL inside a lesion and migrate towards a lymph node which drains the atherosclerotic lesion to present the antigen (e.g. an ApoB100 peptide fragment) to a naïve T cell and induce its activation. Upon its activation, naïve T cells differentiate into effector T cells and migrate towards the atherosclerotic lesion via the blood where they are activated by APCs presenting their cognate antigen, thereby inducing secondary activation (fig. 3).

Through this T cell response, T cells are instructed to resolve lipoprotein accumulation which are actually a modified form of self-antigens, explaining why atherosclerosis can be considered an autoimmune-like disease. T cells which are observed in atherosclerotic lesions are in an activated state [169]. Not surprisingly, $CD4^+$ T cells from the lesion have been shown to respond to oxLDL [170], indicating T cells which are present in the lesion respond in an antigen-specific manner. $CD4^+$ T helper (Th) cells primarily regulate humoral immunity by modulating other immune cells through the secretion of inflammatory mediators such as cytokines and growth factors. Various types of $CD4^+$ T cells have been characterized, but research in atherosclerosis has so far focused primarily on Th1, Th2, Th17 and regulatory T (Treg) cells.

Depending on the environmental signals (primarily cytokines) which naïve T cells receive during activation, T cells differentiate into specialized types of Th cells. Th1 cells have high expression of the transcription factor T-bet and differentiate under the influence of IL-12 and IFNy [168]. Th1 cells are the main subset of T cells observed in murine

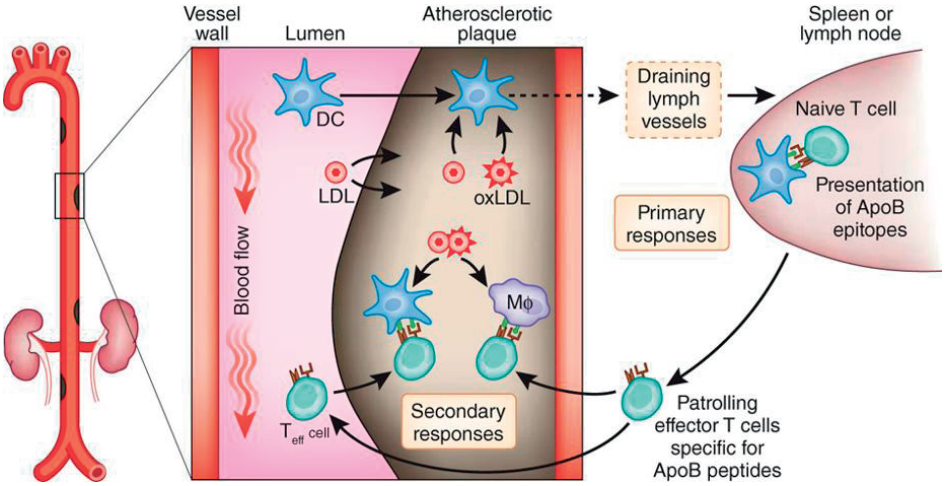

Figure 3 T cell response in atherosclerosis. A T cell response in atherosclerosis is initiated when DCs migrate to the atherosclerotic plaque and engulf native LDL or modified LDL (oxLDL) and subsequently migrate to a lymph node via the draining lymph vessels. Alternatively, blood-borne antigens can be presented by DCs to naïve T cells in the spleen. DCs present the processed antigen (such as peptides from the ApoB100 protein) to naïve T cells. Naïve T cells differentiate into effector T cells and migrate via the blood towards the site of inflammation, which is the atherosclerotic plaque. Here, effector T cells can be activated again locally (secondary response) by DCs and macrophages (MΦ) upon which they exert their effector function. For T helper cells this includes cytokine secretion whereas for cytotoxic T cells this includes cytokine secretion and inducing cell lysis and cell death of their target cells. *Reproduced with permission from Nature Publishing Group (Springer Nature). G.K. Hansson, A. Hermansson, The immune system in atherosclerosis, Nat. Immunol., 2011;12,204-212.*

and human atherosclerotic lesions [169,171,172]. They are considered pro-atherogenic, mainly through the secretion of inflammatory cytokines such as IFNy. IFNy promotes inflammation by enhancing lipid uptake by macrophages, activating ECs and APCs and by reducing collagen production by SMCs [173]. In support of their inflammatory contribution in atherosclerosis, deficiency of T-bet [174] and the inhibition of Th1 differentiation inhibits atherogenesis [175]. In line, deficiency for the IFNy-receptor in *apoE*$^{-/-}$ mice inhibits atherosclerosis [176] and injections of IFNy actually increases atherosclerosis [177].

Th2 cells produce IL-4, IL-5 and IL-13 and are characterized by the expression of GATA-3 [178]. Th2 cells have also been detected in atherosclerotic lesions, although in low numbers [172]. The contribution of Th2 cells to the pathogenesis of atherosclerosis remains controversial as their contribution depends on the stage of the disease and the model which is used [179,180]. IL-4 inhibits the inflammatory Th1 effector function [181] and it has been shown to reduce early lesion formation [180]. Another signature cytokine of Th2 cells, IL-5, reduces atherosclerosis by promoting B1 cells to produce oxLDL-specific IgM antibodies [182]. In a model for atherosclerosis regression, work from our group has shown that OX40-ligand blockade is associated with regression and decreased Th2 cell differentiation and mast

cell activation but increased IL-5 producing T helper cells [183]. The conflicting results come from studies examining IL-4. *IL4*$^{-/-}$ mice actually show reduced atherosclerosis in a bone-marrow transplantation model [184] suggesting Th2 cells promote atherosclerosis, while IL-4 treatment of *apoE*$^{-/-}$ mice had no effect on atherosclerotic lesion size [185].

Another type of Th cell which has been detected in atherosclerotic lesions is the Th17 cell [186]. Th17 cells can be generated by the cytokines transforming growth factor beta (TGFβ) and IL-6 which activates signal transducer and activator of transcription 3 (STAT3) and lead to the expression of the signature transcription factor of Th17 cells: RORyt [187]. Th17 cells are the main source of IL-17 and additionally secrete IL-21 and IL-22. The contribution of Th17 cells to atherosclerosis remains controversial as reports show conflicting results of IL-17 and IL-17 deficiency [188]. Th17 cells are observed in the lesions of unstable angina patients [189] and unstable lesions contain elevated levels of IL-17A [190] compared to stable lesions. In *apoE*$^{-/-}$ mice, blockade of IL-17 reduces atherosclerosis [191] and in line, IL-17A- and IL-17RA-deficiency reduces atherosclerosis [192]. However, other reports have shown that IL-17A deficiency had either no effect on atherosclerosis [193] or even enhanced atherosclerotic lesion size [194]. While the role of Th1 cells in the pathogenesis of atherosclerosis seems clear, future experimental studies using T cell specific genetic blockade of signature transcription factors or cytokines should shed more light on the exact contribution of other Th cell subsets to atherosclerosis.

In contrast to Th cells, Treg cells regulate immune responses by inhibiting other immune cells to maintain self-tolerance and dampen tissue damage during inflammation [195]. Peripheral Treg cells can be thymic-derived or have differentiated in peripheral tissues from naïve T cells under influence of the cytokines TGFβ [196] or through weak TCR stimulation [197]. Treg cells exert their immunosuppressive function through the secretion of anti-inflammatory cytokines such as IL-10 and TGFβ and direct cell-cell contact [198,199]. Upon binding of IL-10 to its receptor IL-10R on their target cells, intracellular signaling induces anti-inflammatory effects [200]. TGFβ has a wide array of effects on immune cells but in the context of Treg cells in atherosclerosis it is considered to be mainly atheroprotective [201]. Treg cells can suppress effector T cells by direct cell-cell contact, partly through the interaction of CTLA-4 which binds to the costimulatory molecule CD80 and CD86 on the surface of target cells, thereby preventing their association with CD28 on the surface of T cells. Other regulatory molecules of Treg cells include GITR and ICOS. Additionally, Treg cells have been described to inhibit multiple atherogenic mechanisms, including EC activation, foam cell formation and the activity of DCs (fig. 4) [202].

Initially, Treg cells were identified as CD4$^+$ T cells with high expression of the IL-2 receptor alpha (CD25). The high expression of CD25 in Treg cells functions as an immunosuppressive mechanism as it has been described to deplete IL-2 from CD8$^+$ effector T cells [203]. Moreover, Treg cells rely on IL-2 for their functional stability [204]. Upon binding of IL-2, CD25 induces intracellular signaling via STAT5 which induces and maintains

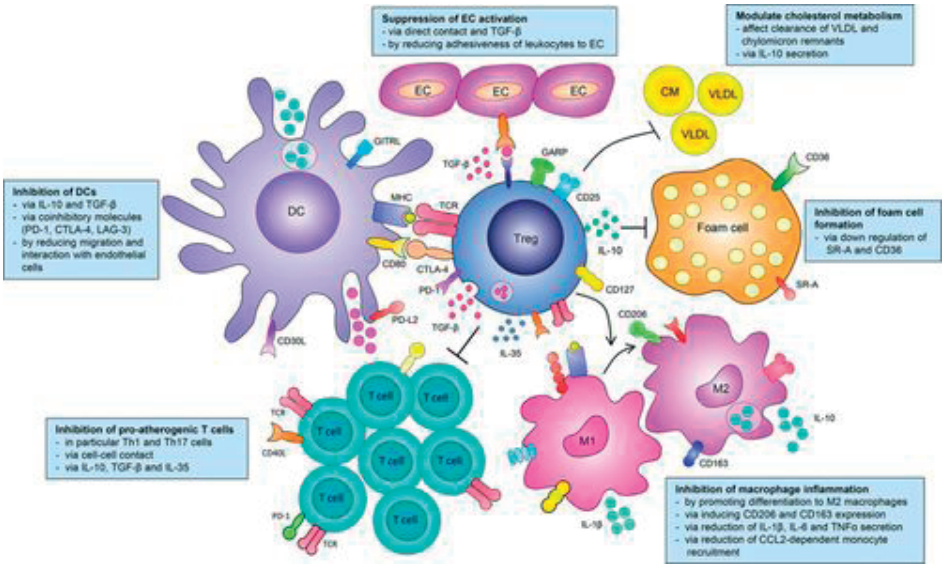

Figure 4 Treg cell suppression of atherogenic immune cell mechanisms. Through cytokine secretion and direct cell-cell interactions, Treg cells inhibit various atherogenic mechanisms and cell types. Treg cells can inhibit EC activation and foam cell formation which are both main mechanisms involved in the early stage of lesion development. Moreover, Treg cells can promote the differentiation into M2 macrophages and inhibit monocyte recruitment. Through inhibition of DCs and Th1 cells, Treg cells inhibit the major inflammatory mechanism in the adaptive immune pathway in atherosclerosis. *Reproduced with permission from Wolters Kluwer Health, Inc. A.C. Foks, A.H. Lichtman, J. Kuiper, Treating Atherosclerosis With Regulatory T Cells, Arterioscler Thromb Vasc Biol. 2015;35:280-287.*

the expression of forkhead box 3 (FoxP3). FoxP3 is a crucial factor for Treg cells and its transcriptional targets maintain the functional integrity of Treg cells through its target genes such as *IL-10*, *CD25*, *CTLA4* [205,206]. The relevance of Treg cells in maintaining immunological tolerance is characterized by scurfy mice which lack FoxP3 and develop an X-linked lymphoproliferative disorder. In humans, dysfunction of the FoxP3 protein leads to the autoimmune disorder IPEX syndrome (immunodysregulation polyendocrinopathy enteropathy X-linked).

In atherosclerosis, Treg cells have been implicated as a promising therapeutic approach to dampen autoimmunity and ameliorate disease. The therapeutic potential to treat atherosclerosis using Treg cells has been elegantly reviewed elsewhere by Foks et al. [202]. Treg cells are an interesting therapeutic point of approach since CVD and atherosclerosis are associated with low numbers and decreased suppressive function of Treg cells [202]. In human atherosclerotic lesions, only 1-5% of all T cells are Treg cells [207] while sufficient suppression by Treg cells is generally attained when ~30% of the T cells are Treg cells [208]. In line, low numbers of Treg cells have been associated with an increased risk of MI [209] and acute coronary syndromes [210]. *In vivo,* Treg cell numbers decrease in *Ldlr*$^{-/-}$ mice as lesions

progress [211]. Depletion of CD25 expressing cells increases atherosclerotic lesion size in *apoE*$^{-/-}$ mice [212]. In another experimental approach, Treg cells were depleted using the depletion of regulatory T cell (DEREG) mice which developed increased atherosclerosis as compared to mice with Treg cells [213]. In line, work from our lab has shown that vaccination of mice against FoxP3 to deplete Tregs significantly increased atherosclerosis [214]. The atherosclerosis ameliorating effect of Treg cells is also shown using the opposite experimental approach. Expansion of Treg cells using an IL-2/anti-IL-2 complex [215] or through adoptive transfer of Treg cells both decrease atherosclerosis [212,216].

Cytotoxic CD8$^+$ T cells primarily regulate cellular immunity through the secretion of cytokines, but also via induction of cell death in target cells through cell lysis and the induction of apoptosis [217] . CD8$^+$ T cells are found in atherosclerotic lesions in all stages [218]. In atherosclerosis, CD8$^+$ T cells mainly exert their cytotoxic function by secreting cytokines and killing target cells, presumably monocytes, macrophages and smooth muscle cells [219]. Herein, IFNy, perforin and granzyme-B are considered to be the most important mechanisms through which they exert their function [219,220]. In experimental atherosclerosis, antibody induced depletion of CD8$^+$ T cells decreases atherosclerosis while adoptive transfer of CD8$^+$ T cells into Rag2 deficient *apoE*$^{-/-}$ mice aggravates atherosclerotic lesion size [219]. These results indicate that CD8$^+$ T cells are detrimental and enhance atherosclerosis. However, *CD8*$^{-/-}$*apoE*$^{-/-}$ mice show no difference in atherosclerotic lesion size as compared to *apoE*$^{-/-}$ mice [221]. The contribution of CD8$^+$ T cells in the pathophysiology is likely dependent on the stage of lesion development and the subset of CD8$^+$ T cells. In early lesions, CD8$^+$ T cells might dampen lesion growth by killing macrophages and thereby help resolve early inflammation. In advanced stages, the killing of SMCs and secretion of IFNy might contribute to decreased lesion stability. In line with a more complex role for CD8$^+$ T cells than experimental work has suggested, a protective type of CD8$^+$ T cells, being Qa-1 restricted CD8$^+$ T cells, have recently been suggested to protect against atherosclerosis [222]. Furthermore, immunization of *apoE*$^{-/-}$ mice with ApoB100 derived peptides protects against atherosclerosis in a CD8$^+$ T cell dependent manner [223]. Altogether, unraveling the role of CD8$^+$ T cells in the pathogenesis of atherosclerosis and their potential targeting for vaccination purposes can significantly contribute to the field of atherosclerosis.

NKT cells are a specialized subset of T cells which are generated in the thymus and, upon their maturation, home to lymphoid tissues and, for a large part, to the liver. Like CD4$^+$ and CD8$^+$ T cells, NKT cells express a TCR composed of TCRα and TCRβ subunits. What distinguishes them from other T cells is that they have typical natural killer cell characteristics such as high membrane expression of NK1.1, Ly49, CD16 and CD122 and the capacity to lyse target cells through granzyme-B and perforin [224]. Moreover, their TCR is unique and does not respond to peptide-MHC complexes but to endogenous and exogenous (glyco)lipid antigens presented on CD1d [225,226]. Upon their activation, NKT cells

secrete a plethora of Th1 and Th2 cell cytokines, including IL-2, IFNy, TNFα, IL-4, IL-5 and IL-10 [227,228]. NKT cells can be activated by foreign lipids and glycolipids. Not surprisingly, NKT cells contribute to the pathogenesis of atherosclerosis in multiple stages of the disease [229,230] which has been extensively reviewed by van Puijvelde et al. [231]. NKT cells can promote atherogenesis through the secretion of cytokines [232] but also in a granzyme-B and perforin-dependent manner [231]. In advanced stages of the disease, NKT cell activation may affect lesion stability through the induction of apoptosis and necrosis of their target cells, like SMCs [233]. Importantly, the ligand for NKT cells in atherosclerosis still remains to be identified and the inflammatory phenotype of NKT cells heavily depends on the ligand which is used for their activation. Therefore, the identification of the NKT cell ligand(s) in atherosclerosis is crucial to investigate the exact contribution of NKT cells to different stages of atherosclerosis.

5. CELLULAR METABOLISM

Immune cells, like all cells, require metabolism to meet their energetic and biosynthetic demand under physiological and pathological conditions. Webster's dictionary defines metabolism as "The chemical changes in living cells by which energy is provided for vital processes and activities and new material is assimilated". In this definition, the two arms of metabolism, catabolism and anabolism, are included. The chemical changes which provide energy is defined as catabolism, i.e. the breakdown of macromolecules to smaller molecules which generates energy in the form of ATP and provides metabolic intermediates that can be used for redox reactions which generate energy. The 'new material' is assimilated in the process of anabolism, in which biosynthetic processes incorporate smaller molecules into macromolecules which can contain energy (such as the synthesis of fatty acids from acetyl-CoA) but do not necessarily do so (such as the synthesis of cholesterol from acetyl-CoA). A nuance which lacks in the definition by Webster's dictionary is the difference between systemic metabolism and cellular metabolism. Systemic metabolism occurs on a tissue and humoral scale and, as previously mentioned, aims to provide peripheral tissues with essential macromolecules and store excess macromolecules in specialized tissues. The sensory and effector mechanism of systemic metabolism takes place at the cellular level, highlighting the importance of cellular metabolism. For example, after a carbohydrate-rich meal, the blood glucose levels rise (systemic) which is sensed by β-cells (cellular level) in the pancreas which respond by secreting insulin. Disturbed systemic metabolism can be pathological on a cellular level. For example, in hepatic steatosis (or fatty liver disease), prolonged dietary intake of excess lipids increases the amount of circulating lipoproteins (systemic level) which leads to abnormal tissue retention of triglycerides. Hepatic steatosis on a cellular level is

reflected by increased uptake of lipoproteins and FFA by hepatocytes and Kupffer cells. These cells subsequently esterify FFA molecules to a glycerol molecule, in the process of triglyceride synthesis, to prevent lipotoxicity.

Immunometabolism is the field which studies how cellular metabolism impacts immune cell function. In immune cells, resting conditions, like those in naïve T cells, require minimal energy expenditure and minimal biosynthetic activity. However, upon their activation by an APC, the bioenergetic and biosynthetic demand changes as cell growth, proliferation and differentiation are required to clonally expand. Cellular metabolism is essential for an immune cell to respond to these kinds of environmental stimuli. These environmental stimuli include the abundance of lipopolysaccharides, cytokines, growth factors, chemokines, costimulatory molecules, antigen-receptor interactions but also changes in substrate abundance [234] and certain neuroendocrine hormones such as leptin [235]. Through the breakdown and synthesis of macromolecules, immune cells meet the metabolic demand which is required to adequately respond to the instructive signals from the (inflammatory) environment. In naïve T cells, the instructive signal is to proliferate, which requires vast amounts of lipids to build cell membranes, proteins to generate organelles, nucleotides to copy the genomic DNA and so forth. Cell growth and proliferation can be the result of instructive signals, but during an immune response, immune cells can also be instructed to migrate or increase protein glycosylation (e.g. antibody production by B cells) [236]. Depending on the cell type and inflammatory process which is required, the activity of a specific metabolic pathway can be increased. This occurs through increases in the expression of substrate transporters at the cell membrane, increases in or activation of the (rate-limiting) enzymes or through altered shuttling processes which facilitate the trafficking of certain metabolites to the correct organelle. The field of immunometabolism is rapidly expanding and the associations between atherosclerosis and cellular metabolism in macrophages, DCs and T cells has recently gained a lot of interest. Details on T cell metabolism in the context of metabolic disease-associated autoimmunity and its potential as a therapeutic target is reviewed in chapter 2.

6. AUTOPHAGY

A cellular process which is tightly linked to metabolism is autophagy, as autophagy degrades intracellular cargo such as proteins and organelles via lysosomal degradation for recycling purposes. There are three types of autophagy; macroautophagy, microautophagy and chaperone-mediated autophagy. Microautophagy is generally a non-selective process in which invaginations in lysosomal membranes directly target cytoplasmic cargo for degradation. It is mostly studied in yeast and the relevance to

mammalian cells is unclear [237]. Chaperone-mediated autophagy is involved in the degradation of cytoplasmic proteins in which specific proteins are bound by chaperone proteins on the lysosomal membrane after which they are directly transported across the lysosomal membrane for degradation [237]. Macroautophagy is the most studied form of autophagy in mammalian cells. Macroautophagy (from henceforth called autophagy) is a well-conserved cellular process in which cytoplasmic cargo is selectively or non-selectively isolated in double-membrane vesicles called autophagosomes and subsequently transported to lysosomes for lysosomal degradation.

Autophagy is induced under various types of stress. Starvation induces autophagy to meet the metabolic demand under nutrient scarcity in an intrinsic manner. On the other hand, autophagy can also be induced by nutrient overload, like is the case during dyslipidemia. In macrophages, autophagy is upregulated to degrade lipid droplets and facilitate reverse cholesterol transport [238]. Autophagy has been proposed to have both protective effects in atherosclerosis, through the degradation of organelles with oxidative stress-induced dysfunction, as detrimental effects, through the deposition of oxidative agents in the microenvironment which promote lipid peroxidation [41]. In vascular SMC, defective autophagy promoted neointima formation and diet-induced atherogenesis [239]. The role of autophagy in adaptive immune cells has also been studied, and its link to cellular metabolism in T cells has recently been reviewed [240]. In specific subsets of T cells, autophagy is upregulated upon activation as the degradation of cytosolic content provides energy when the metabolic demand is high [241]. Genetic blockade of autophagy inhibits the proliferative capacity of T helper cells and reduces memory T cell formation in cytotoxic T cells [241–243]. Moreover, defective autophagy in Treg cells impairs their functional integrity [244,245], highlighting the importance of autophagy in the function of different subsets of T cells. The therapeutic feasibility of pharmacological autophagy inhibition to dampen inflammation and ameliorate atherosclerosis has already been implicated by others [246]. Examining autophagy in T cells in the context of atherosclerosis is required to support this approach and perhaps provide novel therapeutic approaches in CVD.

7. THESIS OUTLINE

Since immune cells have a large contribution to the pathophysiology of atherosclerosis, experimental research has focused on developing immunomodulatory therapies to treat atherosclerosis. Research in atherosclerosis has shown that dyslipidemia drives T cell-mediated immunity by increasing the abundance of antigens derived from native and modified lipoproteins. Cellular metabolism, such as FA oxidation, and intracellular processes linked to metabolism, such as autophagy, are crucial intrinsic processes

involved in T cell-mediated immunity. Interestingly, the antigen-independent immunomodulatory effects of dyslipidemia on cellular metabolism and autophagy in T cells has been unexplored, as has the therapeutic feasibility of targeting these mechanisms to modulate T cell-mediated immunity in atherosclerosis.

The aim of this dissertation is to examine the effects of dyslipidemia-induced nutrient overload in T cells on their cellular metabolism, autophagy and inflammatory phenotype.

In **chapter 2**, the main metabolic pathways and modulators of metabolism in T cells are discussed and how these can be modulated by nutrient overload and used as a therapeutic approach to dampen T cell-mediated autoimmunity.

In **chapter 3**, we report our findings on how diet-induced dyslipidemia affects lipid and glycolytic metabolism of Treg cells. Moreover, we discuss the functional implications of these effects.

In **chapter 4**, we discuss whether and how diet-induced dyslipidemia and lipoproteins can affect autophagy in naïve T cells, prime them to alter their proliferative capacity and skew their differentiation upon activation.

In **chapter 5**, the effect of genetic blockade of autophagy in T cells on the induction of advanced atherosclerotic lesions are discussed.

In **chapter 6**, the contribution of the glycoprotein lipocalin-2 to atherosclerosis is examined as it has been shown to contribute to coronary artery disease as well as to the development of a metabolic syndrome-like phenotype. Hence, Lcn2 might have indirect effects on T cell metabolism and autophagy in the context of dyslipidemia and atherosclerosis.

Finally, we will summarize the data reported in this thesis in **chapter 7** and reflect on how our findings contribute to the knowledge about dyslipidemia and T cells in atherosclerosis.

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CHAPTER 2

T cell metabolism in metabolic disease-associated autoimmunity

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ABSTRACT

This review discusses the relevant metabolic pathways and their regulators which show potential for T cell metabolism-based immunotherapy in diseases hallmarked by both metabolic disease and autoimmunity. Multiple therapeutic approaches using existing pharmaceuticals are possible from a rationale in which T cell metabolism forms the hub in dampening the T cell component of autoimmunity in metabolic diseases. Future research into the effects of a metabolically aberrant micro-environment on T cell metabolism and its potential as a therapeutic target for immunomodulation could lead to novel treatment strategies for metabolic disease-associated autoimmunity.

KEYWORDS

T cells, cellular metabolism, autophagy, diabetes, dyslipidemia, therapeutics

INTRODUCTION

Metabolism is defined as the complex network of (bio)chemical processes occurring in organs and cells required to sustain life. Metabolism is divided into catabolism and anabolism. Catabolism is the degradation/breakdown of macromolecules, generating energy and/or precursors for anabolic processes. Anabolism is the assembly of macromolecules, an energy consuming process. The activity of and (im)balance between these two processes is crucial for various cellular processes, providing energy and building blocks for cellular proliferation, differentiation, function and survival [1]. Tumor cells (and proliferating cells) switch their metabolism from a respiratory towards a glycolytic profile, despite the presence of oxygen [2] to facilitate proliferation [1]. The bioenergetic and biosynthetic requirements of tumor cells may resemble those of proliferating T cells during clonal expansion [3,4]. Naïve T cells (Tn) are in a metabolically dormant state primarily relying on glucose derived pyruvate, fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) to meet a low bioenergetic and biosynthetic demand [5]. After activation of a Tn cell, through mitogens or by T cell receptor (TCR) stimulation, T cells switch to a metabolic state characterized by high rates of glycolysis [4], glutaminolysis [6–8], and OXPHOS [9,10] to provide energy and metabolic intermediates to generate macromolecules and eventually, new effector T cells (Teff) [5,11]. Differentiation into memory T cells (Tm) after clearance of a pathogen is on its turn characterized by a distinct metabolic switch [12–14]. Accordingly, inadequate nutrient availability is a limiting factor for T cell proliferation, differentiation and function [15–17]. In general, metabolism in T cells facilitates an appropriate inflammatory response to specific antigens in a multitude of inflammatory environments with varying nutrient and oxygen availability.

Many diseases (and their complications) challenging the modern health care system are characterized by metabolic disorders and chronic low-grade inflammation [18], including, diabetes [19], atherosclerosis [20], obesity [21] and resulting cardiovascular disease. These metabolic diseases are characterized by systemic metabolic dysregulation, which creates an abnormal metabolic environment for T cells to cope with in the circulation, lymphoid tissue and at the site of inflammation. An abnormal metabolic environment can result from autoimmunity directed to metabolic tissues, as is the case in type 1 diabetes mellitus (T1DM) [22–25]. Vice versa, an abnormal metabolic environment can be a risk factor for disease development. For example, dyslipidemia in familial hypercholesterolemia (FH) patients increases the risk of developing atherosclerosis and cardiovascular disease [26–28]. Nevertheless, these diseases are generally associated with a type 1 autoimmune response [21,25,29,30] and a loss of tolerance by regulatory T cells [31–34]. Currently, it is relatively unknown if and to what extent a metabolically aberrant micro-environment caused by systemic metabolic defects affects T cell metabolism and whether this contributes to

development or progression of diseases hallmarked by both metabolic disease and an autoimmune-like response.

Reviews on T cell metabolism have already identified its potential as a target for therapeutic intervention as well as how altered systemic metabolism in metabolic diseases might affect cellular metabolism in immune cells [35–37]. We now aim to conceive different ways in which a metabolically aberrant micro-environment might affect T cells during metabolic diseases such as diabetes, familial hypercholesterolemia and obesity and how this could contribute to disease progression. Furthermore, we propose different strategies to target T cell metabolism and how existing pharmaceuticals may be implemented to (specifically) modulate T cell metabolism and inhibit disease progression.

SUBSTRATES OF CELLULAR METABOLISM

Glucose

To understand how a metabolically aberrant environment could influence T cell metabolism directly, the most important substrates for T cell metabolism will be discussed. Glucose is one of the vital substrates for T cells to generate ATP [38]. Glucose is mainly taken up by T cells via glucose transporter 1 (GLUT1) [39] and catabolized in the cytoplasm through glycolysis to produce pyruvate and ATP. Pyruvate can subsequently be converted into lactic acid to provide nicotinamide adenine dinucleotide (NAD^+) for redox reactions in the cytosol (e.g. glycolysis). Alternatively, acetyl-CoA is generated in the mitochondria from pyruvate by pyruvate dehydrogenase. Acetyl-CoA can enter the tricarboxylic acid (TCA) cycle to generate the reducing agents NADH and flavin adenine dinucleotide (FADH2) (through a series of biochemical reactions). The latter agents fuel oxidative phosphorylation (OXPHOS) by providing electrons for the electron transport chain (ETC) and creating a proton gradient across the inner mitochondrial membrane for ATP-synthase to convert ADP to ATP. Glycolysis is considered a rapid but relatively inefficient process for energy production whereas OXPHOS is efficient but time- and oxygen consuming [1]. Activated T cells utilize glucose and glycolysis for cell growth and proliferation [40]. The $CD4^+$ T helper (Th) cells Th1, Th2 and Th17, characterized by high levels of interferon-γ (IFNγ), interleukin-4 (IL-4) and IL-17 respectively, are highly glycolytic and depend on high GLUT1 expression for their function [39,41]. Regulatory T cells (Treg), which dampen inflammatory responses, rely less on GLUT1 expression for their function [39] and exhibit relatively high levels of fatty acid oxidation (FAO) and OXPHOS during differentiation and proliferation [41–43]. During T cell proliferation, glycolysis additionally generates metabolic intermediates for anabolic pathways rather than just energy [40,44] while OXPHOS is required for energy generation [9]. In activated T cells, glucose influx via GLUT1 is induced by CD28 co-stimulation in synergy with TCR/CD3 crosslinking [45].

Herein, CD28 acts as an adaptor protein to increase PI3K-Akt signaling which subsequently enhances expression of GLUT1, glucose uptake and glycolysis [45]. The necessity of adequate membrane GLUT1 levels for $CD4^+$ and $CD8^+$ T cell proliferation is illustrated by a decrease in homeostatic and activation-induced proliferation of GLUT1-deficient T cells as compared to their wildtype (WT) control [39]. GLUT1-deficient T cells also show diminished growth and survival upon *in vitro* stimulation indicating glucose influx is required for adequate blast, prior to T cell proliferation [39]. Interestingly, human inducible Tregs (iTreg) have a metabolic program seemingly distinct from murine iTregs. iTregs are highly glycolytic and rely less on FAO for their differentiation and also for their suppressive capacity, human iTregs are more dependent on glycolysis than on FAO [42]. Accordingly, inhibiting glycolysis with the glucose analog 2-deoxyglucose (2-DG) during generation of iTregs decreases their frequency as well as their capacity to suppress $CD4^+$ T helper cells *in vitro* [42]. *Ex vivo* analysis of isolated human Tregs has shown that they primarily rely on glycolysis in rest, whereas both FAO and glycolysis are crucial for proliferation [43]. These findings suggest that Tregs are not ubiquitously skewed towards FAO to meet their metabolic needs and emphasize the requirement of glycolysis for human Tregs. In the context of aforementioned findings from murine Tregs, these data suggest that cellular metabolism can be context-dependent which is an important consideration in assessing and modulating T cell metabolism. Altogether, glycolysis is generally associated with an immunostimulatory T cell response and inhibiting glycolysis using 2-DG seems feasible to dampen a T cell response as characterized by the use of 2-DG to inhibit the $CD8^+$ T cell response in prediabetic NOD mice [46].

Glutamine

Glutamine which is transported into T cells through various solute carrier transporters (SLC), but primarily by a heterodimer of Slc3a2/Slc7a5 (CD98) [47,48], is a crucial amino acid for rapidly proliferating cells [44,49,50]. During glutaminolysis in the cytoplasm, glutamine is primarily hydrolyzed by the rate-limiting enzyme glutaminase-2 to form glutamate and ammonium [48]. Glutamate can enter the mitochondria where it is converted into alpha ketoglutarate (α-KG) by glutamate dehydrogenase (Glud1). α-KG is anaplerotic (i.e. a substrate for the TCA cycle) and can thus facilitate ATP generation by OXPHOS or by pyruvate synthesis as a precursor for acetyl-CoA formation [50,51]. Alternatively, glutamate can be converted to ornithine and eventually polyamines, which are required for biosynthesis during (T cell) proliferation [52,53]. Like glucose, glutamine uptake increases upon activation of T cells with anti-CD3/CD28 [8] or polyclonal mitogens such as concanavalin A [7,38] and glutaminase activity increases correspondingly [48]. Glutaminase inhibition using 6-Diazo-5-oxo-L-norleucine (DON) illustrates the necessity of glutaminolysis for antigen specific T cell expansion. Treating wildtype (WT) mice with DON results in decreased proliferation of adoptively transferred, OVA-challenged OT-II cells as compared

to vehicle controls [48]. Likewise, glutamine starvation diminishes T cell growth and proliferation *in vitro* [48]. Glutamine availability also affects T cell differentiation as glutamine deprivation or DON treatment in Tn cells specifically increases FoxP3 expression in a TGFβ dependent manner, even under Th1 polarizing conditions [54]. Upon administration of the (cell-permeable) α-KG analog DMK, Th1 differentiation is rescued under glutamine deprivation conditions [54], indicating glutaminolysis is involved in both T cell growth and differentiation. Although an elevated level of circulating glutamine is mostly observed in rare diseases (such as chronic kidney disease) [55], inhibiting glutaminolysis in T cells (e.g. using DON) seems feasible to constrain autoimmunity, possibly in combination with other metabolic pathway inhibiting compounds.

Fatty acids

Fatty acids (FA) form a class of substrates with a high energy density for T cells. FAs can enter the cell through various SLC transporters [56], the low-density lipoprotein receptor (LDLr) [57] or the scavenger receptor fatty acid translocase (FAT/CD36) [58]. Before being oxidatively catabolized during FAO, short-, medium-, and long-chain FAs are activated in the cytosol through acylation by acyl-CoA synthetase to facilitate transport through the outer mitochondrial membrane. Carnitine palmitoyl transferase 1 (CPT1) replaces the acyl group by a carnitine group to facilitate transport across the inner mitochondrial membrane so β-oxidation can occur. Inhibiting CPT1 using etomoxir inhibits β-oxidation accordingly. Through β-oxidation, two carbon-units per cycle are cleaved off through a series of biochemical reactions yielding FADH2, NADH and acetyl-CoA, indicating that β-oxidation is a slow, but highly energetic oxidative process. Interestingly, exogenous FA during *in vitro* Th1 differentiation inhibits the production of Th1 cells [41]. Similarly, Th1, Th2 and Th17 cytokine production is decreased by exogenous FA supplementation while FoxP3 expression and suppressive function in Tregs are increased [41]. Thus, extracellular FA abundance can affect Teff function and Treg abundance and function. During murine graft-versus-host disease, Teff cells require upregulation of the FAO machinery and proliferation of allogenic T cells is inhibited by etomoxir accordingly [59], which indicates that inhibition of FAO is feasible to dampen inflammation in some diseases. Tm cells generally have a lower metabolic demand and rely to a large extent on FAO [12–14]. Interestingly, FAO and OXPHOS are of particular importance for the increased inflammatory capacity of Tm cells compared to Teff cells during primary activation. Upon activation of *in vitro* induced Tm cells, high levels of glycolysis, a large mitochondrial mass, and resulting high levels of FAO and OXPHOS facilitate their rapid recall capacity [13]. Etomoxir diminishes proliferation of Tm cells while simultaneously decreasing glycolysis and OXPHOS upon restimulation, as indicated by oxygen consumption- and extracellular acidification rate measurements [13]. In contrast, induction of FAO using metformin or rapamycin increases

Tm generation, providing a useful metabolic compound to improve vaccination efficiency [12,60].

Cholesterol

Besides glucose and glutamine influx, T cells also rapidly upregulate a program for the biosynthesis of fatty acids and cholesterol and increased uptake of lipids after TCR stimulation and mitogen stimulation [61–63]. Cholesterol is a crucial factor as it is required for cellular growth and proliferation as a component of cell membranes [61,64,65]. Moreover, it is important for lipid raft formation by regulating membrane fluidity, which might play a role in T cell activation by regulating immunological synapse stability [66,67]. *De novo* cholesterol is synthesized in the mevalonate pathway in which 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and squalene epoxidase (SQLE) are the rate-limiting enzymes [68]. Statins, a class of drugs which inhibit HMGCR, inhibits TCR-driven T cell expansion accordingly [69]. After TCR stimulation with anti-CD3, T cells acquire a transcriptional program to decrease cholesterol efflux via ATP- binding cassette (ABC) transporter and increase synthesis through *HMGCR* and *SQLE* expression to ensure adequate cholesterol availability for membrane biogenesis [62,63]. Intracellular cholesterol availability is crucial for ER-membrane biogenesis, an important cell-cycle progression checkpoint, again indicating that (sufficient) cholesterol is required for adequate T cell growth and proliferation [63]. For Tregs, cholesterol synthesis through the mevalonate pathway is particularly important for their function. Treating Tregs with simvastatin decreases their suppressive capacity, whereas additional treatment with mevalonate, the product of HMGCR, restores their suppressive capacity [70]. Remarkably, statin treatment in both healthy individuals and hypercholesterolemia patients appears to increase circulatory Treg numbers and FoxP3 expression [71,72]. Atorvastatin increases Treg generation from peripheral blood mononuclear cells *in vitro* from healthy donors, whereas mevastatin and pravastatin do not [71]. Since the Treg promoting effects appear to be statin-type specific further research is required to evaluate the exact effects of specific statins on Tregs. Altogether, not only T cell blast and proliferation, but also the function of specific T cell subsets depends on adequate cholesterol metabolism.

Autophagy

An intrinsic manner through which T cells can acquire nutrients for bioenergetic and biosynthetic purposes is autophagy. Macroautophagy is the predominant and most-studied form of autophagy and is hereafter simply referred to as autophagy. Autophagy is a catabolic recycling process, through which cytosolic macromolecules (e.g. protein aggregates) and damaged or obsolete organelles can be targeted for lysosomal degradation for self-renewal or nutrient reuse [73]. Autophagy starts when double-membrane structures called phagophores are formed *de novo* through a series of complex processes

involving autophagy-related proteins (ATG), among others [73,74], which subsequently enclose cytoplasmic components to form autophagosomes. Thereafter, autophagosomes fuse with lysosomes to form autolysosomes in which macromolecules are degraded [75–77]. The resulting degradation products can be effluxed into the cytosol when the metabolic demand is high, although the mechanisms behind this process are poorly understood [75,78]. Under metabolic stress (e.g. hypoxia, starvation) autophagy is upregulated to intrinsically cope with a changed environment [79,80]. For example, increased autophagy is required to meet the bioenergetic demand in peripheral $CD4^+$ T cells upon TCR stimulation. Atg7-deficient T cells show decreased activation as measured by proliferation and IL-2 secretion [79]. Furthermore, blocking autophagy during stimulation of $CD4^+$ T cells decreases ATP production, lactate generation and FAO [79] which shows that autophagy plays a critical role in metabolic adaptation upon activation. Autophagy is required for quality control of mitochondria in Tn cells to prevent toxicity from reactive oxygen species [81,82]. Interestingly, mitochondria are largely excluded from autophagosomal degradation upon activation in T cells [79] as these need functional mitochondria for proper activation [9]. Interestingly, macrophages and foam cells require autophagy of lipid droplets (lipophagy) when loaded with lipoproteins *in vitro* to degrade cholesteryl-esters and subsequently efflux cholesterol via ABC transporters [83]. Autophagy might play a similar role in T cells for them to cope with a non-physiological micro-environment in metabolic disease and inhibition of autophagy might therefore not be desirable for therapy as will be elaborated further on.

T cells depend on varying substrates to meet the metabolic demand required to transcend from Tn cells into different Teff subtypes and Tm cells in nutrient- and oxygen rich lymphoid tissue and metabolically restricted tissues. Accordingly, different metabolic pathways can be targeted, also simultaneously, to regulate cellular metabolism in T cells to dampen proliferation and skew differentiation (fig. 1). This is an interesting approach and could be especially interesting for treating diseases in which a single substrate is particularly abundant.

REGULATORS OF METABOLISM

To understand the coupling between different metabolic states and inflammatory phenotypes in T cells, the most noteworthy therapeutic targets for T cell metabolism are summarized. A widely studied and important regulator of cellular metabolism during biogenesis and biosynthesis is the serine/threonine protein kinase mammalian target of rapamycin (mTOR). mTOR combines metabolic and environmental signals to regulate a wide range of processes including cell growth, proliferation and autophagy. It is the catalytic subunit of two structurally and functionally distinct protein complexes:

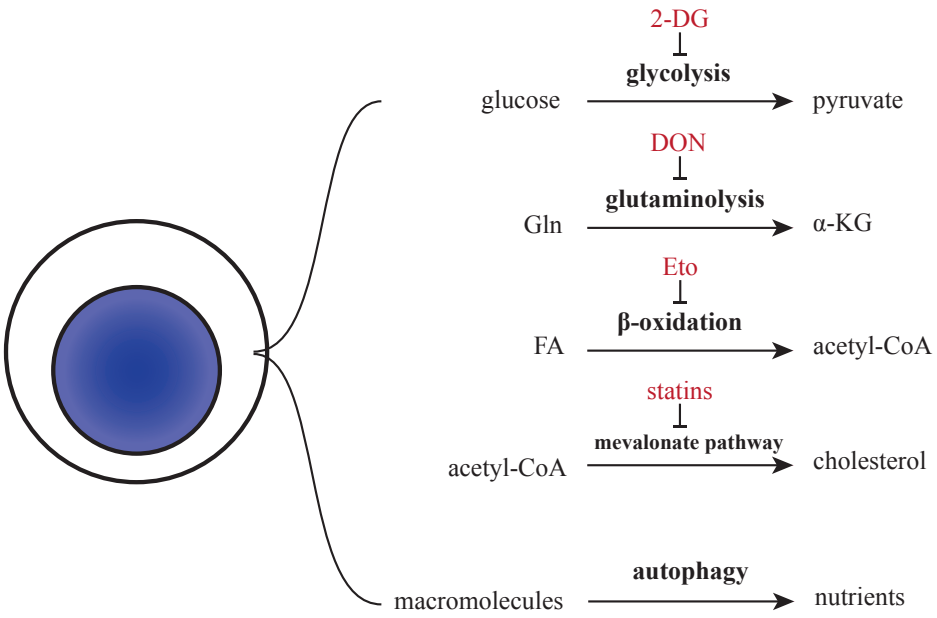

Figure 1 The most relevant metabolic pathways for T cell proliferation and differentiation are depicted with their corresponding inhibitors. The catabolic and anabolic processes which are important for adequate T cell growth, proliferation and differentiation are summarized with the most frequently used corresponding inhibitors. Autophagy is a cellular process T cells upregulate upon activation. Inhibiting T cell autophagy might not be desirable from a therapeutic standpoint. **2-DG**=2-deoxyglucose, **DON**=6-Diazo-5-oxo-L-norleucine, **Eto**=etomoxir.

mTORC1 and mTORC2. In mTORC1, mTOR is in a protein-complex with, among others, regulatory associated protein of mTOR (RAPTOR) while in mTORC2 it is complexed to rapamycin-insensitive companion of mTOR (RICTOR). mTORC1 is involved in nutrient sensing, energy metabolism and protein synthesis, contributing to cell growth, whereas mTORC2 is mainly involved in cytoskeletal rearrangements and cell survival [84]. Based on these properties, it is apparent mTOR signaling is crucial for adequate T cell growth, proliferation and migration [85–87]. Moreover, mTOR is a potent inhibitor of autophagy [88–90], which, together with its role in regulating metabolism, makes it a particularly interesting target for therapy. In regulating glycolysis, mTORC1 can act through hypoxia inducible factor 1 alpha (HIF1α) by increasing its transcriptional activity and its translation, partly by increasing the translation of the 5'UTR sequence of a HIF1α [91,92]. HIF1α regulates the expression of GLUT1 and enzymes required for glycolysis while simultaneously favoring glycolysis over OXPHOS through induction of pyruvate dehydrogenase kinase 1 (PDK1), which prevents pyruvate from entering the TCA cycle [93,94]. HIF1α is upregulated under hypoxic conditions to ensure sufficient energy is generated through anaerobic glycolysis [95]. *In vitro,* HIF1α-deficient T cells show a moderate decrease in expression of hexokinase 2

(HK2) and lactate dehydrogenase A (LDHa) with similar effects on glycolytic rates (within 72h after stimulation) as compared to control T cells, indicating HIF1α is dispensable for acute metabolic programming prior to T cell proliferation. Rather, it is presumed HIF1α plays a role in maintaining glycolysis when T cells enter mitosis [48]. Apart from regulation of glycolysis through HIF1α, mTORC1 can indirectly regulate metabolism through regulation of the YY1-PGC1α complex whose main targets are genes involved in mitochondrial biogenesis and mitochondrial respiration [96,97]. mTORC1 can also promote *de novo* pyrimidine synthesis through its downstream target S6 kinase [98,99]. The role of mTORC2 in metabolism still remains relatively unexplored. Interestingly, PTEN-deficient Tregs show increased glycolysis and decreased mitochondrial fitness suggesting mTORC2 function might also modulate T cell metabolism [100]. mTORC2 is inhibited by long-term rapamycin treatment in various cell types by inhibiting the assembly of the mTORC2 complex [101]. As the function and regulation of mTORC2 are still poorly understood and target-specific compounds are scarce, exploration of the metabolic effector function will be crucial in predicting the therapeutic potential of long-term mTOR modulation.

Myc is another well-recognized protein involved in T cell metabolism and proliferation [48]. Myc is a highly conserved transcription factor, which induces cell cycle progression by regulating p27, cyclins, and CDKs [102,103]. During T cell activation, Myc induces glycolysis and glutaminolysis as Myc is a transcription factor for GLUT1, HK2, pyruvate kinase (PK), and LDHa (which are important for glycolysis) [48,104]. Furthermore, Myc increases the expression of glutamine transporters and mitochondrial glutaminase expression, the latter by repressing microRNAs 23a and 23b [105,106]. The paper of Wang et al. specifically examined the various roles of Myc in the metabolic programming preceding T cell proliferation. Myc-driven glutaminolysis is severely abrogated in *Myc*-deficient T cells with a concomitant decrease in genes and metabolic intermediates involved in polyamine synthesis [48]. Furthermore, Myc-deficient T cells display decreased glycolysis, most likely via decreased expression of HK2 and PK isoform M2 and $CD4^+$ and $CD8^+$ T cell proliferation is severely decreased, indicating Myc-dependent transcriptional programs are required for T cell proliferation [48]. A decrease in the phosphorylation and protein levels of downstream targets of mTOR (4E-BP and S6) is observed in activated Myc-deficient T cells and T cells activated under glutamine starvation conditions [48]. This is particularly interesting as mTOR activity is regulated through CD98 and intracellular L-glutamine levels as L-glutamine efflux induces the influx of essential amino acids and ultimately mTORC1 activation [47]. This suggests that, during T cell activation, Myc-induced glutamine influx is required for mTOR activation and thus crosstalk might exist between the Myc- and mTOR pathways [48]. Myc-inhibition using synthetic inhibitors such as 10058-F4 can prevent Th1 cells from promoting inflammation [107], indicating the feasibility of Myc as a therapeutic target.

Another important sensor and regulator of cellular metabolism is the heterotrimeric

serine/threonine kinase complex AMP-activated kinase complex (AMPK). AMPK can regulate glucose metabolism in various manners, through increased glucose uptake and increased glycolysis [108–110], inhibition of glycogen synthesis and inhibition of gluconeogenesis [111–113]. A major function of AMPK is to inhibit fatty acid synthesis by inactivating acetyl-CoA carboxylase 1 (ACC1) through phosphorylation and inhibiting sterol regulatory element-binding protein 1 (SREBP1) [114,115]. Furthermore, AMPK drives FAO as it inhibits ACC2 by phosphorylation which is in turn an inhibitor of CPT1A expression [116]. Deficiency in *Prkaa1,* encoding the catalytic subunit of AMPK, does not affect T cell proliferation at a high glucose concentration (25 mM) as compared to WT T cells but at low glucose concentrations (3-6 mM) *Prkaa1*-deficient $CD4^+$ T cells are less proliferative, indicating the necessity for adequate AMPK signaling for proliferation in nutrient-restricted conditions [108]. Counterintuitively, IFNγ production in $CD8^+$ T cells after re-stimulation was higher in $Prkaa^{-/-}$ cells at both physiological (5 mM) and high glucose levels (25 mM) compared to control T cells [108]. This is explained by the fact that AMPK inhibits the translation of IFNγ mRNA [108], which supports immunomodulatory effects of stimulation of AMPK in T cells. Another mechanism by which AMPK can induce ATP production is by promoting autophagy through inhibition of mTORC1 and through phosphorylation and subsequent activation of ULK1 [117,118].

Lastly, lipid metabolism is tightly regulated by the counteracting transcriptional regulators liver-X-receptor (LXR) and SREBP. LXR is a transcription factor, which is activated by various oxysterols [119]. Its main action is to induce cholesterol efflux via ABC transporters [120] thereby, effectively counteracting SREBP [121], regulating adequate cholesterol availability under quiescent or activated conditions. Upon T cell activation, simultaneous downregulation of LXR target gene expression and upregulation of target genes of SREBP-1 and -2 ensures cholesterol efflux is decreased and lipid synthesis is increased, respectively [62,63]. Mitogen-induced and TCR-induced proliferation in LXRβ KO mice suggested this particular isoform of LXR is important for proliferation as both $CD4^+$ as $CD8^+$ T cell proliferation was increased compared to WT [62]. LXR activation with natural or synthetic ligand inhibits proliferation accordingly [62,122,123]. SREBP is a zinc finger helicase mainly involved in fatty acid and cholesterol synthesis and uptake during activation through its target genes *Hmgcr*, *Hmgcs*, *Acaca*, *Fasn*, *LDLr* [124]. During T cell activation, SREBP1 and SREBP2 are simultaneously enriched at some of the promotor sites in their target genes indicating SREBP1 and SREBP2 co-regulate lipid synthesis [63]. Interestingly, pretreating T cells with the mTOR inhibitor rapamycin blocked SREBP mediated lipogenesis during TCR mediated stimulation suggesting a crosstalk between mTOR and SREBP pathways [63,91].

In conclusion, multiple modulators of metabolism in T cells represent suitable candidates for therapeutic intervention. As mTOR is involved in the effector function of other metabolic modulators which generally have an important role in facilitating T cell growth,

proliferation and function, including HIF1α, Myc and SREBP, T cell specific inhibition of mTOR appears most feasible. As activation of AMPK has anti-inflammatory effects, partly through inhibition of mTOR, compounds such as metformin are additionally appealing for T cell modulation. Naturally, this depends on which T cell response is to be inhibited or enhanced in which specific micro-environment.

COUPLING METABOLIC TO INFLAMMATORY PHENOTYPE

Differentiation of T cells into specific subtypes of T helper cells is primarily dependent on the inflammatory context and the ability of T cells to adjust their metabolism. $CD4^+$ Th1 are highly glycolytic and display relatively low FAO, a profile similar to the even more glycolytic Th2 cells [41]. As mTOR regulates upregulation of glycolysis upon activation, mTOR deletion in T cells inhibits Th1, Th2 and Th17 generation while simultaneously favoring the induction of $FoxP3^+$ Tregs upon TCR stimulation of $CD4^+$ T cells [125]. Rheb-deficient T cells, which lack mTORC1 activity, fail to differentiate into Th1 and Th17 cells [126]. Interestingly, mTORC2 is important for Th2 development as is characterized by the ability of RICTOR-deficient T cells to differentiate into Th1 and Th17 but not Th2 cells [126]. As Treg differentiation was unaltered in both Rheb- as well as RICTOR-deficient T cells the increased Treg differentiation observed in mTOR-deficient T cells is dependent on inhibition of both mTOR-complexes [126]. mTOR inhibition using rapamycin and DKM1 induces Treg differentiation [127,128], which is indicative of a Treg suppressive function of mTOR, as inhibition of mTOR induces Treg generation both *in vitro* and *in vivo* [129–131].
In Th17 cells, glycolysis is regulated through HIF1α and Th17 generation is highly dependent on HIF1α [132,133]. HIF1α-deficiency impairs the upregulation of genes involved in glycolysis while decreasing the Th17/Treg ratio. Similarly, inhibiting glycolysis in Tn cells using 2-DG or rapamycin shifts T cell differentiation from Th17 cells towards Treg differentiation [132]. Lipid biogenesis is especially crucial for Th17 differentiation during which ACC1 and ACC2 play a central role [134] by regulating *de novo* FA synthesis from acetyl-CoA. Therefore, ACC1 is important for Th17 development as it can couple glycolysis and pyruvate to lipogenesis, thus facilitating membrane biosynthesis. Inhibiting ACC1 and ACC2 using soraphen under Th17 polarizing conditions skews differentiation towards Tregs [134] indicating the intricate link that exists between these $CD4^+$ T cell subtypes. This also emphasizes the potential for metabolic signals to overrule inflammatory signals. The fact that differentiation of Tn cells into Th17 as well as Treg cells largely depends on the pleiotropic cytokine TGFβ certainly explains the potential of metabolic modulation to induce Tregs in the appropriate environment. Sterols represent another class of environmental regulators of Th17 differentiation. Cholesterol uptake and synthesis are increased during Th17 differentiation [135]. During cholesterol synthesis, desmosterol, a

precursor for cholesterol, serves as an endogenous agonist for RORγ, a key transcription factor for Th17 development [135]. This is surprising as desmosterol is a low-affinity LXR agonist [136] and LXR activation with synthetic ligands inhibits Th17 differentiation [137]. During differentiation, Th17 cells increase SULT2B1 expression which catalyzes sulfate conjugation to sterols, thereby inactivating them as LXR agonists [138,139], while desmosterol sulfate retains its RORγ-binding properties. Altogether, the inhibition of glycolysis or induction of sterol efflux seems feasible to skew Tn cells away from Th17 differentiation, thereby dampening inflammation.

The exact potential of autophagy modulation to affect T cell differentiation from Tn cells remains to be elucidated. While increased autophagy is required for adequate $CD4^+$ T cell proliferation and cytokine secretion, induction of autophagy in immune cells is often associated with an anti-inflammatory profile [140] and dysfunctional autophagy disrupts Treg function [141,142]. Atg7-deficient Tregs show impaired cell survival and stability with corresponding increases in apoptosis markers and decreased FoxP3 stability [141]. Moreover, Atg7-deficient Tregs have increased glycolytic metabolism and increased expression of IFNγ and IL-17 as compared to Atg7 WT Tregs [141]. $Foxp3^{Cre}Atg5^{fl/fl}$ mice have higher IFNγ expression in $CD4^+$ and $CD8^+$ T cells and lower Treg percentages compared to $Foxp3^{Cre}Atg5^{+/fl}$ mice, underlining the requirement of functional autophagy for Tregs to maintain immune homeostasis [141]. Likewise, ATG16L-deficient Tregs show decreased survival and increased glycolysis. Presumably, autophagy functions to degrade intracellular lipid droplets (lipophagy) and increase FA abundance for FAO, thereby improving Treg survival [142]. Therefore, (Treg-specific) stimulation of autophagy using metformin or rapamycin might prove a useful approach to stabilize Tregs and increase Treg abundance to diminish autoimmunity. Differentiated Th1 cells require autophagy upon TCR stimulation for cytokine secretion and proliferation as these parameters decrease upon pharmaceutical inhibition of autophagy using 3-MA and NH_4Cl [79]. Likewise, acute deletion of Atg7 after Th1 differentiation results in diminished IFNγ secretion [79]. As ATP production is severely inhibited in Atg7-deficient T cells it is likely that autophagy plays a role in metabolic adaptations for differentiation and function of more T cell subsets. More research on the intricate link between autophagy and metabolism is necessary to predict the outcome of therapeutic intervention in both processes for each type of T cell.

CELLULAR METABOLISM-BASED T CELL MODULATION

Since cellular metabolism is a determining factor in a T cell response, it provides a non-antigen-specific window for T cell therapeutics in the context of diseases characterized by metabolic disease. While a similar phenomenon has been proposed earlier in metabolic and cardiovascular disease [35,36] here we speculate more in detail about the different

mechanisms through which a metabolically altered micro-environment might influence cellular metabolism and immunological phenotype.
We propose five ways through which a metabolically altered micro-environment might fuel T cell metabolism and thereby contribute to disease progression: 1) through increased substrate abundance in the extracellular micro-environment, 2) through increases in intracellular substrate reservoirs, 3) through skewing of substrate dependence, which could alter the activity of bifunctional enzymes or, 4) skew differentiation from Tn cells into Th, Treg or Teff into Tm cells and lastly, 5) through selective metabolic restriction.

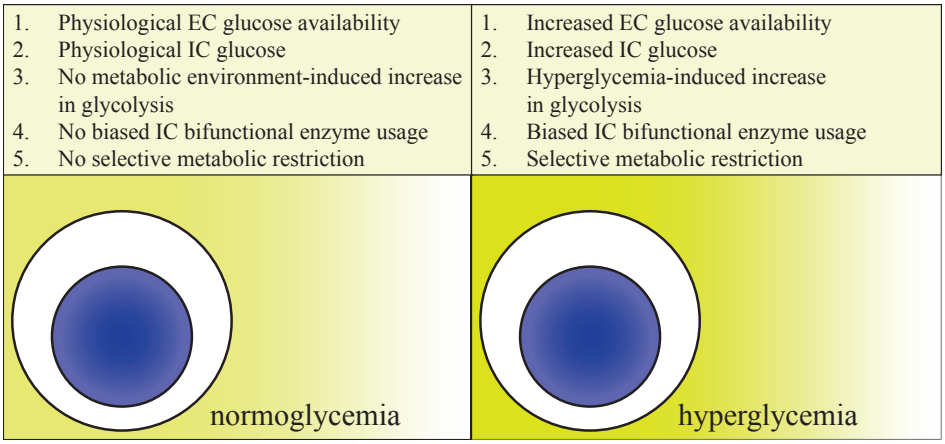

Figure 2 Non-antigen-specific manners through which a metabolically altered micro-environment caused by hyperglycemia might fuel the inflammatory effector functions of T cells and thereby contribute to disease progression. Through these mechanisms, a T cell in a hyperglycemic micro-environment might be more potent in driving inflammation than a T cell with the same cognate antigen in a normoglycemic micro-environment. Similar mechanisms might be applicable to other metabolic diseases, such as dyslipidemia. **EC**=extracellular, **IC**=intracellular.

The proposed mechanisms might be well applicable to diabetes and hyperglycemia (fig. 2). T1DM is a disease in which hyperglycemia caused by $CD4^+$ Th1 and $CD8^+$ T cell mediated autoimmunity against pancreatic islets is one of the hallmarks [22–24]. Insulin insufficiency develops as a result of pancreatic islets degradation, causing T cells to be exposed to prolonged hyperglycemia, which potentially exerts detrimental metabolic effects by one (or several) of the mechanisms described above. As described, *in vitro* hyperglycemia increases IFNy secretion by $CD8^+$ T cells [108]. Although prolonged hyperglycemia in diabetes patients is much milder (fasting plasma glucose ≥ 7 mM) this effect of glucose availability on cytokine secretion does suggest that increased extracellular substrate abundance can indeed fuel T cell mediated autoimmunity. Similarly, culturing T cells under low glucose concentrations decreases the extracellular acidification rate,

a measure for glycolysis, in a dose-dependent manner [108], illustrating the extracellular substrate abundance can dictate the rate at which a certain metabolic pathway is used. Hyperglycemia could increase glycolytic activity in activated T cells which skews GAPDH, an enzyme involved in glycolysis as well as a translational inhibitor of cytokines such as IFNγ [9], availability towards glycolysis. Similarly, increased glycolysis in Tregs might bias enolase-1 (another enzyme from the glycolysis pathway) activity, thereby preventing enolase-1 from binding to the *FoxP3* promotor and FoxP3 CNS2 where it can induce splicing variants of FoxP3 which result in less functional Tregs [42]. Thus, hyperglycemia, and other metabolic diseases, might have anti-inflammatory effects on T cells (or specific T cell subsets) as well, but this review only focuses on the inflammation driving effects for the sake of simplicity. Summarizing, as a result of hyperglycemia, a T cell clone would exhibit increased glycolysis and increased cytokine secretion as compared to the same clone in a normoglycemic environment, thus driving inflammation in a non-antigen-specific manner.

Additionally, selective metabolic restriction might play a pathological role. Metabolic competition exists between progressing tumors and cytotoxic T lymphocytes as highly glycolytic tumors restrict tumor infiltrating lymphocytes from glucose, resulting in a relatively anergic T cell population, unable to properly fight the tumor [143]. A similar discrepancy between supply and demand of certain nutrients for T cells might be present in hypoxic tissues such as atherosclerotic plaques [144–146]. T cell subtypes which are highly glucose consuming with high expression of GLUT1 (e.g. Th1 cells) would benefit more from hyperglycemia than subtypes with an FAO and OXHPOS-dependent profile (e.g. Tregs). Thereby, Th1 cells could deprive Tregs of glucose, an effect which would be particularly pronounced after hyperglycemia has 'primed' Th1 cells through indicated mechanisms and in a micro-environment with nutrient and oxygen scarcity.

Elucidating the mechanisms behind the interplay between altered systemic metabolism and cellular metabolism in T cells would contribute to further understanding T1DM pathology and its comorbidities, for example, an increased risk of atherosclerosis and CVD in T1DM patients [147]. Therapeutic intervention might be possible by inhibiting metabolic pathways, which drive inflammation or by inducing metabolic pathways with an immunomodulatory effect.

Although various immune cells are at the basis of the inflammatory response against pancreatic islets, one central phenomenon contributing to the pathology of T1DM is loss of tolerance by Tregs [31]. Metformin, an AMPK agonist, which is already routinely used as an anti-diabetic is well recognized to modulate T cell metabolism, induce autophagy and induce Treg expansion [41,148,149]. Accordingly, it exerts anti-inflammatory effects on $CD4^+$ T cells in various models of autoimmune disease, including systemic lupus erythematosus, experimental autoimmune encephalomyelitis and arthritis [150–152]. Another AMPK agonist, 5-Aminoimidazole-4-carboxamide ribonucleoside, shows

promising inflammation dampening properties by decreasing T cell proliferation and secretion of pro-inflammatory cytokines, including IFNγ and tumor necrosis factor α [153]. Besides metabolism modulation and its anti-inflammatory effects, metformin might induce autophagy in T cells through inhibition of mTOR, which might show additional beneficial effects as compared to metabolism modulators primarily targeting one specific metabolic pathway (e.g. glycolysis).

Dyslipidemia is another metabolic disease, which might affect T cell metabolism. Dyslipidemia in the form of (familial) hypercholesterolemia and/or hypertriglyceridemia is a major risk factor for atherosclerosis [154], an inflammatory disease characterized by lipid accumulation and subsequent leukocyte infiltration in the wall of medium and large sized arteries. It is the main underlying pathology of CVD and, as a chronic autoimmune-like disease, it has a large component which is $CD4^+$ T cell (mainly Th1) mediated [29,155]. Dyslipidemia in FH patients is characterized by hypercholesterolemia, mainly caused by elevated low-density lipoproteins [156]. Hypercholesterolemia might drive T cell-mediated autoimmunity by providing substrate for membrane synthesis, as cholesterol supplementation *in vitro* is known to drive T cell proliferation [157]. Moreover, diet-induced dyslipidemia could alter the lipid content of T cells intracellularly as these lipids are stored in lipid droplets, or shuttled to the cell membrane, thereby potentially driving membrane raft formation which affects stability of the immunological synapse [158]. Interestingly, prolonged diet-induced dyslipidemia in low density lipoprotein receptor knockout mice altered the membrane lipid composition in T cells which increased T cell activation status [159]. Modulation of T cell lipid metabolism in dyslipidemia patients might therefore help dampen T cell mediated autoimmunity.

Statins might be particularly successful doing this as these are quite successful in primary prevention of coronary heart disease [160]. While the main therapeutic effect of statins is aimed at inhibition of hepatic cholesterol synthesis, T cells are also directly modulated, although the underlying mechanism is sometimes unclear. Atorvastatin inhibits T cell proliferation in mice, an effect, which is overruled by the addition of mevalonate or its precursor farnesyl-PP [161,162]. Moreover, cerivastatin, simvastatin, lovastatin, and atorvastatin induce a Th2 biased differentiation and a decreased Th1 differentiation, as measured by cytokine secretion, although the effects of atorvastatin are not observed in human primary T cells [163,164]. Simvastatin inhibits *ex vivo* T cell proliferation in CVD patients on simvastatin [165]. Modulating LXR through synthetic agonists in T cells, thereby depriving them of cholesterol, might prove successful as well. Systemic administration of LXR agonists GW3965 or T0901317 dampens atherosclerosis development in experimental models of disease [166–168].

mTOR inhibitors such as sirolimus (rapamycin), everolimus and other rapalogs (functional analogs of rapamycin) are well known for their immunosuppressive capacity in graft-versus-host disease and have shown to reduce plaque size in various animal models, as

was extensively reviewed by others [169]. Moreover, mTOR inhibitors, for example in the form of drug-eluting stents with everolimus, are used in the clinic after percutaneous coronary intervention to inhibit restenosis [170].

mTOR signaling also plays a role in obesity-associated low grade inflammation [21]. Inflamed visceral adipose tissue (VAT) contains a disproportionally low amount of Tregs displaying a dysfunctional phenotype, as opposed to Tregs from lean VAT [34]. A significant part of the dysfunction of VAT Tregs is through an insulin induced decrease in IL-10 expression and secretion via the Akt/mTOR pathway [171]. Increasing the Treg population in inflamed VAT has been shown to reduce HFD-induced obesity and insulin resistance [172]. Rapamycin abolishes the negative effects of insulin on mTOR signaling in Tregs, suggesting also in obesity related insulin resistance, T-cell specific modulation of mTOR may have beneficial effects [171]. Direct exposure during priming of $CD4^+$ T cells to the saturated fatty acid palmitate modulates the PI3K-p110 -Akt axis thereby causing a decrease in C-C chemokine receptor type 7 and L-selectin [173]. This contributes to biased differentiation of inflammatory $CD4^+$ T cells which is observed during chronic low grade inflammation in obesity [173].

Although long-term rapamycin treatment improves the metabolic state in animal models [174], a potential side-effect of rapamycin is dyslipidemia which occurs in 40-75% of the patients receiving rapamycin treatment [175–177]. Detrimental effects of rapamycin in mice are found early in treatment [174] indicating treatment duration might be an important factor. Ongoing research and developing other rapalogs should further improve the applicability of these compounds for preventative therapy.

A final important point to address is the implementation of modulators to target T cell metabolism in the pathologies, which were discussed. Systemic administration is most readily available as some of the compounds discussed (e.g. metformin, rapamycin) are already used in the clinic for other therapeutic means. The United Kingdom Prospective Diabetes Study showed the use of metformin as an antidiabetic drug in T2DM patients decreased the risk of myocardial infarction with 33% as compared to a conventional-intervention group, 10 years after cessation of randomized intervention [178]. Although the immune-mediated part of this beneficial effect of metformin remains speculative, it indicates the feasibility of this compound to diminish cardiovascular disease in an aged population. Combined therapy of lifestyle intervention and metformin in newly diagnosed T2DM patients has been shown to decrease serum IL-17 levels, indicating metformin dampens Th17 cells [179]. In patients with multiple sclerosis and metabolic syndrome, disease activity of multiple sclerosis as measured by brain magnetic resonance imaging was decreased in metformin treated patients as compared to non-treated control patients [180]. Additionally, circulating Treg percentage was increased in metformin treated patients, as well as Treg suppressive capacity and IL-10 secretion [180]. However, the possible off-target effects during long-term treatment that could be detrimental

to disease progression call for a more T cell-specific approach. Although metformin is generally considered a safe and low-cost compound for treatment of diabetes, detrimental gastrointestinal side-effects are observed relatively frequently [181]. Lactic acidosis is an infrequently observed complication of metformin usage but is potentially lethal [182]. To overcome potential off-target effects as the ones discussed above for metformin

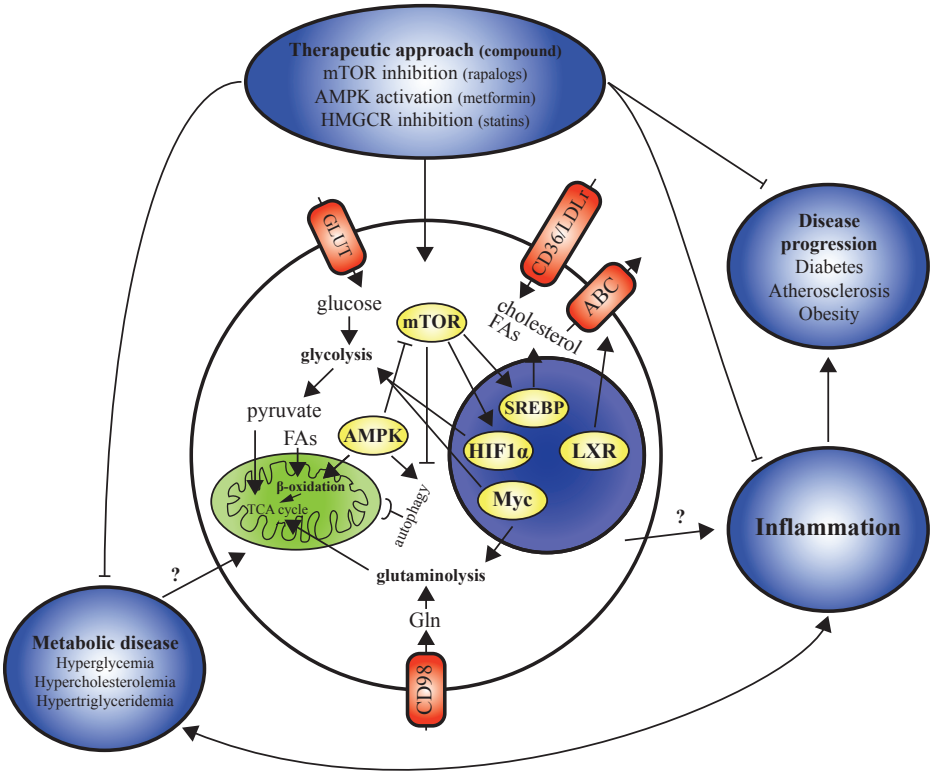

Figure 3 T cell metabolism as a potential driving force of inflammation and target for immunomodulation in metabolic disease-associated autoimmunity. A simplified scheme of T cell metabolism is presented as a hub whose (specific) modulation could prove a valuable therapeutic target. Whether T cell metabolism is modulated by a metabolically aberrant micro-environment (e.g. during hyperglycemia) and how this affects the inflammatory response remains elusive but probably provides an interesting novel therapeutic target. Systemic administration of clinically available compounds (such as rapalogs) could be used to therapeutically modulate T cell metabolism and subsequently dampen T cell-mediated autoimmunity and inflammation. This approach might also contribute to disease through off-target effects. T cell specific ex vivo therapy requires further investigation but would overcome possible off-target effects. Additionally, inhibition of one or multiple metabolic pathways might also be feasible, depending on the specific metabolic micro-environment T cells are exposed to (e.g. 2-DG in hyperglycemia). **GLUT**=glucose transporter, **CD36**=cluster of differentiation 36/fatty acid translocase, **LDLr**=low-density lipoprotein receptor, **ABC**= ATP-binding cassette transporter, **CD98**=cluster of differentiation 98/dimer Slc3a2/Slc7a5, **mTOR**=mammalian target of rapamycin, **HIF1α**=hypoxia inducible factor 1 alpha, **AMPK**= 5' AMP-activated protein kinase, **LXR**=liver-X-receptor, **SREBP**=sterol regulatory element-binding protein.

and rapamycin, *ex vivo* treatment of T cells isolated from peripheral blood with the suggested compounds, followed by an adoptive transfer, seems feasible as T cells are specifically targeted and any patient heterogeneity in off-target effects are abolished. $CD4^{+}CD127^{lo/-}$ T cells from peripheral blood of recent-onset T1DM patients can be successfully expanded *in vitro* into Tregs in the presence of rapamycin [183]. A disadvantage of this approach is that multiple time-consuming treatments would likely be necessary. *In vivo*, specifically targeting T cells using micro- or nanoparticles seems challenging due to the limited phagocytic capacity T cells have. A possibility lies in the endocytotic processes which ensue in T cells upon cytokine stimulation, for example after binding of IL-7 to CD127 [184]. Although this remains speculative and this approach is not entirely T cell specific, drug-cytokine tandems have been described in literature [185] and their development and therapeutic application might be achievable in the future. Regardless, modulation of T cell metabolism could form the hub in the treatment of diseases characterized by metabolic disease and autoimmunity (fig. 3).

CONCLUSION

The importance of cellular metabolism for T cell proliferation, differentiation and function is indisputable. For T cell metabolism-based therapy, there are multiple approaches to modulate metabolic pathways directly or to activate/inhibit modulators of metabolism to ultimately treat metabolic disease-associated autoimmunity. Examination of the metabolic pathways, which are likely to be modulated in T cells, during metabolic disease might reveal novel therapeutic targets for treatment of prevalent diseases such as diabetes, atherosclerosis, obesity and resulting cardiovascular disease. T cell-specific *in vivo* or *ex vivo* treatment might improve the general applicability of clinically available and novel compounds. Herein, a challenge lies in translating the findings from experimental (disease) models to human disease.

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CHAPTER 3

Diet-induced dyslipidemia induces metabolic and migratory adaptations in regulatory T cells

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ABSTRACT

A hallmark of advanced atherosclerosis is inadequate immunosuppression by regulatory T (Treg) cells inside the atherosclerotic lesion. Here we show that this impairment is not due to a reduction in migratory capacity of Treg cells as diet-induced dyslipidemia, one of the major risk factors for atherosclerosis, actually skewed their migration towards sites of inflammation instead of lymph nodes. Mechanistically, we discovered that diet-induced dyslipidemia altered mTOR signaling and induced PPARδ activation, thereby increasing fatty acid (FA) oxidation in Treg cells. Treatment with a synthetic PPARδ agonist increased the migratory capacity of Treg cells in an FA oxidation dependent manner. Furthermore, diet-induced dyslipidemia enhanced Treg cell influx into atherosclerotic lesions indicating that enhanced FA oxidation drives Treg cell migratory function during atherosclerosis. Altogether, our findings implicate that a decrease in Treg cell immunosuppression is not due to impairment in their migratory capacity but due to an unfavorable microenvironment inside atherosclerotic lesions.

KEYWORDS

Dyslipidemia, atherosclerosis, Treg cell, mTOR, PPARδ, migration

INTRODUCTION

Dyslipidemia as exemplified by hypercholesterolemia and/or hypertriglyceridemia is a driving force for the development of atherosclerosis. Atherosclerosis is an autoimmune-like disease affecting the arterial wall in which (modified) lipoproteins such as low-density lipoprotein (LDL) accumulate in the subendothelial space and elicit an adaptive immune response involving $CD4^+$ T cells [1]. Regulatory T (Treg) cells represent a subset of $CD4^+$ T cells which maintains tolerance to self-antigens and regulates inflammation to dampen tissue damage [2]. Treg cells are thus considered a promising therapeutic target to treat autoimmune-like disorders, including atherosclerosis [3]. Accordingly, as Treg cell abundancy is low in advanced atherosclerotic lesions in mice [4] and humans [5–7], a local loss of tolerance to lipoproteins is speculated to be causal in atherosclerosis progression. Previously, it has been shown that the capacity of splenic Treg cells to bind to activated endothelium is inversely related to the degree of diet-induced dyslipidemia and this may be due to a decreased expression of ligands for P- and E-selectin on Treg cells [4]. Thereby, fewer Treg cells which egress from secondary lymphoid organs (SLOs) and enter the circulation would subsequently migrate towards atherosclerotic lesions, thereby contributing to a local loss of tolerance.

In the last decade, intricate adaptations in the metabolism of glucose and lipids have been shown to be crucial for Treg cells to properly exert their function during inflammation [8–11]. Recently, it was discovered that Treg cells require glycolysis to generate sufficient ATP for their migration [12]. Interestingly, inhibition of the glycolytic enzyme PFKFB3 in $CD4^+$ T cells has been shown to induce ATP-depletion, which causes lipid droplet formation and activates the transcriptional program for migratory machinery, thereby generating pro-inflammatory, tissue invasive T cells [13]. Thus, cellular metabolism affects T cell migration in different manners. Whether dyslipidemia causes adaptations in cellular metabolism in Treg cells inside SLOs and whether this affects their migration in the context of atherosclerosis has not yet been investigated.

It seems feasible that dyslipidemia affects cellular metabolism in Treg cells as cholesterol accumulation in ATP-binding cassette G1 (ABCG1)-deficient Treg cells inhibits mammalian target of rapamycin complex 1 (mTORC1) [14]. mTORC1 is a protein complex in which the kinase mTOR can regulate cellular metabolism by promoting glycolysis in activated T cells through its downstream targets hypoxia inducible factor-1α (HIF1α) and Myc [15,16]. Moreover, mTORC1 inhibits fatty acid (FA) oxidation, possibly by inhibiting the rate-limiting enzyme carnitine-palmitoyl transferase 1A (Cpt1a) [17,18].

Furthermore, diet-induced dyslipidemia likely also affects Treg cell migration through a distinct mechanism. Specifically, obesity-induced metabolic stress primes $CD4^+$ T cells to acquire an effector phenotype by altering the activity of the PI3K-p110δ-Akt kinase signaling pathway upon activation. This alteration lowers the expression of CD62L and

C-C chemokine receptor type 7 (CCR7) [19] which are membrane-associated proteins involved in the homing of T cells to lymph nodes (LN) through high endothelial venules. Hence, these reports indicate that key regulators of cellular metabolism and migration in T(reg) cells can be affected by perturbations in the levels of extra- and intracellular lipids.

In this paper we investigated the dyslipidemia-induced effects on migration and cellular metabolism in Treg cells. Mechanistically, we discovered that dyslipidemia induced intrinsic changes in mTORC2 signaling, inhibited mTORC1 and glycolysis but increased FA oxidation in Treg cells. We furthermore showed that the latter was not entirely mTORC1 mediated. Mass spectrometry analysis of dyslipidemic serum uncovered increases in peroxisome proliferator activated receptor delta (PPARδ) ligands, which contributed to increased FA oxidation in Treg cells. Dyslipidemia also increased the capacity of Treg cells to migrate towards sites of inflammation and the PPARδ agonist GW501516 increased their migration in an FA oxidation-dependent manner indicating that diet-induced dyslipidemia can affect Treg cell migration partly by skewing their metabolism.

MATERIALS & METHODS

Mice

Wildtype C57BL/6J mice and LDL-receptor deficient B6.129S7-Ldlrtm1Her/J (*Ldlr*$^{-/-}$) mice were purchased from the Jackson Laboratory and further bred in the Gorlaeus Laboratory in Leiden, The Netherlands. Diet-induced dyslipidemia and atherosclerosis were established by feeding *Ldlr*$^{-/-}$ mice from 9-12 weeks of age a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services) for 16-20 weeks. At sacrifice, the mice were sedated and their blood was collected via orbital blood collection. Subsequently, their vascular system was perfused with PBS at a continuous low flow via heart puncture in the left ventricle after which the spleens and lymph nodes were collected for further processing. Apolipoprotein E deficient B6.129P2-Apoetm1Unc/J (*Apoe*$^{-/-}$) mice were purchased from the Jackson Laboratory and at 20 weeks of age were fed a WTD for 8 weeks to promote atherosclerosis formation. *Apoe*$^{-/-}$ mice were sacrificed similar to LDLr$^{-/-}$ mice and the aortic arch and thoracic aortas were harvested additionally. B6.SJL-PtprcaPepcb/BoyCrl (CD45.1) mice were purchased from Charles River. The animals were kept under standard laboratory conditions and were fed a normal chow diet (NCD) and water *ad libitum*. All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and the experimental work was approved by the Animal Ethics committee of Leiden University.

Flow cytometry

Spleens and lymph nodes were mashed through a 70 µm cell strainer after isolation. Erythrocytes were subsequently eliminated from the splenocyte suspension by incubating the cells with ACK erythrocyte lysis buffer to generate a single-cell suspension prior to staining of surface markers. Blood samples were also lysed with ACK erythrocyte lysis buffer to prepare them for staining. For the staining of surface markers, cells were stained at 4°C for 30 minutes in staining buffer (PBS with 2% (vol/vol) fetal bovine serum (FBS)) in which we diluted the antibodies. Intracellular transcription factors were stained for by following the FoxP3 staining protocol (eBioscience). All antibodies used for staining of surface markers or transcription factors were from eBioscience, BD Biosciences or BioLegend (table 1). For staining of unesterified cholesterol, lymphocytes were stained after surface staining with 50µg/mL filipin III (Cayman) at room temperature for 45 minutes and subsequently washed with PBS twice before sample analysis. For staining of lipid droplets using BODIPY^{TM} 493/503 (Invitrogen), cells were stained with 1,3µg/mL BODIPY in pre-warmed PBS at room temperature for 10 minutes. For staining of mitochondria, lymphocytes were incubated with 10 nM MitoTracker Deep Red (Life Technologies) for 30 minutes at 37°C. To stain for Glut1, cells were fixed in 1% formaldehyde and incubated overnight in 100% methanol at -80°C. Subsequently, the cells were washed with PBS with 2% FCS, incubated with blocking solution and subsequently incubated with anti-Glut1 antibody (Abcam) at room temperature for 1h. After extensive washing, the cells were stained for 30 min. at room temperature with antibodies for surface markers and a goat-anti-rabbit antibody conjugated to allophycocyanin (Abcam). All samples were washed with staining buffer and resuspended in staining buffer prior to flow cytometric analysis.

Flow cytometric analysis was performed on a FACSCantoII (BD Biosciences) or a Cytoflex S (Beckman Coulter) and data was analyzed using Flowjo software (TreeStar).

Phosflow analysis

For detection of phosphorylated signaling proteins using flow cytometry, splenocytes from NCD or WTD fed mice or diet-switch mice were rested in complete RPMI-1640 for 2h. After this, splenocytes were stimulated with 100U/mL of recombinant IL-2 (Peprotech) prior to staining for surface markers on ice in the dark for 30 minutes. A control without IL-2 induced stimulation of the mTORC1 and mTORC2 pathways was used. Stimulation was followed by fixation with BD Phosflow™ Lyse/Fix Buffer (BD Biosciences), subsequent permeabilization with Phosflow Perm buffer III (BD Biosciences) and staining with Alexa Fluor® 488-conjugated phospho-S6 (Ser235/236), Alexa Fluor® 647-conjugated phospho-4E-BP1 (Thr37/46) (Cell Signaling Technologies) or V450 conjugated phospho-Akt (Ser473) antibodies (BD Biosciences) at room temperature for 1h. The cells were then washed and prepared for flow cytometric analysis.

Cell culture

$CD4^+$ T cells or $CD4^+CD25^+$ Treg cells were isolated from spleens from $LDLr^{-/-}$ mice using MACS microbeads (Miltenyi Biotec). Samples were excluded when the purity was below 93% as assessed by flow cytometry. Alternatively, Treg cells were flow sorted on a FACS Aria III by gating on viable $CD3^+CD4^+CD25^{hi}$ cells. Treg cells were stimulated using plate-coated anti-CD3e (5µg/mL; Ebioscience), anti-CD28 (0,5µg/mL; Ebioscience) and 200U/mL recombinant mIL-2 (Peprotech) and cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 100U/mL pen/strep and 10% FBS (all from Lonza). For *in vitro* experiments, Treg cells were isolated from the spleen, mediastinal, mesenteric and inguinal lymph nodes using MACS microbeads or flow sort. *In vitro* lipid loading experiments were performed by supplementing culture medium with 10% mouse serum from $LDLr^{-/-}$ mice with normolipidemia or dyslipidemia. Alternatively, lipid loading was achieved by culturing Treg cells with β-very low density lipoprotein particles which were isolated from rat serum through KBr density gradient ultracentrifugation. GW501516 (Enzo Life Sciences) was used at 0.1µM in complete RPMI-1640 with 10 mM D-glucose. Dimethyl sulfoxide (DMSO) was used as a vehicle control in the GW501516 compound experiments. For the GSK compound study, GSK0660 (Sigma) was used *in vitro* on isolated NCD- and WTD-Treg cells under plate-coated anti-CD3e, anti-CD28 and 200U/mL recombinant mIL-2 stimulation for 4 hours at a concentration of 1µM. DMSO was used as a vehicle control. Rapamycin (LC Laboratories) was used at a concentration of 100 nM and DMSO was used as a vehicle control.

RNA and immunoblot analysis

mRNA was extracted from freshly isolated or cultured Treg cells using the guanidium isothiocyanate (GTC) method after which cDNA was generated using RevertAid M-MuLV reverse transcriptase per manufacturer's instructions (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to 2-3 housekeeping genes (table 1). Immunoblots were performed and quantified as described previously[8], using the following antibodies: p-S6, p-Foxo1 (all from Cell Signaling Technology), HIF1α (Cayman Chemical) and β-actin (Sigma). Immunoblot results were quantified using Fiji biological-image analysis software.

FA oxidation assay

Freshly isolated or cultured Treg cells were resuspended in minimal DMEM supplemented with 10 mM D-glucose (Sigma), 2mM L-glutamine (Lonza), 10% FBS, HEPES, sodium bicarbonate and 5µCi [9,10-^{3}H]-palmitic acid (PA) (Perkin Elmer). Cells were incubated for 2h (unless otherwise stated) at 37°C after which the supernatant was transferred to 1.5 mL microcentrifuge tubes. After centrifugation, the supernatant was transferred to

20ml scintillation vials which were sealed with a rubber stopper containing Whatmann filtration paper pre-equilibrated in milliQ. After 48 hours of incubation at 37°C, the Whatmann filtration paper containing the metabolized ^3H_2O was harvested. Per assay, three cell-free samples containing only 5µCi [9,10-^{3}H]-PA were used as a background control. Mitochondrial FA oxidation (simply referred to as FA oxidation) was determined by the difference between the oxidation rate in the absence or presence of 100µM etomoxir (Sigma).

Metabolic flux assay

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using an XF96e Extracellular Flux Analyzer (Seahorse Bioscience). Freshly isolated Treg cells were stimulated with plate-bound anti-CD3e, soluble anti-CD28 and IL-2 for 4h. After this, Treg cells were replated in XF medium (nonbuffered DMEM supplemented with 10 mM glucose, 2mM L-glutamine and 1 mM sodium pyruvate). An XF96e Extracellular Flux Analyzer was used to measure OCR and ECAR in response to 1µM oligomycin, 1µM FCCP, 1.25 µM rotenone, 2.5 µM antimycin A and 100 mM 2-deoxyglucose (all from Sigma).

Treg cell peritoneum migration

Treg cell peritoneal homing experiments were based on Fu et al [50]. Briefly, CD45.1 mice were injected i.p. with 600U interferon gamma (Ebioscience) 72h prior to an i.v. adoptive transfer of $15x10^6$ CD45.2 $CD4^+$ T cells or $1.5x10^6$ $CD45.2^+CD4^+CD25^+$ Treg cells. After 16-18 hours, the mice were sacrificed and peritoneal cells were collected by performing a peritoneal lavage and the spleen and mesenteric lymph nodes were excised. Peritoneal cells, splenocytes and lymphocytes were subsequently used for flow cytometry detection of $CD45.2^+$ Treg cells.

Aorta influx

In vitro Treg cell homing was assessed by adapting a protocol from Li et al. [43]. The aortic arch and thoracic aorta were surgically removed from WTD-fed *Apoe*$^{-/-}$ mice with atherosclerosis and dissected transversally into six pieces. Freshly isolated Treg cells from the spleens of mice which were fed an NCD or WTD were pooled in an NCD and a WTD fraction and subsequently labeled with 5 µM CellTrace™ Violet (Invitrogen) as per manufacturer's instructions. After this, the NCD- and WTD-Treg cell fractions were split in two and were treated with 100µM etomoxir or maintained in medium for 1h. After incubation, the cells were washed and counted using a hemocytometer before adding 200,000 Treg cells per aorta. The Treg cells were left to migrate overnight in complete RPMI-1640 with 10 mM D-glucose. Subsequently, the aortic fragments were washed extensively in ice-cold PBS containing 2% FCS and 2 mM EDTA. After this, the aortic fragments were

cut up into small pieces and digested by incubating them with a digestion mix containing 450 U/mL collagenase I, 250 U/mL collagenase XI, 120 U/mL DNAse and 120 U/mL hyaluronidase (all from Sigma) for 30 minutes at 37˚C under agitation. Subsequently, the digested aortas were strained over a 70-µm strainer to prepare them for antibody staining for flow cytometry to detect migrated Treg cells.

Transmigration assay

Treg cells were treated with 0.1µM GW501516 or vehicle for 16-18h in complete RPMI supplemented with 10 mM glucose. Subsequently, Treg cells were treated with 100 µM etomoxir for 1 hour and then seeded into transwell tissue culture well inserts (5µm pore-size) and left to migrate towards 250 ng/mL CCL21 (Peprotech) for 6-8 hours. The number of migrated cells was determined manually using a hemocytometer. The results are depicted as a percentage of migrated cells.

Serum analysis

Concentrations of total cholesterol and triglycerides in the serum were determined by an enzymatic colorimetric assay (Roche Dagnostics). Precipath (standardized serum, Roche Diagnostics) was used as an internal standard. Concentrations of free fatty acids in the serum were quantified using the Free Fatty Acid Quantification Kit (Sigma) as per manufacturer's instructions. For measurement of blood glucose levels, mice were fasted for 4 hours prior to blood collection. Blood samples were taken from the tail vein and directly applied to an Accu-Check glucometer (Roche Diagnostics).

Serum lipidomics

Ldlr$^{-/-}$ mice were fed a WTD or maintained on an NCD for 8 weeks and upon sacrifice, serum samples were collected and frozen at -80˚C until use. The operating procedures of the targeted lipidomics platform are optimized from the previously published method [51]. Polar lipids are extracted using methanol to precipitate proteins from serum samples and this method covers low abundance lipid species, including free fatty acids and lysophospholipids—lysophosphatidylcholines (LPCs) and lysophosphatidylethanolamines (LPEs). Chromatographic separation was achieved on an ACQUITY UPLC™ with a HSS T3 column (1.8 µm, 2.1 * 100 mm) coupled to a Q-TOF (Agilent 6530) high resolution mass spectrometer using reference mass correction. Lipids were detected in full scan in the negative ion mode. The leukotrienes, hydroxyl-fatty acids, epoxy-fatty acids and lipoxins were analysed using a fully targeted method as as previously described [52]. Oxylipid enrichment was achieved using a hydrophilic-lipophilic balance (HLB) SPE cartridge (Oasis). Oxylipid analysis used high-performance liquid chromatography (Agilent 1260) coupled to a triple-quadrupole mass spectrometer (Agilent 6460), using an Ascentis® Express column (2.7 µm, 2.1 × 150 mm).

A heatmap was generated with the software R (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.). To this end, the add-on package ggplo2 (v2.1.0) was used to run its heat map 2.0 function.

Suppression assay

Treg cells were isolated and co-cultured in complete RPMI with splenocytes labeled with 5 µM CellTrace Violet. The cells were stimulated with anti-CD3e (1µg/mL; Ebioscience), anti-CD28 (0,5µg/mL; Ebioscience) and 100U/mL recombinant mIL-2 (Peprotech). The suppressive capacity of Treg cells was determined by flow cytometry by measuring the proliferation of CellTrace Violet labeled $CD4^+$ T effector cells after 72 hours in different Treg:splenocyte ratios in which the amount of splenocytes per well were set at 50,000 cells.

Statistical analysis

Data are expressed as mean ± SD. A two-tailed student's T-test was used to compare individual groups with Gaussian distributed data. Correction for multiple comparisons was performed using Bonferroni correction. Non-parametric data was analyzed using a Mann-Whitney U-test. Data from three groups were analyzed using a one-way ANOVA with a subsequent Tukey's multiple comparison test. A p-value below 0.05 was considered significant. In the figures * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ and **** indicates $p<0.0001$.

RESULTS

Diet-induced dyslipidemia decreases Treg cell homing to lymph nodes through intrinsic changes in mTORC2 signaling

Germline mutations in the *Ldlr* gene are a frequent cause of familial hypercholesterolemia and premature cardiovascular disease [20] which makes the low density lipoprotein-receptor knock-out (*Ldlr*$^{-/-}$) mouse a commonly used model to study atherosclerosis. To study alterations in Treg cell metabolism and migration in the context of dyslipidemia-induced atherosclerosis, normal chow diet (NCD) fed *Ldlr*$^{-/-}$ mice were compared to *Ldlr*$^{-/-}$ mice which were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter for 16-20 weeks. In this setup, WTD-fed mice develop advanced atherosclerotic lesions with a low abundance of Treg cells [4] and metabolic dysregulation in the form of hypercholesterolemia (fig. S1A) and hypertriglyceridemia (fig. S1B) but not hyperglycemia (fig. S1C).

During inflammation, Treg cells exert part of their immunosuppressive function in draining LNs of inflammatory sites [21] to which they migrate through the high-endothelial venules or lymphatic system. Since obesity-induced metabolic stress affects the migration of $CD4^+$ T cells towards LNs, we examined whether WTD-induced dyslipidemia induces intrinsic changes in Treg cells which affects their migration via a similar mechanism. We examined the splenic Treg cell population since prolonged diet-induced dyslipidemia is associated with changes in the expression of certain selectins in Treg cells, one of which is a decrease in CD62L expression (in this population) [4].

First, the number of splenic Treg cells to migrate towards the mesenteric LN in a peritoneal homing experiment was assessed. Isolated $CD4^+$ T cells from NCD- or WTD-fed donor *Ldlr*$^{-/-}$ mice were injected into CD45.1 acceptor mice and in both groups, the number of donor-derived Treg cells in the mesenteric LNs was quantified. Treg cells derived from WTD-fed mice (WTD-Treg cells) migrated less efficiently towards mesenteric LNs compared to Treg cells from NCD-fed mice (NCD-Treg cells) as the number of retrieved WTD-Treg cells was lower as compared to NCD-Treg cells (fig. 1A). During obesity-induced metabolic stress, altered PI3K activity leads to increased Akt phosphorylation at serine 473, an amino acid residue on Akt which is targeted by mTORC2. We postulated that the observed decrease in LN homing was caused by WTD-induced metabolic stress in Treg cells which increased mTORC2 activity. Through phosphorylation of Akt kinase at the serine 473 residue and subsequent phosphorylation of forkhead Box O1 (Foxo1), increased mTORC2 activity would ultimately cause decreased expression of *Klf2, CD62L*, *CCR7* and *S1pr1* in WTD-Treg cells (fig. 1B). *Klf2* is the gene encoding Krüppel-like factor 2 which, like Foxo1, is a transcription factor whose target genes include proteins that are crucial for T cells to home towards LNs, including CD62L, CCR7 and sphingosine-1-phosphate receptor 1 (S1Pr1) [22]. p-Akt S473 levels as measured by flow cytometry were confirmed to be elevated in WTD-Treg cells as compared to NCD-Treg cells (fig. 1C). Accordingly, p-Foxo1 levels as measured by immunoblot analysis were elevated as well (fig. 1D) indicating less transcriptionally active Foxo-1. In addition, *Klf2* expression was slightly decreased (fig. 1E) which, together with less functional Foxo1, resulted in decreased expression of *CD62L*, *Ccr7* and *S1pr1* (fig. 1F). This also resulted in decreased protein levels of CD62L and CCR7 (fig. 1G) as measured by flow cytometry and a decreased percentage of Treg cells expressing CD62L and/or CCR7 (fig. 1H). The percentage of $CD62L^+$ Treg cells in the circulation was also lower during dyslipidemia which, although CCR7 expression was unchanged (fig. 1I), indicates that metabolic stress in Treg cells in the spleen affects the migratory phenotype of Treg cells which egress there from and reenter the circulation.

Overall, these data indicate that, similar to diet-induced obesity, WTD-induced dyslipidemia caused intrinsic changes in mTORC2 activity in Treg cells which ultimately decreased the capacity of Treg cells to migrate towards lymph nodes.

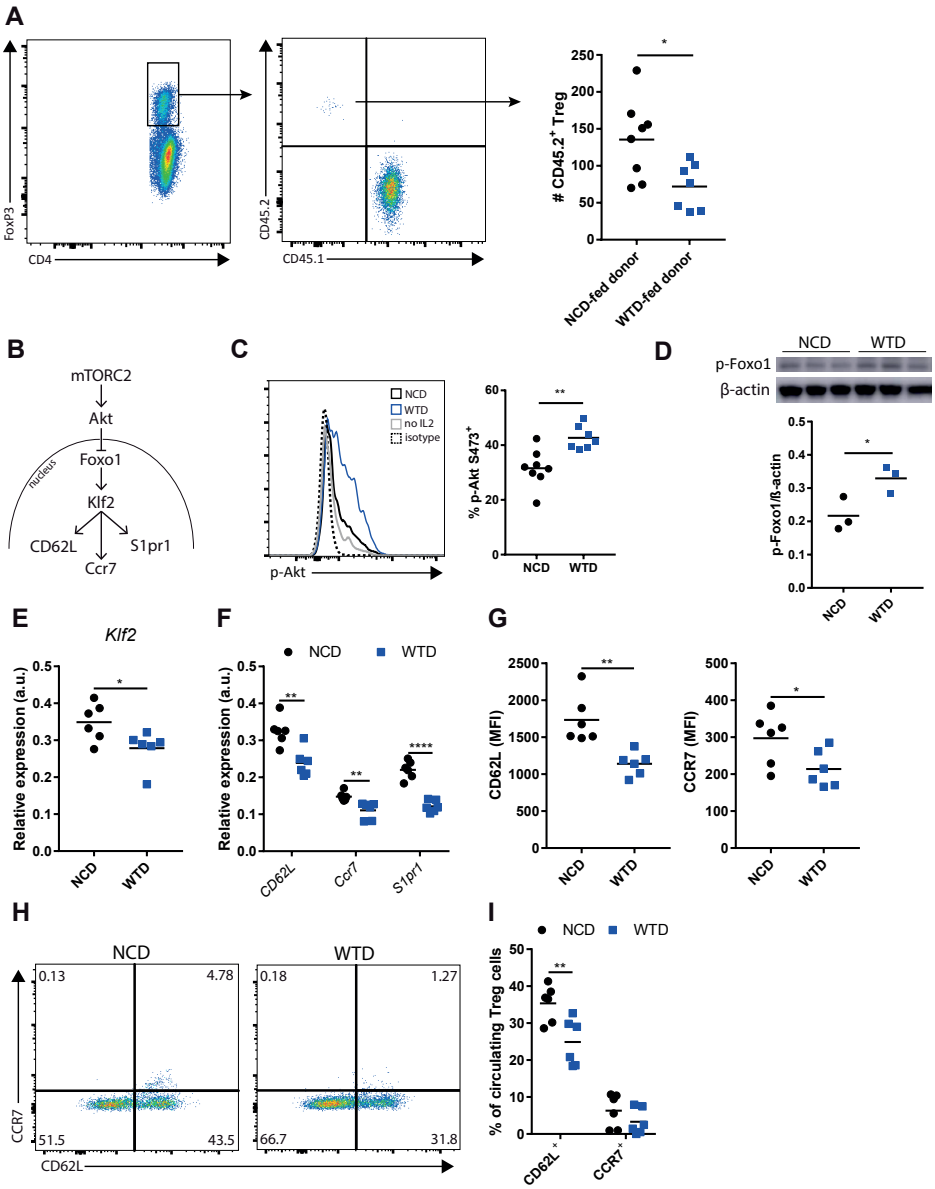


Figure 1 Diet-induced dyslipidemia in *Ldlr*$^{-/-}$ is associated with intrinsic changes in mTORC2 activity and lymph node homing of Treg cells (A) Gating strategy to identify adoptively transferred Treg cells derived from normal chow diet (NCD) or Western-type diet (WTD) fed *Ldlr*$^{-/-}$ mice which had migrated to mesenteric LN in a peritoneal homing experiment. (B) mTORC2-Akt-Foxo1-Klf2 axis. (C) p-Akt levels in splenic Treg cells derived from NCD-fed mice (NCD-Treg cells) or WTD-fed mice (WTD-Treg cells). (D) Representative data for p-Foxo1 levels in NCD- and WTD-Treg cells. (E) *Klf2* expression in NCD- and WTD-Treg cells. (F) Expression of *CD62L*, *Ccr7* and *S1pr1* in NCD- and WTD-Treg cells. (G) MFI for CD62L and CCR7 in NCD- and WTD-Treg cells. (H) representative plots of percentages of CD62L^{+} and CCR7^{+} in NCD- and WTD-Treg cells. (I) CD62L and CCR7 expression in Treg cells in the blood of NCD- and WTD-fed mice. A represents one experiment. C-H are representative data for two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. MFI = median fluorescence intensity

Diet-induced dyslipidemia increses lipids and inhibits mTORC1 activity and cholesterol synthesis in Treg cells

As diet-induced dyslipidemia caused metabolic stress in splenic Treg cells, which skewed their migration, we further characterized lipid accumulation of Treg cells in other SLOs and the circulation. Diet-induced dyslipidemia potentially affects the intracellular lipids of Treg cells in various SLOs. Lipoproteins in the blood can infiltrate LNs through the high-endothelial venules or the lymphatics system and can actually enter the spleen more easily as the spleen is a very well-vascularized SLO organ.

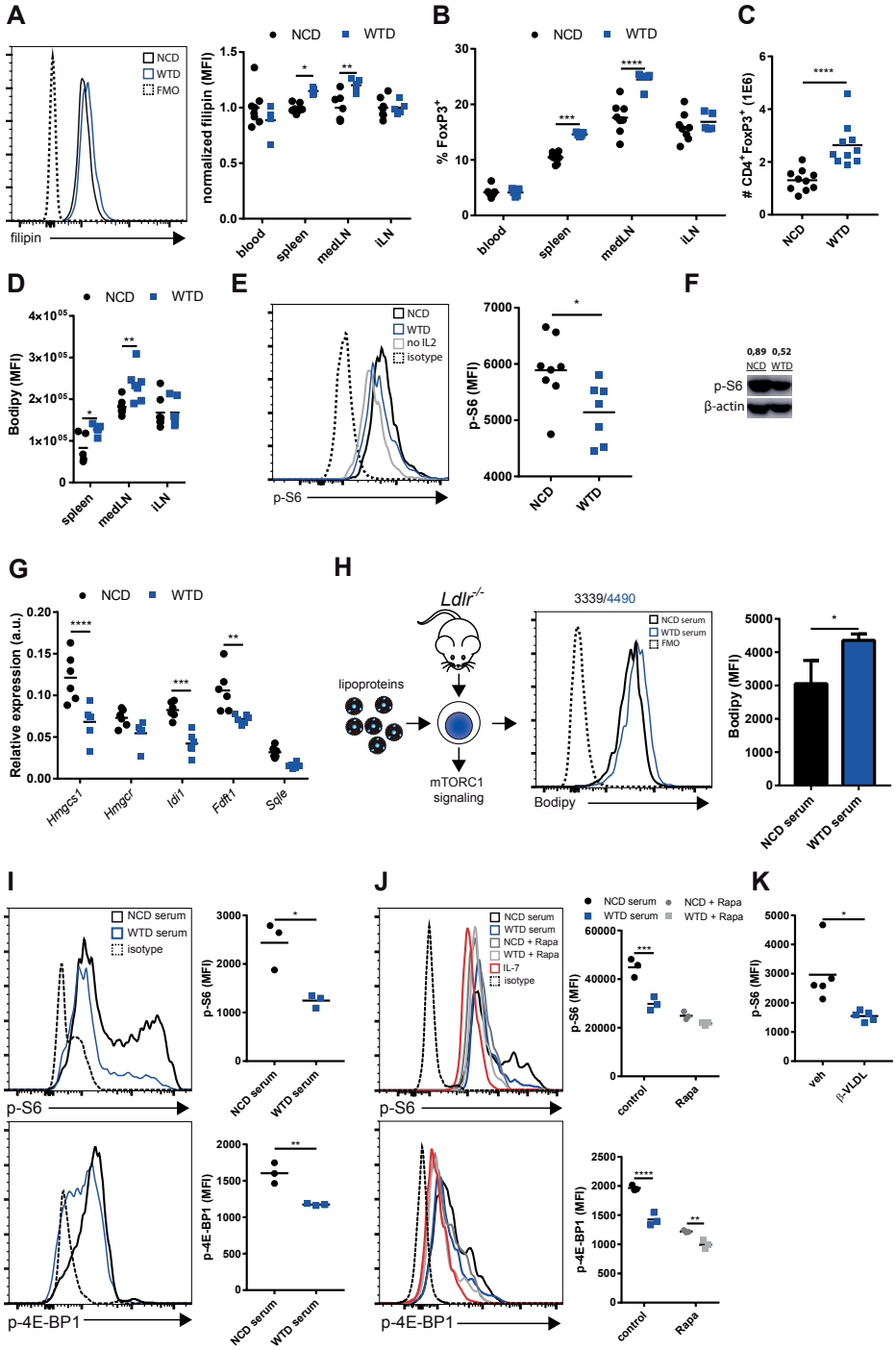
First, we examined if cholesterol accumulation in Treg cells in the blood, spleen, draining LN and non-draining LNs is equally affected when comparing WTD-fed *Ldlr*$^{-/-}$ mice to age-matched NCD-fed control mice. The mediastinal LN (medLN) drains atherosclerotic lesions in the proximal part of the aorta while the inguinal LN (iLN) drains hind limbs which do not develop diet-induced atherosclerosis in this experimental setup. After 16 weeks of WTD, Treg cells in the spleen and mediastinal LN (medLN) showed elevated cholesterol accumulation whereas Treg cells in the blood and inguinal LN (iLN) were unaffected (fig. 2A). Next, we measured the amount of Treg cells in these organs and observed an increase in Treg cells specifically in the spleen and medLN (fig. 2B). In the spleen, this increase in the percentage of Treg cells resulted in a strongly expanded splenic Treg cell population (fig. 2C). A lipid droplet staining showed that besides cholesterol accumulation in Treg cells, dyslipidemia was also associated with lipid droplet accumulation in Treg cells in the spleen and medLN (fig. 2D). These findings are especially relevant as splenic Treg cells encounter blood-borne antigens (e.g. derived from modified LDL [23]). Accordingly, antigen-specific Treg cells can be found in the spleen during atherosclerosis [24]. Presumably, the increase in Treg cells and their lipid content in the medLN is due to ongoing inflammation in atherosclerotic lesions which contains copious amounts of (modified) lipoproteins which are drained to the medLN .

Interestingly, the level of cholesterol which accumulated in WTD-Treg cells was sufficient to decrease mTORC1 activity as compared to NCD-Treg cells (fig. 2E). The decrease in mTORC1 activity was confirmed by measuring phospho-S6 levels in isolated Treg cells using immunoblot analysis (fig. 2F). In addition, the expression of *Srebp1* and *Srebp2*, whose expression is decreased by inhibition of mTORC1 [25,26], was diminished in WTD-Treg cells (fig. S1D). Concomitantly, the expression of enzymes which are crucially involved in cholesterol synthesis through the mevalonate pathway was decreased, particularly that of *Hmgcs1, Idi1* and *Fdft1,* whose expression was decreased by approximately 50% (fig. 2G). Treg cells which lack Raptor, an essential protein in mTORC1, lose their suppressive capacity which is mostly due to reduced activity of the mevalonate pathway in proliferating Treg cells [8]. As compared to NCD-Treg cells, WTD-Treg cells had similar suppressive function as measured by their capacity to suppress proliferation of $CD4^+$ T effector cells (Fig. S1E). mTORC1 activity as measured by p-S6 levels was also decreased

in $CD4^+FoxP3^-$ conventional T (Tconv) cells from WTD-fed mice (fig. S1F) which indicates diet-induced dyslipidemia also exerts metabolic stress on non-Treg cells.
To confirm that the observed changes were specifically caused by dyslipidemia, we mimicked it *in vitro* by culturing Treg cells in culture medium supplemented with serum from NCD or WTD-fed mice (fig. 2H) or supplemented with isolated β-very low density lipoprotein (β-VLDL) particles. Lipid loading *in vitro* through serum supplementation mimicked the effects of hypercholesterolemia on mTORC1 activity as measured by p-S6 and p-4E-BP1 (an additional mTORC1 target) levels in Treg cells (fig. 2I). Moreover, this effect of serum supplementation also occurred in Treg cells isolated from C57/BL6 mice and additional treatment of Treg cells with the mTOR inhibitor rapamycin severely diminished the WTD-serum induced inhibition of mTORC1 (fig. 2J). Additionally, the p-S6 levels were reduced by approximately 50% when incubating Treg cells with isolated β-VLDL particles as compared to the vehicle control (fig. 2K).
Altogether, these results showed that diet-induced dyslipidemia induced lipid accumulation in Treg cells, which reduced mTORC1 activity and the expression of genes crucially involved in the mevalonate pathway without altering their suppressive capacity.

WTD-Treg cells have impaired glycolysis but increased FA oxidation

Next, we reasoned that dyslipidemia-induced mTORC1 inhibition would change the bioenergetic metabolism in Treg cells. mTOR kinase is a crucial regulator of energetic metabolism which can regulate glycolysis by increasing the transcription and translation of HIF1α [26,27]. We measured glycolysis in NCD-Tregs and WTD-Tregs using an XF analyzer (fig. 3A) and observed a small decrease in basal extracellular acidification rate (ECAR), which is a measure for lactate producing glycolysis. Furthermore, when compared to NCD-Treg cells, WTD-Treg cells had decreased glycolytic reserve and glycolytic capacity upon exposure of Treg cells to the complex I inhibitor oligomycin (Fig. 3B). As we observed a decrease in mRNA expression of the target genes of HIF1α (*Glut1, Pgk1, LDHa, Pkm2*) when culturing Treg cells with WTD serum in vitro (fig. S2A) we speculated that WTD-induced dyslipidemia decreased HIF1α expression in Treg cells, thereby decreasing glycolysis. However, immunoblot analysis revealed that HIF1α levels were unchanged (Fig. S2B) as were the mRNA expression levels of *Glut1, Pgk1, LDHa, Pkm2* (fig. S2C) in freshly isolated WTD-Treg cells as compared to NCD-Treg cells. Similar to HIF1α, through signaling down-stream of mTORC1, Myc can transcriptionally regulate the expression of glycolytic genes [16]. Myc expression was also equal between NCD-Treg and WTD-Treg cells (fig. S2D). As the Treg cells in which we measured glycolysis were activated with anti-CD3/CD28 and IL-2 prior to the measurements it is likely that mTORC1 inhibition by excess intracellular cholesterol only affected the expression of downstream target genes of HIF1α or Myc upon activation as mTORC1 activity is increased upon activation[8]. mTORC1 activation also promotes mitochondrial biogenesis [28]. The mitochondrial mass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Hmgcr
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Ldlr-/-
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NCD serum
WTD serum
FMO
Bodipy
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β-VLDL

Figure 2 mTORC1 activity is diminished in Treg cells from WTD-fed *Ldlr*$^{-/-}$ mice. (A) Filipin staining for cholesterol in Treg cells from blood, spleen, mediastinal lymph node (medLN) and inguinal lymph node (iLN). (B) Percentage of Treg cells in same tissues as in (A). (C) Number of Treg cells in spleen. (D) Bodipy staining for lipid droplets in Treg cells. (E) p-S6 levels as measured by flow cytometry (F) p-S6 level in isolated splenic Treg cells using immunoblot. p-S6 levels as assessed by immunoblot were normalized for β-actin levels as shown above the lanes. (G) Expression of genes from mevalonate pathway in isolated Treg cells (H) *In vitro* lipid loading of Treg cells to study mTORC1 signaling (I) p-S6 and p-4E-BP1 levels after 48h *in vitro* lipid loading with serum. (J) p-S6 and p-4E-BP1 levels after 48h *in vitro* lipid loading of wildtype Treg cells with rapamycin and/or serum. Interleukin-7 (IL-7) was used to sustain Treg cells without anti-CD3 stimulation. (H) p-S6 levels after lipid loading with β-VLDL. A-E and G-I are representative data for 3 individual experiments. J represents 1 experiment. K is representative for 2 individual experiments. MFI = median fluorescence intensity. FMO = fluorescence minus one control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The data in H represents the mean±standard deviation.

in Treg cells was equal in both groups (fig. 3C) and when measuring oxygen consumption rate (OCR) as a measure for oxidative phosphorylation, we observed no differences between NCD-Treg cells and WTD-Treg cells (fig. 3D), which implicated that diet-induced dyslipidemia did not induce changes in mitochondrial function.

As mTORC1 can also regulate FA oxidation through inhibition of the rate-limiting enzyme carnitine palmitoyltransferase 1 (CPT1), we measured the detritiation of ^{3}H-palmitic acid as a measure for FA oxidation. Pharmacological mTOR inhibition *in vitro* with rapamycin increased the rate of mitochondrial FA oxidation (calculated by subtracting the detritiation of ^{3}H-palmitic acid with etomoxir from the total ^{3}H-palmitic acid detritiation) with or without etomoxir in Treg cells as compared to the control (fig S2E). In line with dyslipidemia-induced mTORC1 inhibition, Treg cells from WTD fed mice showed about twice the level of mitochondrial FA oxidation (fig. 3E) and a comparable increase in *Cpt1a* expression (fig. 3F). FA oxidation in Tconv cells was unaffected by diet-induced dyslipidemia, suggesting that metabolic modulation by dyslipidemia were Treg cell-specific (fig. S2F). Mitochondrial FA oxidation has been shown to be crucial for Treg cell proliferation [9] although it must be noted that CPT1 deficient Treg cells proliferate normally [29]. We compared the proliferation of WTD-Treg cells to NCD-Treg cells by measuring the percentage of Ki-67^{+} Treg cells but this was unaltered (fig. S2G). To examine whether the metabolic adaptations in WTD-Treg cells were induced by diet-induced dyslipidemia and not by the chronic low-grade inflammation which is associated with atherosclerosis [30], we performed a diet-switch experiment in which we reverted WTD-fed mice to an NCD. Ten weeks after reverting WTD-fed mice to an NCD, atherosclerotic plaque size is similar as compared to prior to the diet-switch [31,32]. Thus, a diet-switch normalizes the circulating lipid levels without decreasing the degree of atherosclerosis, thereby uncoupling the contribution of circulating lipids from atherosclerosis-associated low-grade inflammation in the effect that diet-induced dyslipidemia has on FA metabolism in Treg cells 18 days after reverting the mice to an NCD, total cholesterol levels in the serum were

normalized in the DS group (fig. 3G). Accordingly, cholesterol levels were normalized in Treg cells from diet-switch mice (hereafter referred to as DS-Treg cells) (fig. 3H). Gene expression of liver-X-receptor (which is activated by cholesterol-derivatives) target genes *Abca1* and *Abcg1* were increased in WTD-Treg cells but not in DS-Treg cells (fig. 3I), confirming that the cholesterol levels in Treg cells were normalized by a diet-switch. Counterintuitively, flow cytometric analysis revealed that mTORC1 activity in DS-Treg cells was diminished as compared to NCD-Treg cells, suggesting that normalization of mTORC1 activity might occur gradually after normalization of cellular cholesterol levels (fig. 3I). Strikingly, despite mTORC1 activity being attenuated, mitochondrial FA oxidation in DS-Treg cells did not differ significantly from NCD-Treg cells, and *Cpt1a* expression was also equal between these groups (fig. 3J).
Collectively, these data indicate that glycolysis and FA oxidation are modulated by dietary lipids during dyslipidemia but that the latter effect of dyslipidemia is not exclusively mediated by mTORC1.

WTD increases PPARδ ligands in serum and increases the expression of PPARδ target genes

As we observed that a decrease in mTORC1 activity alone was not sufficient to increase mitochondrial FA oxidation we sought to determine which additional mechanism(s) could be responsible. Since FA oxidation in Treg cells was affected by the composition of the diet, we reasoned that peroxisome proliferator activated receptors (PPAR) were involved as these nuclear receptors are activated by dietary lipids and can modulate cellular metabolism.
The PPAR proteins, PPARα -δ and –γ, have limited overlap in their natural ligands and biological function [33,34]. We were unable to detect *PPARα* expression in NCD- or WTD-Treg cells (fig. S4A). PPARδ and PPARγ share some of their target genes. Since PPARγ target genes are involved in the uptake and biosynthesis of lipids and *PPARδ* expression was about 10-fold higher compared to *PPARγ* expression in Treg cells (Fig. S3A), it seemed plausible that any PPAR-mediated effects were mainly PPARδ mediated. In support of this, the mRNA expression of PPARγ target genes *Scd1* and *Dgat* was unaltered between NCD- and WTD-Treg cells (fig. S3B).
In skeletal muscle, PPARδ activation using a synthetic ligand increases FA oxidation by increasing the expression of genes involved in FA catabolism (including *Cpt1*) and reduces glucose catabolism by reducing the expression of genes involved in glycolysis [35]. To examine whether diet-induced dyslipidemia activated PPARδ in our study we first treated isolated Treg cells with GW501516, a PPARδ agonist, *in vitro.* GW501516 treatment increased the expression of *Cpt1a, Slc25a20* and *Plin2* while decreasing *Lipe* expression (fig. 4A). *Slc25a20* and *Plin2* expression were increased and *Lipe* expression was decreased in WTD-Treg cells as compared to NCD-Treg cells (fig. 4B), indicating that

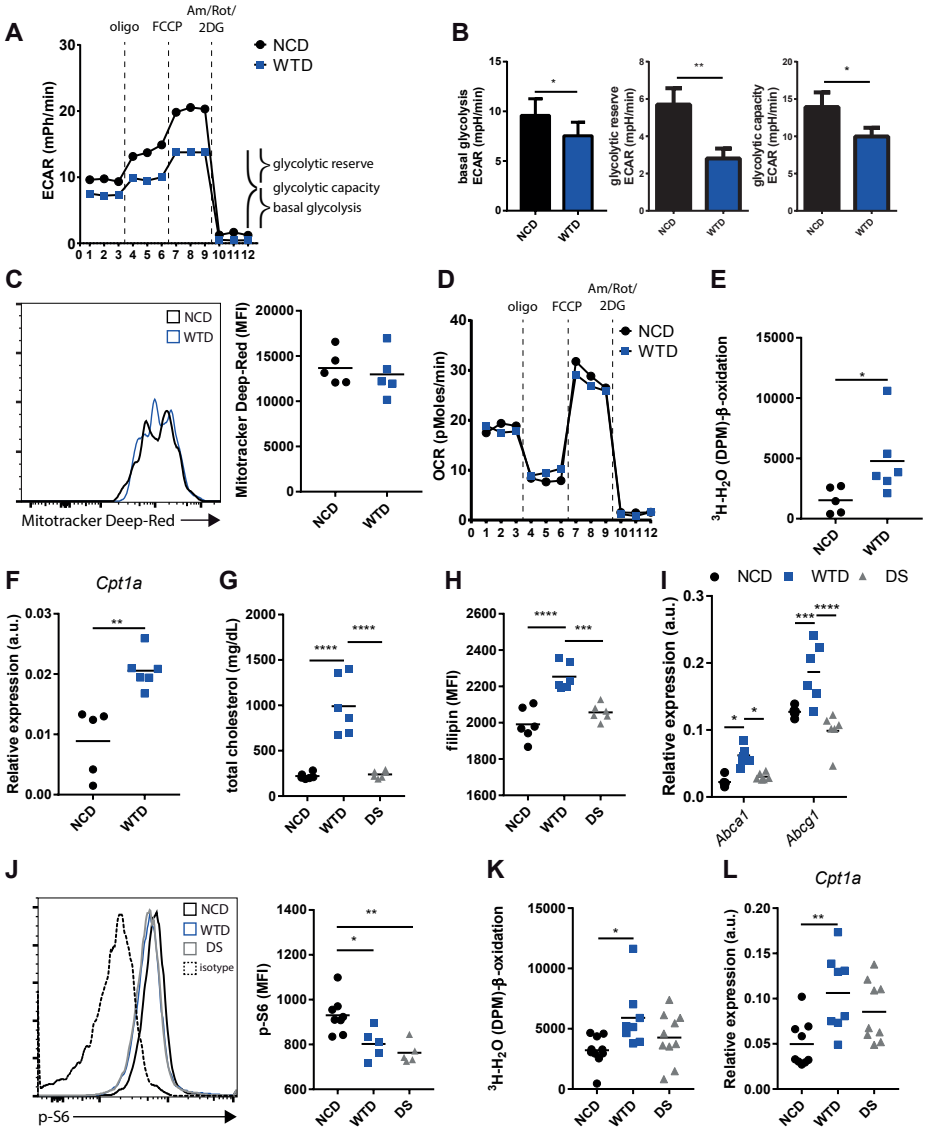
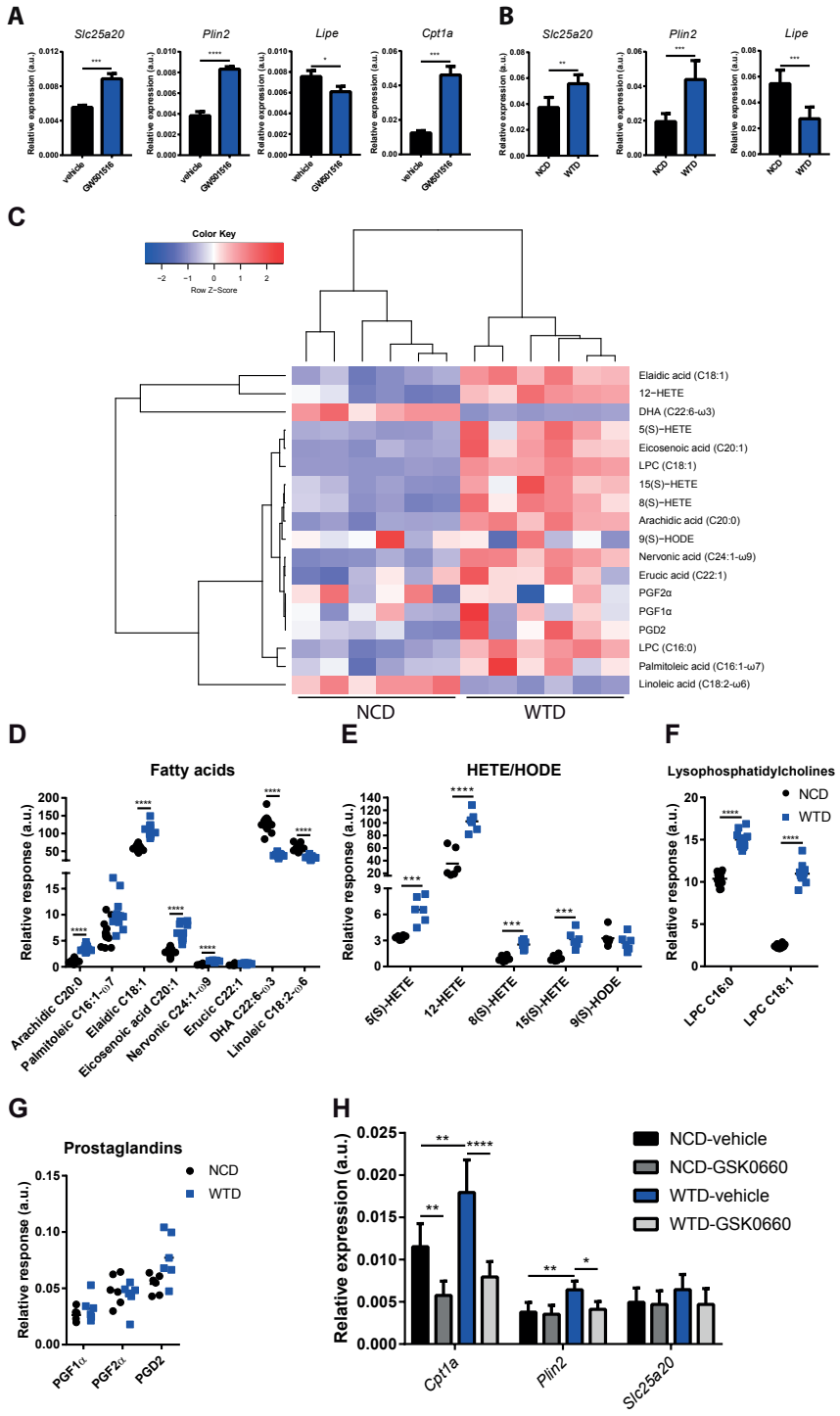


Figure 3. Diet-induced dyslipidemia in *Ldlr*$^{-/-}$ mice impaired glycolytic metabolism but enhanced mitochondrial FA metabolism in WTD-Treg cells. (A) Extracellular acidification rate (ECAR) during of NCD-Tregs and WTD-Tregs in response to oligomycin (oligo), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), antimycin-A (Ant), rotenone (Rot) and 2-deoxyglucose (2-DG). (B) Basal glycolysis, glycolytic reserve and glycolytic capacity quantified from (A). (C) Mitochondrial mass in Treg cells (D) Oxygen consumption rate (OCR) in same assay as in (A). (E) ^{3}H-palmitic acid detritiation in isolated Treg cells from indicated groups (F) *Cpt1a* expression in isolated Treg cells. (G) Total serum cholesterol levels from diet-switch experiment (H) Cellular cholesterol staining in Treg cells (I) Expression of cholesterol efflux transporters *Abca1* and *Abcg1*. (J) p-S6 levels as measured by flow cytometry (K) ^{3}H-palmitic acid detritiation after 4h incubation with ^{3}H-palmitic acid (L) *Cpt1a* expression in diet switch experiments. A,-J represents data from 2 independent experiments. Data in K and L is pooled from two independent experiments showing similar effects. DS = diet switch group *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The data in B represents the mean±standard deviation.


A
Slc25a20
Plin2
Lipe
Cpt1a
Relative expression (a.u.)
vehicle
GW501516
B
Slc25a20
Plin2
Lipe
NCD
WTD
C
Color Key
Row Z-Score
Elaidic acid (C18:1)
12−HETE
DHA (C22:6-ω3)
5(S)−HETE
Eicosenoic acid (C20:1)
LPC (C18:1)
15(S)−HETE
8(S)−HETE
Arachidic acid (C20:0)
9(S)−HODE
Nervonic acid (C24:1-ω9)
Erucic acid (C22:1)
PGF2α
PGF1α
PGD2
LPC (C16:0)
Palmitoleic acid (C16:1-ω7)
Linoleic acid (C18:2-ω6)
NCD
WTD
D
Fatty acids
Relative response (a.u.)
Arachidic C20:0
Palmitoleic C16:1-ω7
Elaidic C18:1
Eicosenoic acid C20:1
Nervonic C24:1-ω9
Erucic C22:1
DHA C22:6-ω3
Linoleic C18:2-ω6
E
HETE/HODE
Relative response (a.u.)
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12-HETE
8(S)-HETE
15(S)-HETE
9(S)-HODE
F
Lysophosphatidylcholines
NCD
WTD
Relative response (a.u.)
LPC C16:0
LPC C18:1
G
Prostaglandins
NCD
WTD
Relative response (a.u.)
PGF1α
PGF2α
PGD2
H
Relative expression (a.u.)
NCD-vehicle
NCD-GSK0660
WTD-vehicle
WTD-GSK0660
Cpt1a
Plin2
Slc25a20

Figure 4 Diet-induced dyslipidemia in *Ldlr*$^{-/-}$ mice increases circulating PPARδ ligands and elevates PPARδ target gene expression in Treg cells (A) Expression of PPARδ target genes in Treg cells treated with GW501516 or vehicle *in vitro* (B) Expression of same genes as in (A) minus *Cpt1a* in NCD- and WTD-Treg cells (C) Heatmap of natural PPARδ ligands in serum from NCD and WTD-fed mice. (D) Relative abundance of PPARδ ligands from fatty acids subclass. (E) Relative abundance of PPARδ ligands from hydroxyeicosatetraenoic acid (HETE) and hydroxyoctadecadienoic acid (HODE) subclasses (F) Relative abundance of PPARδ ligands from lysophosphatidylcholine subclass. (G) Relative abundance of PPARδ ligands from prostaglandin subclass. (H) Expression of PPARδ target genes after *in vitro* treatment with GSK0660 or vehicle (DMSO) treatment. *<0.05, **p<0.01, ***p<0.001, ****<p<0.000. The data in A, B and H represent the mean±standard deviation.

specific target genes of PPARδ which are involved in FA catabolism (*Slc25a20*, *Lipe*) and lipid droplet formation (*Plin2*) are modulated by dyslipidemia.

CD36 is a scavenger receptor involved in the uptake of FAs and (modified) lipoproteins. It is a transcriptional target of PPARγ [36] and CD36-mediated uptake of FAs can modulate the activity of PPARδ whose target genes are involved in FA- and glucose metabolism [37]. Compared to NCD-Treg cells, CD36 expression was increased in WTD-Treg cells but not in DS-Treg cells (fig. S3C), suggesting that elevated CD36 expression might contribute to the increased mitochondrial FA oxidation in WTD-Treg cells by increasing the uptake of lipids. CD36 expression in Tconv cells from WTD-fed mice was also increased but remained far lower as compared to Treg cells (fig. S3D), possibly explaining why the latter are more sensitive to perturbations in environmental lipid levels.

To confirm WTD-induced dyslipidemia increased the levels of circulating PPARδ ligands, we performed metabolomics profiling by high-performance liquid chromatography and mass spectrometry of the free and total oxidized lipids in sera of NCD- and WTD fed mice. We selected previously described PPARδ ligands [38–40] which were included in our lipidomics platform and examined relative increases or decreases in the abundance of these lipids. In general, PPARδ ligands were increased in sera of mice which were fed a WTD compared to NCD fed *Ldlr*$^{-/-}$ mice (Fig. 4C). Of the eighteen PPARδ ligands we examined, only two were less abundant in sera of WTD fed mice while eleven were more abundant. Especially lipids from the saturated- and monounsaturated FAs (fig. 4D) , hydroxyeicosatetraenoic acid (HETE) (fig. 4E) and lysophosphatidylcholine (fig. 4F) subclasses were relatively increased after 8 weeks of WTD as compared to NCD. There were no changes in the abundance of serum prostaglandins (fig. 4G). HETEs can be synthesized from various polyunsaturated FAs, including arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) or eicosapentaenoic acid (EPA) through similar pathways. AA (20:4 ω-6) showed a small relative decrease in WTD serum as compared to NCD serum (fig S3E). Relative EPA abundance was decreased as well (fig. S3F) but DGLA (fig. S3G) was increased, suggesting that an increase of DGLA in the serum contributed to increased HETE synthesis and circulating HETEs. Specific triglyceride-derived FAs, which were identified as potent natural ligands for PPARδ but not for PPARγ in macrophages [38], were

increased during WTD-induced dyslipidemia, including palmitoleic, elaidic, eicosenoic, and erucic acid. Reverting the WTD group to an NCD normalized the free fatty acid levels (FFA) in the serum (Fig. S3H) as well as serum triglycerides (fig. S3I). Lastly, we treated isolated Treg cells *in vitro* with the PPARδ antagonist/inverse agonist GSK0660 to confirm that the increase in *Cpt1a*, *Plin2* and *Slc25a20* was PPARδ-mediated. The increased expression in *Cpt1a* and *Plin2* but not of *Slc25a20* in WTD-Treg cells was sensitive to a 4h treatment with GSK0660 *in vitro* (fig. 4H) suggesting that PPARδ was indeed activated by WTD-induced dyslipidemia but that pharmacological inhibition of PPARδ activity did not abolish this entirely.

The presented data show that dyslipidemia increased the abundance of PPARδ ligands in the circulation, thereby increasing PPARδ activity in Treg cells and contributing to alterations in their bioenergetic metabolism.

Treg cells with high rates of FA oxidation migrate more efficiently towards sites of inflammation

Since PPARδ contributed to metabolic adaptations in WTD-Treg cells and we hypothesized that the changes in glycolytic and FA metabolism could affect their migration, we mimicked that effect of dyslipidemia by treating purified Treg cells *in vitro* with the PPARδ agonist GW501516 and assessed Treg cell migration. Similar to WTD-Treg cells, mitochondrial FA oxidation was increased in GW501516-treated Treg cells (fig. 5A). Additionally, GW501516 treatment decreased Glut1 expression on Treg cells on an mRNA (fig. 5B) and protein level (fig. 5C) without affecting the expression of membrane proteins involved in migration, including CCR5, CCR7, CXCR3, CD62L and LFA-1 (fig. S4A). A peritoneal homing experiment showed that GW501516-treated Treg cells migrated more efficiently towards the inflamed peritoneum as compared to vehicle control (fig. 5D). Moreover, this effect was FA oxidation-dependent as pre-incubating GW501516-treated Treg cells with a 100 μM of the irreversible CPT1 inhibitor etomoxir abolished their enhanced migratory capacity. A 100 μM etomoxir concentration is relatively high, but has no off-target effects on oxidative phosphorylation [29]. Etomoxir treatment did not affect the viability of Treg cells treated with dimethyl sulfoxide (DMSO) or GW501516 indicating the decreased number of Treg cells pre-treated with etomoxir, which we recovered from the inflamed peritoneum. was not due to increased cell death (fig. 5E). To validate these findings *in vitro*, a transwell migration assay with CCL21 was performed which confirmed that GW501516-treated Treg cells displayed more potent migration, again in an FA oxidation dependent fashion (fig. 5F). As mentioned above, Treg cells from WTD-fed *Ldlr*$^{-/-}$ mice are less capable to bind to activated endothelium as compared to Treg cells from NCD-fed *Ldlr*$^{-/-}$ mice [4]. As we unraveled a metabolic phenotype in WTD-Treg cells that may promote their migratory capacity, we assessed this in a peritoneal homing assay. Although the number of migrated WTD-Treg cells was equal compared to NCD-fed

donor derived Treg cells (fig. 5G) the percentage of WTD-Treg cells in the peritoneal Treg cell population was higher than NCD-Treg cells (fig. 5H). Supposedly, this was because the total number of Treg cells which were recruited towards the inflamed peritoneum in the WTD-Treg cell-injected mice was lower but the migratory capacity of WTD-Treg cells was higher as compared to the CD45.1^{+} acceptor Treg cells. To further examine WTD-Treg cell migration in the context of atherosclerosis, we performed an *in vitro* aorta homing experiment with NCD- and WTD-Treg cells with or without pre-incubation with etomoxir. We incubated Treg cells with isolated atherosclerotic aortas from apolipoprotein E deficient mice, which had previously developed advanced atherosclerotic lesions. Interestingly, WTD-Treg cells migrated more efficiently into atherosclerotic lesions as compared to NCD-Tregs (fig. 5I). Surprisingly, as opposed to GW501516-treated Treg cells, the increased migratory capacity of WTD-Treg cells was only mildly affected by pre-treatment with etomoxir, suggesting that despite dyslipidemia-induced skewing of bioenergetic metabolism WTD-Treg cells were sufficiently flexible to generate the required amounts of ATP using alternative fuel sources.
In conclusion, these results indicate that activation of PPARδ and FA oxidation increases Treg cell migration and that dyslipidemia does not reduce but actually promotes migration of Treg cells towards sites of inflammation.

Diet-induced dyslipidemia affects the cellular lipid content of T cells inside atherosclerotic lesions of *Ldlr*$^{-/-}$ mice

As we established that Treg cells in specific SLOs were affected by dyslipidemia-induced lipid accumulation, we next focused on Treg cells which had migrated into atherosclerotic lesions as their function is crucial for immunosuppression at the site of inflammation. Using flow cytometry, we assessed the amount of cholesterol and lipid droplets in CD4^{+}CD25^{hi} T cells from atherosclerotic lesions of the aortic arches of NCD- and WTD-fed *Ldlr*$^{-/-}$ mice (fig. 6A). In atherosclerotic aortic arches, similar to the spleen and medLN, the amount of cholesterol (fig. 6B) and lipid droplets (fig. 6C) was higher in CD4^{+}CD25^{hi} T cells from WTD-fed mice as compared to NCD-fed mice. It must be noted that it is unlikely that, as opposed to the spleen and medLN, aortic CD4^{+}CD25^{hi} T cells predominantly represent Treg cells as these can also be activated Tconv cells. We measured CD36 expression in these cells to get an indication of whether the extent of lipid-induced metabolic stress was sufficient to induce PPAR activation. Again, similar to the Treg cell populations of the spleen and medLN, WTD-induced dyslipidemia was associated with increased expression of CD36 in CD4^{+}CD25^{hi} T cells from the atherosclerotic aortic lesions as compared to NCD controls (fig. 6D).
In conclusion, the T(reg) cell population in atherosclerotic lesions from WTD-fed *Ldlr*$^{-/-}$ mice contained more cholesterol and lipid droplets as compared to NCD-fed mice which is similar to what we observed in the spleen and medLN.

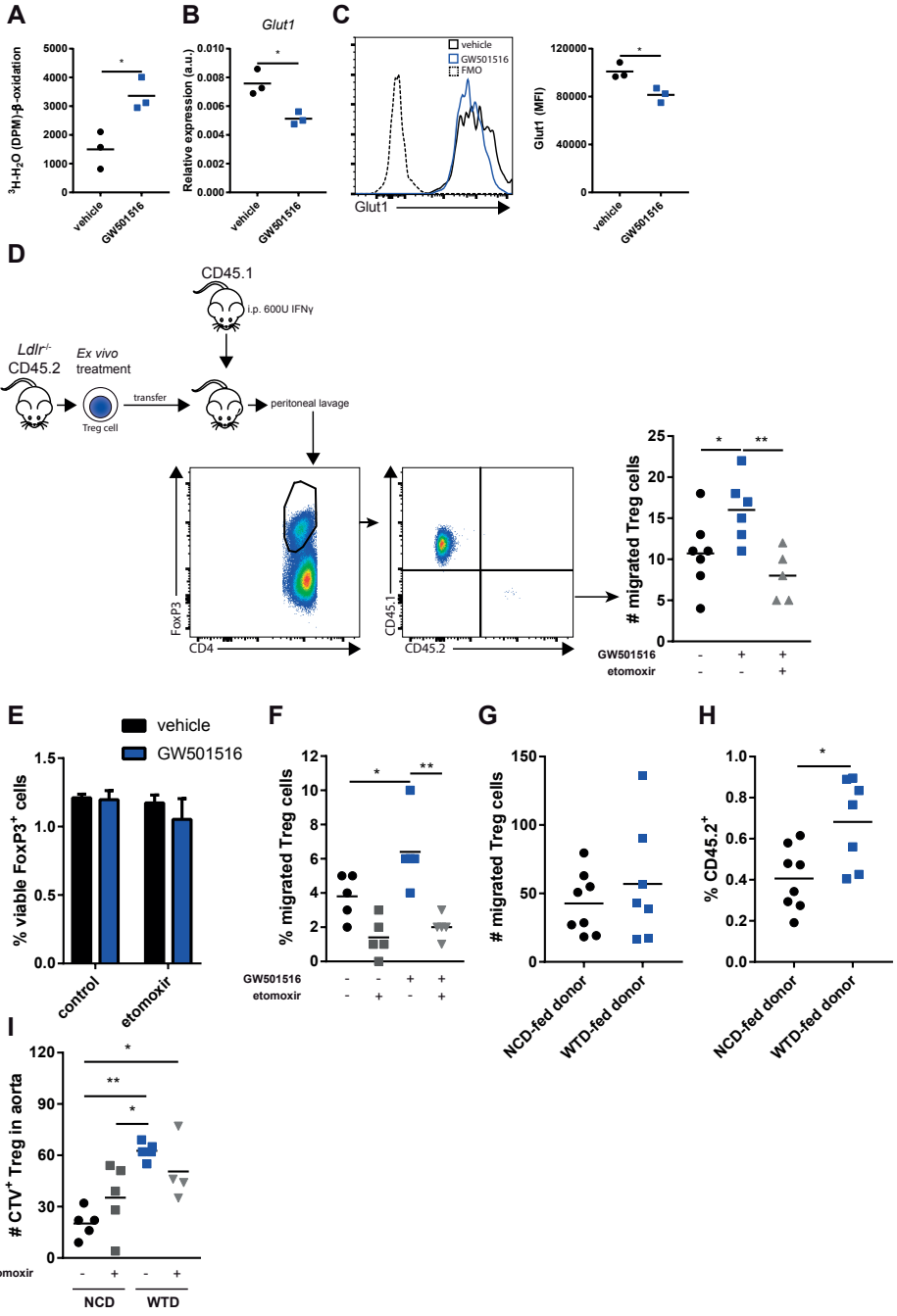
A
B
Glut1
C
vehicle
GW501516
FMO
Glut1
Glut1 (MFI)
^{3}H-H$_2$O (DPM)-β-oxidation
Relative expression (a.u.)
D
CD45.1
i.p. 600U IFNγ
Ldlr$^{-/-}$
CD45.2
Ex vivo
treatment
transfer
Treg cell
peritoneal lavage
FoxP3
CD4
CD45.1
CD45.2
migrated Treg cells
GW501516
etomoxir
E
vehicle
GW501516
% viable FoxP3$^+$ cells
control
etomoxir
F
% migrated Treg cells
G
migrated Treg cells
NCD-fed donor
WTD-fed donor
H
% CD45.2$^+$
I
CTV$^+$ Treg in aorta
NCD
WTD

Figure 5 Increased FA oxidation increases *Ldlr*$^{-/-}$ Treg cell migration (A) ^{3}H-palmitic acid detritiation in GW501516 treated Treg cells. (B) RT-qPCR analysis of *Glut1* expression. (C) Flow cytometry analysis of Glut1 expression. (D) Peritoneal homing experiment of GW501516 treated Treg cells. (E) Viability of Treg cells with indicated treatments (F) Transmigration assay towards 250ng/mL CCL21 of Treg cells treated as indicated. (G) Peritoneal homing experiment of NCD-Tregs versus WTD-Tregs. CD4^{+} T cells from NCD or WTD mice were injected i.v. and the number of Treg cells retrieved from the peritoneum were normalized for the number of Treg cells which were injected in the different CD4^{+} fractions. (H) Percentage of transferred NCD- and WTD-Treg cells relative to total number of peritoneal Treg cells (I) In vitro homing assay of NCD-Treg cells versus WTD-Treg cells with or without pre-incubation with 100 μM etomoxir. Treg cells were left to migrate towards atherosclerotic aortas from *Apoe*$^{-/-}$ mice. A-C and F represents data of 2/3 independent experiments. D represents data from 2 pooled experiments which showed similar effects. G-I represents data from one experiment. *p<0.05, **p<0.01. The data in E represents the mean±standard deviation.

DISCUSSION

A decrease in Treg cells in atherosclerotic lesions is associated with the degree of dyslipidemia. We showed that Treg cells accumulate (free) cholesterol and other neutral lipids during dyslipidemia which, through intrinsic changes in mTORC1/mTORC2 signaling and PPARδ activity, skewed their migration towards sites of inflammation instead of LNs. Pharmacological activation of PPARδ with GW501516 mimicked the effects of dyslipidemia on FA oxidation in Treg cells and increased their migration towards sites of inflammation. These findings suggest that the decrease in Treg cell immunosuppression in advanced atherosclerosis is not due to dyslipidemia-induced impairments in migratory capacity as dyslipidemia biased migration towards sites of inflammation.

An important point to address is how Treg cell-mediated immunosuppression is decreased in atherosclerotic lesions while diet-induced dyslipidemia induces intrinsic (metabolic) changes which skews their migratory phenotype, presumably in a beneficial manner. We propose that diet-induced dyslipidemia enhances the capacity of Treg cells to migrate towards sites of inflammation but that the local environment inside atherosclerotic lesions is unfavorable for Treg cells, thereby disrupting their immunosuppressive capacity. In support of this, Treg cells inside murine atherosclerotic lesions become increasingly apoptotic as lesions progress during diet-induced atherosclerosis an effect, which is counteracted by restoration of normocholesterolemia [4]. The main culprit lipoprotein in atherosclerosis is the cholesterol-rich LDL particle, which becomes oxidized in the vessel wall. Oxidized LDL (oxLDL) can dose-dependently induce apoptosis in human Treg cells [41] and has been suggested to induce apoptosis in murine Treg cells as well [7]. This suggests that dyslipidemia itself contributes to a microenvironment inside lesions which is especially unfavorable for Treg cells.

Another feasible explanation for a loss of Treg cells inside lesions, in addition to increased local apoptosis, is that Treg cells might lose expression of FoxP3 inside atherosclerotic lesions and are therefore not identifiable as Treg cells. Indeed, oxLDL can increase meth-

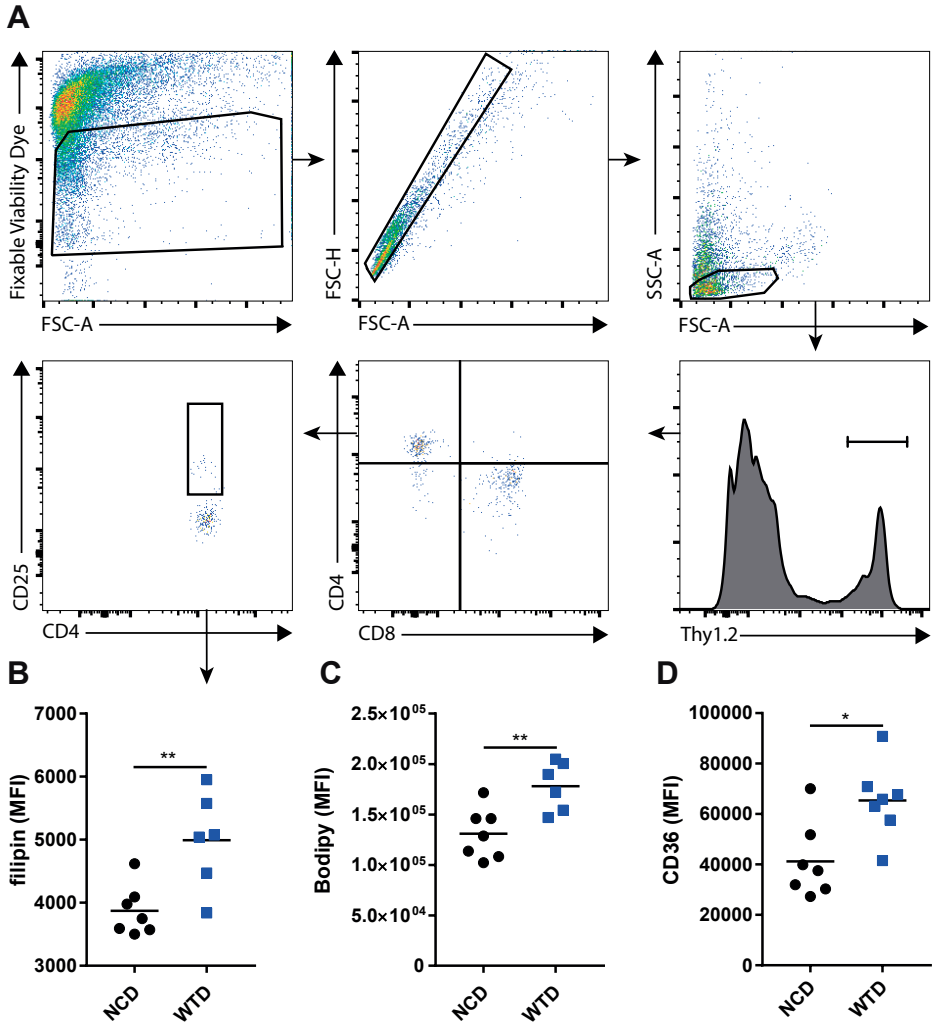

Figure 6 The effects of WTD-induced dyslipidemia on CD4^{+}CD25^{hi} T cells in atherosclerotic lesions from *Ldlr*$^{-/-}$ mice (A) The gating strategy for CD4^{+}CD25^{hi} T cells in atherosclerotic lesions from the aortic arch. (B) Filipin staining in CD4^{+}CD25^{hi} T cells. (C) Bodipy staining in CD4^{+}CD25^{hi} T cells. (D) CD36 staining in CD4^{+}CD25^{hi} T cells. *<0.05, **p<0.01.

ylation of the demethylated regions in the promotor of the *FoxP3* gene in Treg cells from healthy subjects [42], resulting in decreases of *FoxP3* expression in Treg cells. Also in the microenvironment of murine atherosclerotic lesions, a CD4^{+} T cell population expressing both FoxP3 and Tbet has been described [43] although it is unclear whether these cells originated from Treg cells. Moreover, WTD-induced atherogenesis was recently shown

to decrease FoxP3 expression in Treg cells and induce their differentiation to follicular helper T cells [44].

Therefore, data from previous reports suggest that dyslipidemia contributes to a microenvironment in lesions which is especially hostile for Treg cells, indicating that decreased immunosuppression by Treg cells in atherosclerotic lesions is likely due to local apoptosis and differentiation to T helper cell subsets but not due to decreased migration of circulating Treg cells towards lesions.

Diet-induced dyslipidemia increased the levels of cholesterol and lipid droplets in Treg cells in the spleen, medLN and atherosclerotic lesions of the aorta but not in the blood and iLN. It is unclear whether the increase in lipids in Treg cells from the medLN and atherosclerotic lesions occurred *in situ* or whether Treg cells which accumulated lipids elsewhere preferentially migrated towards atherosclerotic lesions and (subsequently) to draining LNs. Further investigation is required to examine whether Treg cells from atherosclerotic lesions can efficiently migrate towards draining LNs as migration from sites of inflammation towards draining LNs via afferent lymph vessels is regulated in part by CCR7 and S1Pr1 [45].

Regardless of the tissue in which lipid accumulation occurs in Treg cells during dyslipidemia, it is intriguing to investigate whether lipid loaded Treg cells which reside in the microenvironment of atherosclerotic lesions also have altered migratory and metabolic phenotypes and how these contribute to aberrations in their immunomodulatory function and contribute to increased apoptosis and local differentiation.

mTORC2 activity was increased in WTD-Tregs, which, through the mTORC2-Akt-Foxo1-Klf2 axis, resulted in decreased expression of markers which T cells require to home towards LNs. As a result of decreases in expression of CCR7, CD62L and S1Pr1, WTD-Tregs were less able to migrate towards dLNs. These findings confirmed to some extent a report describing obesity-induced metabolic stress causing altered PI3K-p110δ activity which, via increased mTORC2 activity, skews $CD4^+$ T cell migration towards sites of inflammation [19]. As we observed a large increased in FFAs in the serum of WTD-fed mice as compared to NCD-fed mice, it is probable that a feeding *Ldlr*$^{-/-}$ mice a WTD increased circulating palmitate levels as well which altered PI3K and mTORC2 activity in Treg cells through similar mechanisms. Thereby, perturbations in systemic lipid metabolism had profound effects on the migratory markers which WTD-Treg cells express.

Dyslipidemia led to elevated cholesterol in Treg cells which mildly decreased mTORC1 activity and led to decreased expression of genes from the mevalonate pathway without affecting the levels of HIF1α and its transcriptional targets. The effects of diet-induced dyslipidemia on the mevalonate pathway in Treg cells are reminiscent of the effects Treg cell-specific *Raptor* deletion has on cholesterol synthesis [8]. However, the effects we observed were most likely also due to liver-X-receptor (as indicated by increased expression of Abca1 and Abcg1 in WTD-Treg cells) and sterol regulatory element bind-

ing protein (SREBP) directly responding to intracellular cholesterol accumulation to prevent lipotoxicity. In this study, endocytosis of lipoproteins resulted in large amounts of cholesterol in lysosomes which could be sensed by mTORC1. Lysosomal cholesterol accumulation can specifically activate the mTORC1 complex through the SLC38A9–Niemann-Pick C1 signaling complex [46]. Instead, our data suggested cholesterol overload in Treg cells decreased mTORC1 activity. This is also supported by literature describing Treg cell specific genetic deletion of *Abcg1* in mice with normolipidemia and dyslipidemia resulted in an increase in free cholesterol levels and decreased mTORC1 activity in Treg cells [14]. Teleologically, metabolic stress during prolonged dyslipidemia requires a cell intrinsic response to prevent lipotoxicity and decreased lipid synthesis, partly regulated by decreased mTORC1 and SREBP activity, establishes this.

Dyslipidemia increased the mitochondrial FA oxidation rate and reversion to normolipidemia through dietary intervention abolished this effect, which suggested that systemic lipid metabolism is tightly linked to cellular lipid metabolism in Treg cells. Treg cells, which unlike foam cells are not historically recognized as lipid scavengers, adapt their cellular metabolism most likely to prevent lipotoxicity. This had a profound effect on their migratory function. Although glycolysis and glycolytic capacity were slightly impaired, most likely as a result of cholesterol-mediated inhibition of mTORC1 activity, increased ATP generation through FA oxidation might have compensated for these defects when large amounts of ATP are required for cytoskeletal actin rearrangements during cell migration [47,48]. In Treg cells, glucokinase has been shown to be crucial for glycolysis-derived ATP generation to facilitate Treg cell migration upon migratory stimuli [12]. However, the Treg cells used in that report were primarily generated or treated *in vitro* meaning that these cells probably depended mainly on glycolysis to generate ATP. In support of this, in $CD8^+$ T cells, the ECAR dose-dependently increases with the concentration of glucose in the culture medium [49]. As dyslipidemia and GW501516 treatment augmented FA oxidation and migration but not glycolysis in Treg cells, our study suggests that the dominant ATP-generating catabolic pathway is crucial for Treg cell migration and how bioenergetic metabolism is skewed by which environmental stimuli determines which catabolic pathway is dominant.

Treatment of Treg cells with GW501516 resulted in higher migratory capacity of Treg cells towards sites of inflammation. As this was dependent on FA oxidation both *in vitro* and *in vivo*, treatment with this compound presumably decreased the metabolic flexibility of Treg cells. In contrast, pre-treatment of WTD-Tregs with etomoxir only showed mild effects on their migratory capacity, suggesting that WTD-Treg cells were better capable of switching to alternative catabolic pathways to generate ATP. Further characterization of the dependence of WTD-Treg cells on other metabolic pathways besides glycolysis and FA oxidation would deepen our understanding of how dyslipidemia can affect Treg

cell metabolism, possibly leading to new therapeutic opportunities to modulate Treg cell function and dampen inflammation.

Altogether, our observations suggest that dietary lipids can alter Treg cell metabolism and migratory function. It is expected that pharmacological intervention to increase Treg cell migration alone will not suffice to dampen atherosclerosis or other autoimmune-like diseases if the microenvironment at the site of inflammation is not suitable for Treg cells. Furthermore, our findings suggest that in other metabolic diseases which are characterized by nutrient excess and autoimmune-like chronic inflammation (such as obesity) cellular metabolism in T(reg) cells might be altered and that these adaptations might be exploited to alter their migration for therapeutic purposes.

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SUPPLEMENTARY FIGURES

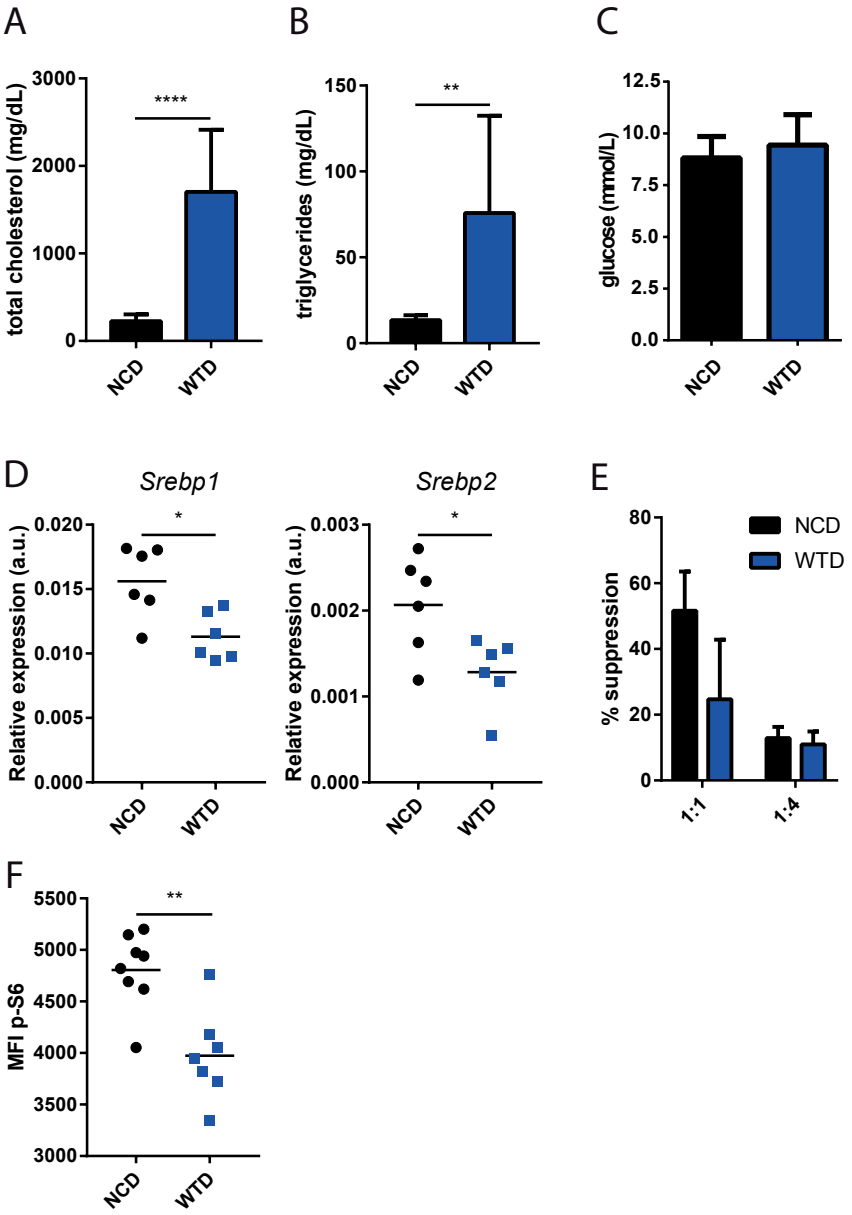

Figure S1 Diet-induced dyslipidemia affects mTORC1 signaling and cholesterol metabolism (A) Total cholesterol levels in serum (B) Triglyceride levels in serum (C) Blood glucose levels after 4h of fasting (D) *Srebp1* and *Srebp2* in isolated Treg cells. (E) Suppression assay with Treg cells and effector T cells in two different ratios (Treg cell:splenocytes) (F) mTORC1 activity in conventional T cells as measured by assessing p-S6 levels. Data in A-D and F are representative for two individual experiments. Data in E represents one experiment. Data in A-C and E represent mean ± standard deviation. *p<0.05, **p<0.01, ****p<0.0001.

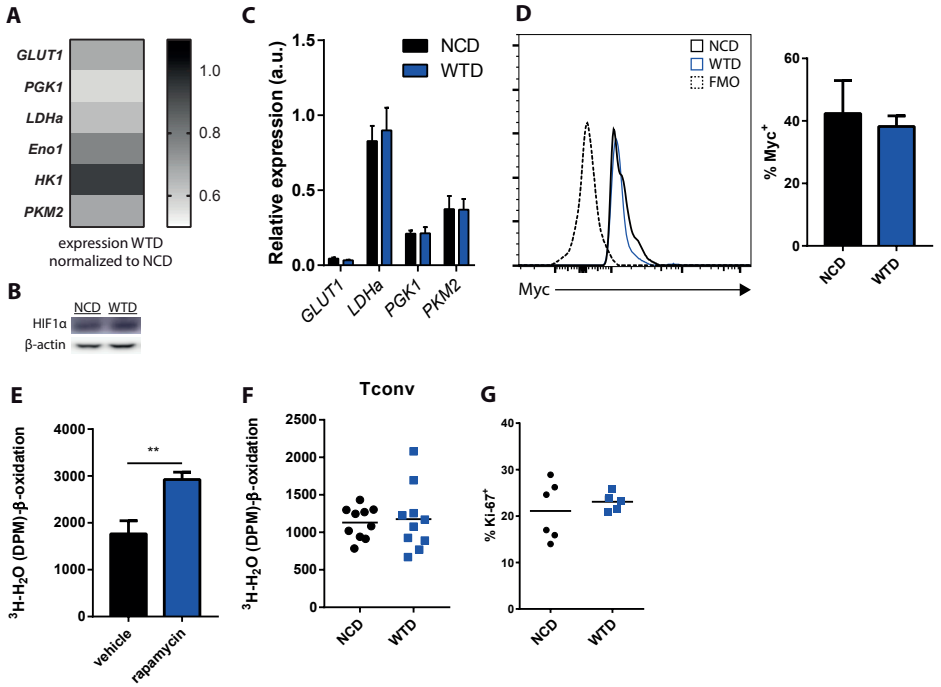

Figure S2 Metabolic effects of mTOR inhibition (A) Expression of HIF1α targets in Treg cells cultured with NCD- or WTD-serum. (B) HIF1α immunoblot on freshly isolated NCD- or WTD-Treg cells. (C) Expression of HIF1α targets in NCD- and WTD-Treg cells (D) Myc expression in NCD- and WTD-Treg cells (E) FA oxidation in Treg cells after rapamycin treatment (F) ^{3}H-palmitic acid detritiation in conventional T (Tconv) cells from NCD- or WTD-fed *Ldlr*$^{-/-}$ mice. (G) Percentage of proliferating Treg cells. **p<0.01. The data in C-E represent the mean±standard deviation.

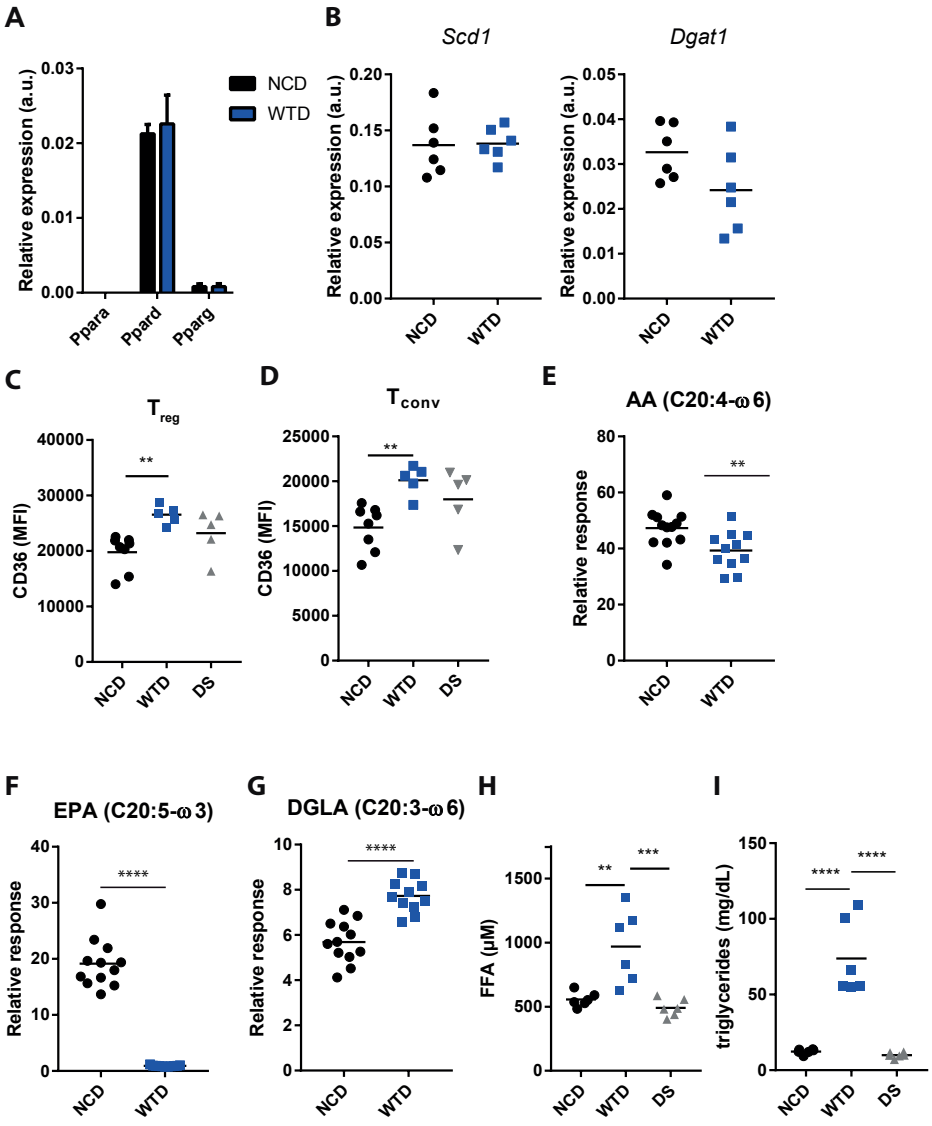

Figure S3 PPAR ligands and target gene expression during diet-induced dyslipidemia. (A) Expression of different PPARs in NCD-Treg cells and WTD-Treg cells. (B) Expression of the PPAR γ target genes *Scd1* and *Dgat1* (C) CD36 expression (MFI) in Treg cells from diet switch experiments (D) CD36 expression (MFI) in Tconv cells from diet switch experiments (E) Relative abundance of arachidonic acid (AA) (F) eicosapentaenoic acid (EPA) and (G) dihomo-γ-linolenic acid (DGLA) in serum of *Ldlr*$^{-/-}$ mice fed an NCD or WTD. (H) Free fatty acid (FFA) levels in serum during diet switch experiments (I) Serum triglyceride levels in the serum**.** *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The data in A represents the mean±standard deviation.

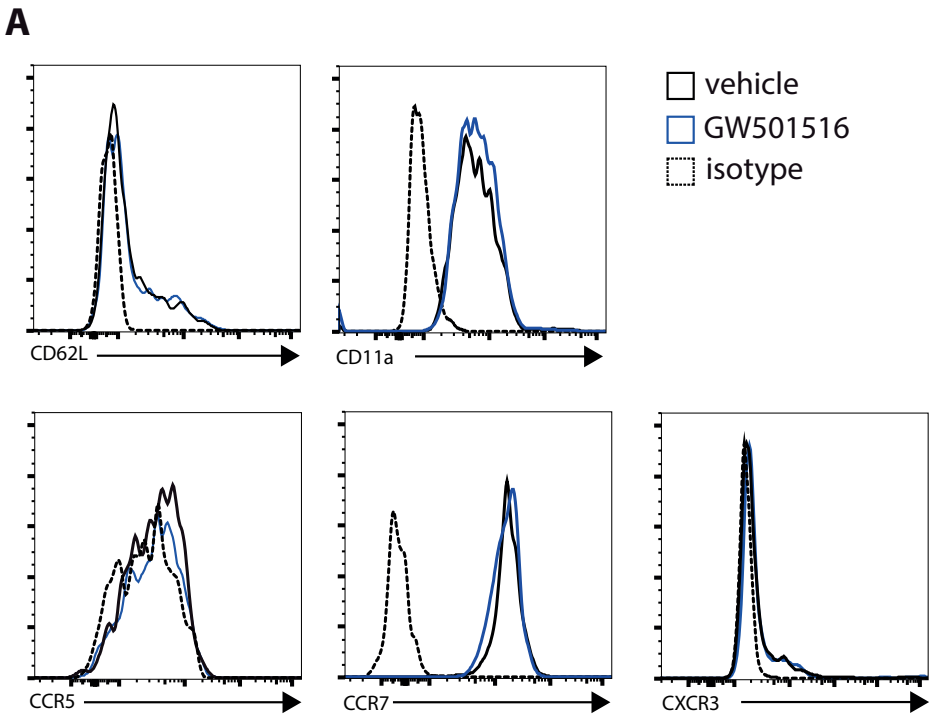

Figure S4 GW501516 treatment and migratory markers. (A) *In vitro* treatment of Treg cells with GW501516 did not affect the expression of CD62L, CD11a (also known as lymphocyte function-associated antigen 1, or LFA1), C-C chemokine receptor type 5 (CCR5), CCR7 or C-X-C motif receptor 3 (CXCR3).

SUPPLEMENTARY TABLES

Table 1 Antibodies/dyes used for flow cytometry

antigen	label	clone	manufacturer
p-4-EBP1 Thr37/46	AF647	236B4	Cell Signaling Technology
CCR5	PE-Cy7	HM-CCR5	BioLegend
CCR7	APC	4B12	eBioscience
CD11a	BV650	2D7	BD Biosciences
CD25	FITC	PC61.5	eBioscience
CD25	APC	PC61.5	eBioscience
CD3	APC	145-2C11	eBioscience
CD36	PE	CRF D-2712	BD Biosciences
CD4	FITC	GK1.5	eBioscience
CD4	PE	GK1.5	eBioscience
CD4	eFluor 405	GK1.5	eBioscience
CD45.1	PB	A20	eBioscience
CD45.2	APC	104	eBioscience
CD62L	PerCP-Cy5.5	MEL-14	eBioscience
CXCR3	PE	CXCR3-173	BioLegend
FoxP3	eFluor 405	FJK-16s	eBioscience
FoxP3	APC	FJK-16s	eBioscience
FoxP3	PE	NRRF-30	eBioscience
Ki-67	FITC	SolA15	eBioscience
p-Akt Ser473	V450	M89-61	BD Biosciences
p-S6 Ser235/236	AF488	2F9	Cell Signaling Technology
Thy1.2	PE-Cy7	53-2.1	eBioscience
eFluor 780 viability dye	APC-Cy7	-	eBioscience

Table 2 List of primers used for qRT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (3′-5′)
Hmgcs1	aaaacacagaaggacttacgcccgg	gttgcagggagtcttggcactttct
Hmgcr	cgagccacgacctaatgaagaatg	tgcatcactaaggaactttgcacc
Idi1	cattggtgtgaagcgagcagcaaag	caccccagataccatcagattgggc
Fdft1	aacatgcctgccgtcaaagctatca	gcttgatgatgggtctgagttgggg
Sqle	gagtgtgtgaccggtcctgttgg	actgaaaagggcccgtggttttgta
Srebp1	tctgaggaggagggcaggttcca	ggaaggcaggggggcagatagca
Srebp2	ccagctcctgggtgagacctac	caggcgcacagtggcttcat
Abca1	agagcaaaaagcgactccacatagaa	cggccacatccacaactgtct
Abcg1	ttgacaccatcccagcctac	cagtgcaggtcttctcggt
Klf2	gccgccacacatacttgcagct	tccagccgcatccttcccagtt
CD62L	tcattcctgtagccgtcatggtc	agcacttcatggctttcctttcac
Ccr7	cgtgctggtggtggctctcct	accgtggtattctcgccgatgtagtc
S1pr1	gtcagtcgccgacagcagcaag	acagcaaagccaggtcagcgag
Hif1a	aggagccttaacctgtctgccac	cctgctgcttgaaaaagggagcc
Glut1	ggtgtgcagcagcctgtgt	cacagtgaaggccgtgttga
Pgk1	gtgggtcgagctaagcagattgtttgg	tgctcacatggctgactttatcctctgt
Ldha	acgtggcttggaaaatcagtggctt	ggcaacattcacaccactccacaca
Eno1	cgcctggccaagtacaatcagatcc	tctccggtccatgctttatttggcc
Hk1	acgggacgctctacaaactccatc	gaggaaggacacggtacactttggt
Pkm2	ccattaccagcgaccccacagaag	agacttggtgagcacgataatggcc
Cpt1a	ggttgctgatgacggctatggt	tggcttgtctcaagtgcttccc
Ppara	tgacatttccctgtttgtggctgct	tgcacaatcccctcctgcaacttc
Ppard	gaccagaacacacgcttccttc	ccgacattccatgttgaggctg
Pparg	aagccctttggtgactttatggagcc	tgcagcaggttgtcttggatgtcc
Slc25a20	cagagatgtgttgagagagctgatccg	tgtccagcatccacaggtcttgaag
Plin2	gcacagtgccaaccagaaaattcagg	cagtctggcatgtagtctggagctg
Lipe	ctgacaataaaggacttgagcaactc	aggccgcagaaaaaagttgac
Taf7	agtctgggcatgtcaacctgaa	cgtaacacaaggcaaatcgacca
actin	cttctttgcagctccttcgttgccg	aatacagcccggggagcatcgtc
Rpl27	cgccaagcgatccaagatcaagtcc	agctgggtccctgaacacatccttg
Rpl37	agagacgaaacactaccgggactgg	cttgggtttcggcgttgttccctc
36B4	ctgagtacaccttcccacttactga	cgactcttcctttgcttcagcttt

CHAPTER 4

Modulation of lipid metabolism during dyslipidemia primes naïve T cells and affects their effector phenotype

Manuscript in preparation

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ABSTRACT

Lipid metabolism is a determining factor during the proliferation and differentiation of $CD4^+$ naïve T (Tn) cells into T helper and regulatory T (Treg) cells. An integral process in lipid metabolism during lipid overload is lipophagy, which is a specialized form of autophagy in which lipid droplets are targeted for lysosomal degradation. It is unclear whether dyslipidemia and modulation of lipid metabolism or lipophagy induce intrinsic changes in $CD4^+$ Tn cells which affect their effector phenotype.

Diet-induced dyslipidemia and dyslipidemia-like conditions, as mimicked by culturing $CD4^+$ Tn cells in the presence of excess lipoproteins or serum, induced liver-X-receptor (LXR) activation. Priming of $CD4^+$ Tn cells with atherogenic lipoproteins increased their proliferative capacity only in response to suboptimal antibody-induced stimulation but not antigen-induced stimulation. In line, priming of $CD4^+$ Tn cells under dyslipidemia-like conditions with serum from WTD-fed mice did not affect their proliferation in response to antigen stimulation. Interestingly, pharmacological LXR activation using T0901317 during priming inhibited the proliferation of $CD4^+$ T cells and induced Treg cell differentiation only after priming in dyslipidemia-like conditions. In line, modulation of lipid metabolism using the lysosomal inhibitor chloroquine during priming of $CD4^+$ Tn cells under dyslipidemia-like conditions decreased proliferation and decreased T helper 1 cell differentiation. *In vivo*, diet-induced dyslipidemia was not associated with increased autophagy in $CD4^+$ T cells. Interestingly, treatment of mice with chloroquine, which inhibits endolysosomal processing of lipoproteins as well as lipophagy, induced Treg cell differentiation but also increased $CD4^+$ T cell proliferation in mice with diet-induced dyslipidemia but not normolipidemia.

Altogether, modulation of lipid metabolism by LXR activation or lysosomal inhibition during the priming of $CD4^+$ Tn cells under dyslipidemia-like conditions affects the effector phenotype.

KEYWORDS

Naïve T cell, priming, lipids, LXR, autophagy, chloroquine

INTRODUCTION

A major risk factor for atherosclerosis is dyslipidemia in the form of hypercholesterolemia, as this is characterized by increased amounts of atherogenonic lipoproteins, such as low density lipoprotein (LDL). Native and modified LDL contain epitopes in its core-protein ApoB-100 which act as antigens for $CD4^+$ T cells inside atherosclerotic lesions [1,2] and accordingly promote atherosclerosis [3]. After activation by antigen-presenting cells (APC), $CD4^+$ naïve T (Tn) cells undergo a phase of cell blast, cell division and differentiation into a T helper cell subset. Cell blast and division require a distinct metabolic program to meet the biosynthetic demand which facilitates the generation of daughter cells from a single T cell clone [4]. Cell membranes consist, in part, of a phospholipid bilayer and free cholesterol molecules. Hence, lipid synthesis is among the first proteomic clusters to be upregulated upon activation of Tn cells [5], which is regulated by liver-X-receptor (LXR) [6] and sterol-regulatory element binding protein (SREBP) [7]. Activated LXR modulates cholesterol metabolism through its target genes, such as the cholesterol-efflux transporters ATP binding cassette A1 (ABCA1), which essentially promotes cholesterol efflux [8]. Hence, LXRβ-deficient T cells are hyperproliferative and pharmacological LXR activation inhibits T cell proliferation [6]. In line, T cells deficient for ABCG1, another cholesterol efflux transporter which regulates intracellular cholesterol trafficking and presumably localizes to the cell membrane [9], accumulate cholesterol and are hyperproliferative [10]. ABCG1 deficiency has also been shown to promote regulatory T (Treg) cell differentiation from naïve T cells [11]. Thus, cholesterol metabolism in T cells is a determining factor in the proliferative capacity and differentiation skewing of activated $CD4^+$ T cells.

In atherosclerosis, T helper (Th)1 cells are the main subset of $CD4^+$ T cells found in atherosclerotic lesions [12–14]. Th1 cells are characterized by high expression of the transcription factor T-bet and the secretion of interferon-gamma (IFNγ) via which Th1 cells drive atherosclerosis [15]. Another type of $CD4^+$ T cells, the Treg cell, is actually specialized in immunosuppression, through cell-cell interactions and the secretion of anti-inflammatory cytokines such as interleukin-10 (IL-10), and inhibit atherosclerosis [16]. Thus, hypercholesterolemia (or dyslipidemia) elevates the amount of antigen inside atherosclerotic lesions for T cells to respond to and cholesterol metabolism is an important factor in the inflammatory potency of T cells.

Interestingly, hypercholesterolemia has been suggested to modulate the inflammatory potency of T cells in an antigen-independent manner as well. For example, the supplementation of culture medium with cholesterol increases the proliferative capacity of wild-type T cells [10]. The majority of a cell's free cholesterol is located in the cell membrane where it modulates membrane rigidity [17,18] and lipid raft formation [19]. $CD8^+$ T cells deficient in acetyl-CoA acetyltransferase 1, the rate-limiting enzyme for cholesterol esterification, have elevated levels of free cholesterol and a more rigid cell membrane

which results in a more stable immunological synapse, enhanced T cell receptor (TCR)-signaling and increased T cell proliferation and inflammatory cytokine secretion [20]. Furthermore, lipid loaded $CD4^+$ T cells which are activated *in vitro* via antibody-mediated TCR stimulation are prone towards Th1 differentiation [21]. Additionally, as compared to $CD4^+$ T cells from normolipidemic mice, $CD4^+$ T cells from *Ldlr*$^{-/-}$ mice with diet-induced hypercholesterolemia contain different lipids and have an altered membrane lipid composition, which is associated with a more rigid cell membrane and increased T cell proliferation *in vivo* [22].

An essential organelle in the endolysosomal uptake of lipoproteins and degradation of intracellular lipid droplets is the lysosome [23]. Lysosomes contain lysosomal acid lipases (LAL), which degrade the triglycerides and cholesteryl from lipoproteins and deposit their content, being free fatty acids and cholesterol, into the cytoplasm. Autophagy is a cellular process in which intracellular cargo is targeted for lysosomal degradation through the selective or nonselective isolation of organelles by autophagosomes [24]. Lipophagy is a specific form of autophagy in which lysosomes degrade lipid droplets (LD) which have been targeted for lysosomal degradation by autophagosomal isolation membranes. In foam cells, atherogenic lipoprotein-induced LD accumulation is decreased by lipophagy-mediated LD degradation which enhances cholesterol efflux to Apo-A1 [25]. Most reports describe antigen-independent effects of dyslipidemia on T cells in the effector phase or the total $CD4^+$ T cell population. Presumably, dyslipidemia also modulates Tn cells which could affect their effector phenotype after TCR-stimulation. We define this as 'priming' of Tn cells as lipid accumulation could induce phenotypic changes in Tn cells prior to activation, which could affect the effector phenotype, as the strength of TCR-signaling and the induction of specific transcriptional programs, which drive lipid synthesis, could be altered. Through this mechanism, dyslipidemia primes Tn cells to have an altered inflammatory phenotype in their effector phase. We postulate here that lysosomal lipid handling and LXR-mediated cholesterol metabolism are an integral part of the priming effect of dyslipidemia on naïve T cells.

MATERIALS AND METHODS

Mice

All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and the experimental work was approved by the Animal Ethics committee of Leiden University. LDL receptor deficient (*Ldlr*$^{-/-}$) mice were purchased from the Jackson Laboratory and further bred in the Gorlaeus Laboratory in Leiden, The Netherlands. Diet-induced dyslipidemia was established by feeding mice from 9-12 weeks of age a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa

butter (Special Diet Services) for 2, 4, 8 or 16 weeks. At sacrifice, the mice were sedated and their blood was collected via orbital blood collection. Subsequently, their vascular system was perfused with PBS at a continuous low flow via heart puncture in the left ventricle after which the spleens were collected. For studies examining antibody-induced stimulation of T cells, male *Ldlr*$^{-/-}$ mice were used. For studies using antigen-induced T cell stimulation, we used male transgenic OT-II mice, purchased from the Jackson Laboratory and further bred in-house. OT-II mice have T cells which have a TCR recognizing chicken ovalbumin 323-339 peptide in the context of major histocompatibility complex II molecules. The animals were kept under standard laboratory conditions and were fed a normal chow diet (NCD) and water ad libitum.

Chloroquine treatment in vivo

The effect of chloroquine treatment on CD4$^+$ T cells was studied by feeding *Ldlr*$^{-/-}$ mice from 9-12 weeks of age an NCD or a WTD for 4 weeks. In the last three days of the experiment, mice from both diet groups were split into two groups which received a daily injection of either PBS or chloroquine (60 mg/kg) via intraperitoneal injection. After three days of chloroquine treatment, the mice were sacrificed as described above. Spleens were harvested, mashed through a 70 µm cell strainer to generate a single-cell suspension and used for CD4$^+$ T cell isolation.

Flow cytometry

Spleens were isolated and mashed through a 70 µm cell strainer. Erythrocytes were subsequently eliminated from the spleen by incubating the cells with ACK erythrocyte lysis buffer to generate a single-cell suspension prior to staining of surface markers. For analysis of surface markers, cells were stained at 4°C for 30 min. in PBS with 2% (vol/vol) fetal bovine serum (FBS). Intracellular transcription factors were stained for by following the FoxP3 staining protocol (eBioscience). All antibodies used for staining of surface markers or transcription factors were from eBioscience, BD Biosciences or BioLegend (table 1). For staining of LD using Bodipy™ 493/503 (Invitrogen), cells were stained with 1,3µg/mL Bodipy in pre-warmed PBS at room temperature for 10 minutes. Proliferation was assessed by flow cytometry by measuring the dye dilution in stimulated CD4$^+$ T cells which were labeled with 5 µM CellTrace™ Violet (Invitrogen) as per manufacturer's instructions. Flow cytometry analysis was performed on a FACSCantoII (BD Biosciences) or a Cytoflex S (Beckman Coulter) and data was analyzed using Flowjo software (TreeStar).

Cell culture

CD4$^+$ T cells or CD4$^+$CD62L$^+$CD44$^-$ naïve T (Tn) cells were isolated from spleens and peripheral lymph nodes (inguinal and mesenteric) from *Ldlr*$^{-/-}$ mice using MACS microbeads (Miltenyi Biotec). T cells were cultured in RPMI-1640 medium supplemented with

2 mM L-glutamine, 100U/mL pen/strep and 10% FBS (all from Lonza). Lipid loading of Tn cells was established *in vitro* by supplementing the medium with 5% serum from NCD- or WTD-fed mice for 48h. Alternatively, lipid loading was achieved by the addition of lipoproteins (10 µg/mL oxLDL and 50 µg/mL very low density lipoprotein (VLDL)) which were isolated from human serum through KBr density gradient ultracentrifugation as described below. Antibody-induced activation after lipid loading in Tn cells was performed using plate-bound anti-CD3e (1µg/mL; Ebioscience), anti-CD28 (1 µg/mL; Ebioscience) and 100U/mL recombinant murine IL-2 (Peprotech). The LXR agonist T0901317 (Sigma) was dissolved in dimethylsulfoxide and used at 5µM. The LXR antagonist GSK2033 (GlaxoSmith Klein) was used at 5µM. The lysosome inhibitor chloroquine diphosphate (ThermoFisher) was used at 50 µM.

OVA323 induced proliferation

Bone-marrow derived dendritic cells (BMDCs) were generated and cultured as described before [26] with the exception that 8% FCS was used. 24 hours before $CD4^+$ Tn cells were added to the BMDCs, the BMDCs were incubated with different concentrations of OVA323 peptide (Genscript) and 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech). Tn cells were isolated from male OT-II mice and primed with WTD-serum with or without T0901317 for 48 hours. After 48 hours, Tn cells were washed twice and added to GM-CSF-stimulated BMDCs to induce antigen-specific proliferation.

RNA and immunoblot analysis

mRNA was extracted from freshly isolated $CD4^+$ cells using the guanidium isothiocyanate (GTC) method after which cDNA was generated using RevertAid M-MuLV reverse transcriptase per manufacturer's instructions (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to 2-3 housekeeping genes (table 2). For immunoblot analysis, whole-cell lysates were prepared using radioimmunoprecipitation assay buffer (Cell Signaling Technology) supplemented with protease inhibitor (Roche). Protein lysates were resolved on a 15% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane with 0.2 µm pore-size. The membranes were probed with anti-LC3B or anti-β-actin antibodies (both from Novus Biologicals) and the proteins were detected using chemiluminescence.

Lipoprotein isolation and modification

The lipoproteins very-low density lipoprotein VLDL and LDL were isolated from non-fasted human serum using KBr-density gradient ultracentrifugation as described previously [27]. Oxidized LDL (oxLDL) was generated through copper oxidation by incubating LDL with $CuSO_4$ overnight at 37°C.

LC3 immunofluorescence

Isolated $CD4^+$ T cells were fixed with 3.7% formaldehyde in PBS and adhered to Superfrost adhesion slides (Thermo Scientific). Subsequently, the cells were permeabilized for 15 minutes at RT using 0.2% Triton-X, washed and then incubated with blocking solution containing 2% BSA and 0.2% Tween-20. Thereafter, LC3B was visualized by incubating the cells with a rabbit-anti-LC3B antibody (Thermo Scientific) and subsequently with a goat anti-rabbit Alexa Fluor 647 secondary antibody. Finally, the slides were mounted with Fluoroshield containing DAPI (Sigma). Images were taken on a confocal microscope Nikon Eclipse Ti with a 60x oil objective. The number of cells containing LC3B, were quantified manually and plotted relative to the total number of cells.

Automated segmentation of lipid droplet-lysosome colocalization

$CD4^+$ Tn cells were incubated with 50 µM chloroquine-diphosphate for 2 hours prior to the addition of 5% serum from NCD- or WTD-fed *Ldlr*$^{-/-}$ mice. The cells were incubated with serum and or chloroquine for 24 hours. After 24 hours, lipid droplets were stained using Bodipy as described above. Subsequently, the cells were stained with 60 nM Lysotracker Red DND-99 in complete RPMI-1640 medium for 30 min. at 37 °C. Hereafter, the cells were washed with PBS and stained with live-Hoechst (Sigma) for 45 min. at room temperature. Subsequently, the cells were washed and seeded on a plate coated with 5mg/mL poly-D-lysine (Sigma). Bodipy, lysotracker and Hoechst staining as well as transmitted light were monitored using a Nikon Eclipse Ti2 C2+ confocal laser microscope (lasers 540, 488 and 408 nm), equipped with a 20X (NA 0.75) , an automated stage and perfect focus system at 37 °C with humidified atmosphere and 5% CO2/air mixture from Okolab. Image preprocessing was performed in ImageJ [28] (rolling ball background subtraction and Gaussian smoothing). Foci detection was performed using the FociPicker3D ImageJ plugin [29]. An in house developed macro was used to automate the image preprocessing and foci detection. CellProfilerv2.22 [30] was used for nuclei segmentation, counting the number of Bodipy and lysosome foci and establishing parent-child relationships (which foci belong to which cell).

Image analysis results were exported as csv files and read into R (version 3.4.1) for further downstream analysis. Eventually, the fraction of overlapping foci were calculated as compared to total bodipy or lysosomes and normalization of these fractions per number of cells.

Statistical analysis

Bar graphs are expressed as mean ± SD. A two-tailed student's T-test was used to compare individual groups with Gaussian distributed data. Non-parametric data was analyzed using a Mann-Whitney U-test. Data from three or more groups were analyzed using a one-way ANOVA with a subsequent Tukey's multiple comparison test. A p-value

below 0.05 was considered significant. In the figures a * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001 and **** indicates p<0.0001.

RESULTS

Total and naïve CD4$^+$ T cells accumulate lipids during dyslipidemia

Presumably, Western-type diet (WTD)-induced dyslipidemia in mice increases the amount of lipoproteins to which CD4$^+$ Tn cells are exposed to, as compared to normal chow diet (NCD) fed mice (with normolipidemia). This increase would result in enhanced endolysosomal uptake of lipoproteins and increased lipid droplets (LD) content, despite LXR activation and increased ABC transporter expression.

We examined whether CD4$^+$ Tn cells actually contain more LD when exposed to excess lipoproteins *in vitro* by mimicking dyslipidemia with atherogenic lipoproteins. Alternatively, dyslipidemia was mimicked using serum from WTD-fed mice and compared to NCD-serum control, to mimic lipoprotein excess in the context of inflammatory factors associated with dyslipidemia-induced atherosclerosis.

First, we incubated isolated CD4$^+$ Tn cells with the atherogenic lipoproteins oxLDL and VLDL, which mimic hypercholesterolemia and hypertriglyceridemia, respectively, and assessed LD accumulation through flow cytometry. OxLDL and VLDL increased the median fluorescent intensity (MFI) of Bodipy, a neutral lipid dye which can stain LD, in CD4$^+$ Tn cells as compared to control incubated T cells (fig. 1A). Similar to incubation with isolated oxLDL and VLDL, incubating CD4$^+$ Tn cells with serum from WTD-fed *Ldlr*$^{-/-}$ resulted in a higher LD stain as compared to serum from NCD-fed *Ldlr*$^{-/-}$ mice (fig. 1B).

To confirm that dyslipidemia is indeed associated with increased LD content and LXR activation *in vivo*, we examined whether CD4$^+$ T cells have enhanced LD formation during dyslipidemia. To this end, we fed *Ldlr*$^{-/-}$ mice a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter for 4 weeks to induce dyslipidemia and quantified the degree of LD formation in isolated CD4$^+$ T cells. CD4$^+$ T cells isolated from WTD-fed mice more frequently contained one or more LD than CD4$^+$ T cells from normal chow diet (NCD) fed control mice (fig. 1C). In line, the mRNA expression of *Plin2*, which is involved in LD formation, was increased in CD4$^+$ T cells from WTD-fed mice (fig. 1D). *CD36* mRNA expression was not modulated by WTD (fig. 1E) suggesting that prolonged exposure of CD4$^+$ T cells to excess lipoproteins did not result in downregulation of scavenger receptors which mediate the uptake of lipoproteins, comparable to lipid loaded macrophages [31]. Actually, we have shown that CD36 expression on a protein level is increased in conventional CD4$^+$ T cells isolated from *Ldlr*$^{-/-}$ mice with diet-induced dyslipidemia, as compared to normolipidemic mice (J. Amersfoort, unpublished).

In $CD4^+$ T cells from WTD-fed mice we determined, however, an increased mRNA expression of *Abca1* which encodes the ABCA1 cholesterol efflux transporter but not of *Abcg1* (fig 1F), indicating that LXR is activated in $CD4^+$ T cells *in vivo* by dyslipidemia. Next, we assessed whether there are dynamic changes in LXR activation in $CD4^+$ T cells during prolonged WTD-induced dyslipidemia. Again, the mRNA expression of *Abca1* was elevated after 4 weeks of WTD as compared to the NCD controls (fig. 1G). Moreover, it remained increased after 8 and 16 weeks of WTD to a similar extent suggesting that LXR remained elevated during prolonged WTD feeding. The expression of *Abcg1* was significantly increased after 4 weeks of WTD as compared to the NCD control, but did not remain significantly higher at 8 and 16 weeks of WTD (fig. 1H). To establish that the increase in *Abca1* and *Abcg1* was LXR-dependent, we isolated $CD4^+$ T cells and mimicked dyslipidemia *in vitro* using human VLDL and simultaneously treated the cells with the LXR antagonist GSK2033. *Abca1* and *Abcg1* expression was almost abolished in GSK2033 treated $CD4^+$ T cells (fig. 1I), suggesting that *Abca1* and *Abcg1* expression in CD4+ T cells in the presence of excess atherogenic lipoproteins is LXR-dependent.

Altogether, these results indicated that WTD-induced dyslipidemia induced lipid accumulation in total $CD4^+$ T cells and an LXR-dependent increase in the expression of cholesterol efflux transporters and that dyslipidemia-like conditions induced lipid accumulation in $CD4^+$ Tn cells *in vitro*.

Priming of $CD4^+$ Tn cells with isolated lipoproteins increases proliferation of antibody- but not antigen-stimulated T cells

As we were interested in the priming of $CD4^+$ Tn cell by lipoproteins, we isolated $CD4^+$ Tn cells, induced lipid loading *in vitro* using oxLDL and VLDL and subsequently studied cell growth and proliferation after TCR stimulation with antibodies or antigen (fig. 2A). We used suboptimal antibody-induced TCR stimulation (1 µg/mL plate-bound αCD3) as we reasoned that any dyslipidemia-mediated effects on proliferation would be less apparent during strong TCR stimulation. Alternatively, we assessed proliferation in response to strong, antigen-induced TCR stimulation, which has the advantage that bone marrow-derived dendritic cells (BMDC) provide a more physiological co-stimulatory signal and cytokine stimulation of primed $CD4^+$ Tn cells.

We also studied the effect of lipoprotein-induced priming by activating the cells in the absence or presence of oxLDL and VLDL. We hypothesized that the cell blast (or cell growth) phase which precedes cell division within 24 hours after antibody-induced activation [32] is increased after lipoprotein-induced priming of $CD4^+$ Tn cells. However, T cell blast after 18 hours of activation was not affected by lipoprotein-induced priming since the cell size, which was measured by the forward scatter (FSC), was not enhanced in the primed $CD4^+$ T cells as compared to the vehicle-treated $CD4^+$ T cells. (fig. 2B). Interestingly, T cell blast was diminished when $CD4^+$ Tn cells were activated in the pres-

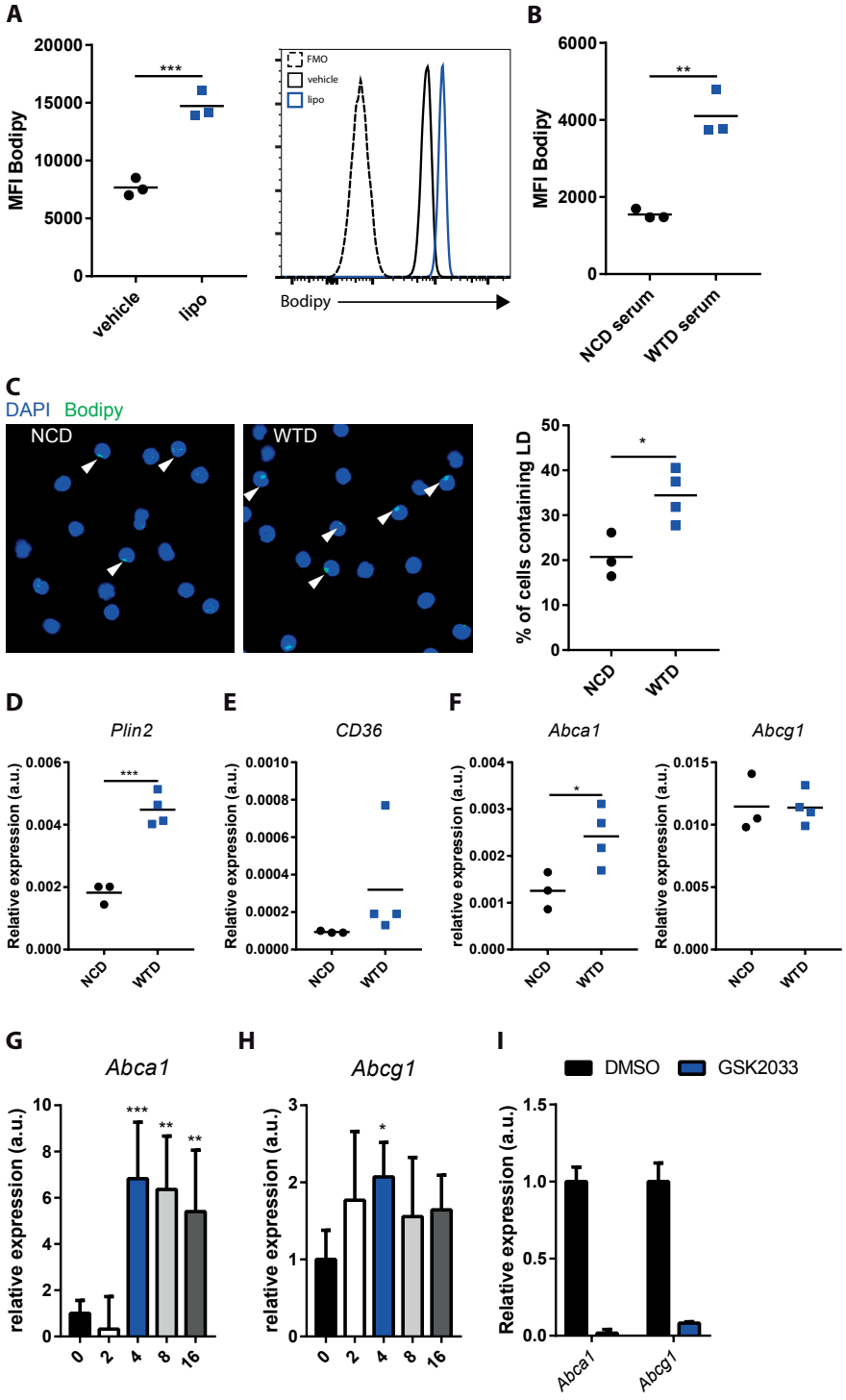
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Figure 1 CD4$^+$ T cells from *Ldlr*$^{-/-}$ mice accumulate lipids *in vivo* and *in vitro* which elicits an LXR response. (A) Quantification of Bodipy median fluorescent intensity (MFI) in CD4$^+$ T cells loaded with lipoproteins (lipo) *in vitro*. (B) Quantification of LD MFI in Tn cells incubated *in vitro* with serum from NCD- or WTD-fed mice. (C) The percentage of T cells containing LD as assessed by confocal microscopy. (D) *Plin2* expression in isolated CD4+ T cells. (E) *CD36* expression in isolated CD4$^+$ T cells. (F) *Abca1* and *Abcg1* expression in isolated CD4$^+$ T cells. (G) Expression of *Abca1* in T cells from mice fed a normal chow diet (NCD, t=0) or a WTD for 2-16 weeks. (H) Expression of *Abcg1* in same experiment. (I) Expression of *Abca1* and *Abcg1* after LXR inhibition with GSK2033 *in vitro* in the presence of atherogenic lipoproteins. Asterisks in 1G and 1H indicate a significant difference as compared to 0 weeks WTD. White arrows in 1C indicate a CD4$^+$ T cell with LD.

ence of lipoproteins, independent of the priming condition. We also measured CD4$^+$ T cell activation as reflected by the percentage of IL-2 producing CD4$^+$ T cells after 18 hours of antibody-induced activation. In line with equal cell growth between vehicle- and lipoprotein-primed CD4$^+$ T cells, the percentage of IL-2$^+$ cells within the CD4$^+$ T cell population was equal between vehicle- and lipoprotein-primed T cells (fig. 2C). Activation of CD4$^+$ Tn cells in the presence of lipoproteins also reduced the percentage of IL-2 producing CD4$^+$ T cells. Besides the effect of priming on T cell activation, we wanted to examine the effect of lipoprotein-induced priming on the proliferation of CD4$^+$ T cells. To this end, we activated CD4$^+$ Tn cells for 72 hours with αCD3 and αCD28 antibodies as initial cell division occurs after approximately 48 hours after TCR stimulation and priming effects might be diluted after longer stimulation. Interestingly, lipoprotein-induced priming of CD4$^+$ Tn cells increased the percentage of proliferated cells after 72 hour of antibody-induced TCR stimulation but this effect of lipoprotein-induced priming was abolished when the cells were activated in the presence of excess lipoproteins (fig. 2D). Furthermore, activation of vehicle-primed CD4$^+$ Tn cells in the presence of excess lipoproteins increased the percentage of proliferated cells as compared to CD4$^+$ T cells activated without excess lipoproteins. The effects of lipoprotein-induced priming and activation of CD4$^+$ Tn cells in the presence of excess lipoproteins were reflected by the percentage of IL-2 producing CD4$^+$ T cells after 72 hour stimulation (fig. 2E).

Next, we wanted to assess the effect of lipoprotein-induced priming on T cell proliferation by activating CD4$^+$ Tn cells in an antigen-specific manner. To this end, we induced proliferation of primed OT-II T cells in a co-culture of these cells with BMDC which were previously loaded with various concentrations of OVA-323 peptide. We did not include a condition in which we activated vehicle- or lipoprotein-primed CD4$^+$ OT-II T cells in the presence of excess lipoproteins as lipoprotein uptake by BMDCs induces phenotypic changes, leading to enhanced activation of T cells [33]. Moreover, lipoprotein-induced priming was counteracted by the presence of excess lipoproteins during antibody-induced activation of CD4$^+$ T cells, suggesting that activation in the context of dyslipidemia might mask any priming effect which we were interested in. Lipoprotein-induced

priming of CD4$^+$ Tn cells did not enhance the antigen-induced proliferation of OT-II cells as compared to vehicle-induced priming (fig. 2F).
Altogether, these data showed that priming of CD4$^+$ Tn cells with lipoproteins enhanced proliferation after suboptimal, antibody-induced T cell activation but not after antigen-induced T cell activation.

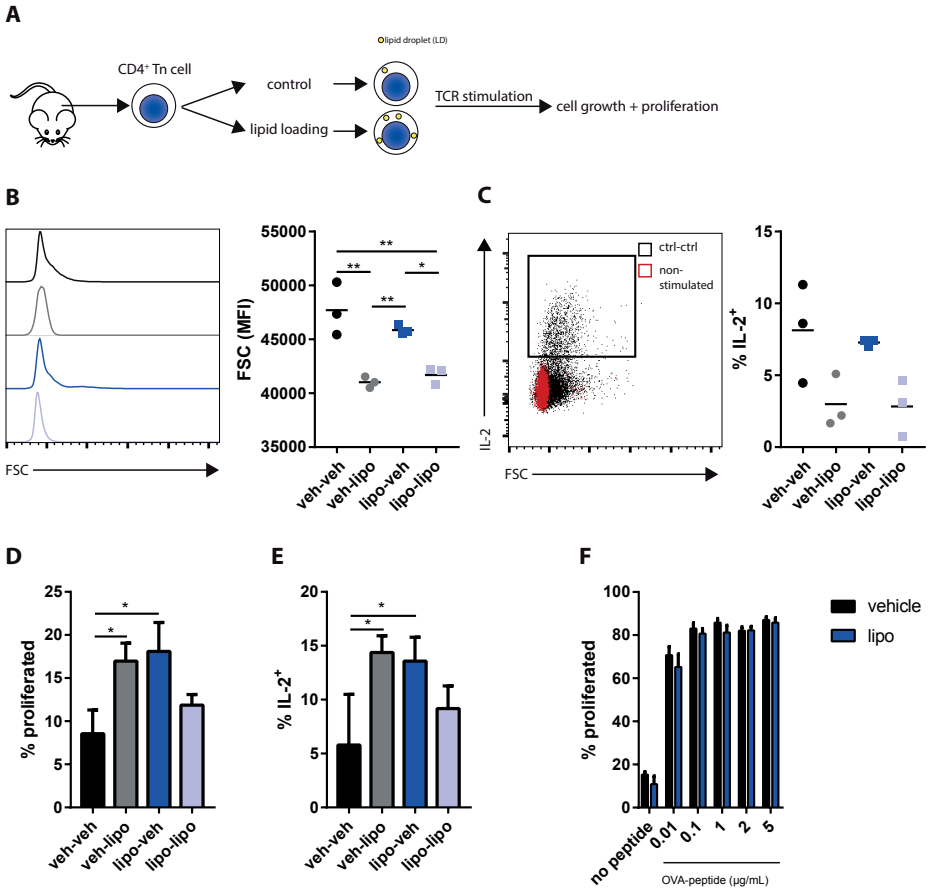


Figure 2 Lipoprotein-induced priming of CD4$^+$ *Ldlr*$^{-/-}$ Tn enhanced proliferation in response to antibody-mediated TCR stimulation. (A) Experimental setup for *in vitro* evaluation of effect of CD4$^+$ Tn cell priming on proliferation. (B) Cell growth as assessed by flow cytometry (median fluorescent intensity, MFI) after 18 hours of antibody-induced T cell activation. (C) Percentage of IL-2 producing CD4$^+$ T cells after 18 hours of antibody-induced T cell activation. (D) Proliferation after 72h stimulation with antibody-induced T cell activation. (E) Percentage of IL-2 producing CD4$^+$ T cells after 72 hours of antibody-induced T cell activation. (F) Antigen-induced proliferation of OT-II T cells in a co-culture with BMDC as measured by CellTrace dilution. Groups in B-E are indicated as priming condition-activating condition, thus, veh-lipo indicates vehicle-primed T cells which are activated in the presence of excess lipoproteins.

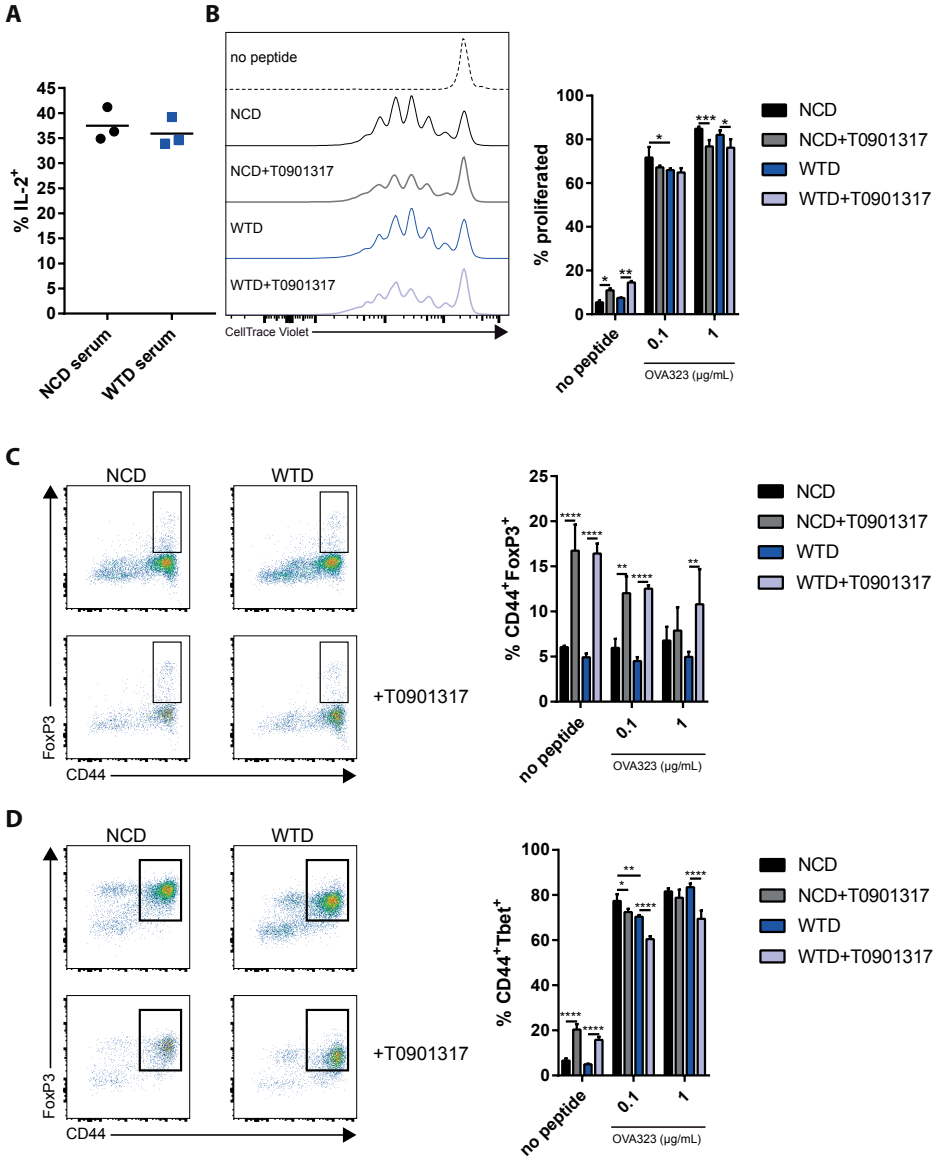


Figure 3 Pharmacological LXR activation during the priming of CD4$^+$ Tn cells with WTD-serum decreased proliferation and promoted Treg cell differentiation. (A) Percentage of IL-2 producing CD4$^+$ T cells after 18 hours incubation of OT-II CD4$^+$ Tn cells with BMDCs loaded with various concentrations of OVA323. (B) Antigen-induced proliferation as assessed by CellTrace dilution after 96 hours of incubation with indicated preceding priming conditions. (C) Treg cell percentage after 96 hours of incubation. (D) Th1 cell percentage after 96 hours of incubation.

LXR activation during WTD serum-induced priming of CD4$^+$ Tn cells has anti-inflammatory effects

Since LXR activation and Abcg1 are crucial factors during T cell proliferation and differentiation and lipid accumulation in T cells induced LXR activation, we postulated that activation of LXR in CD4$^+$ Tn cells under dyslipidemic conditions would affect their effector phenotype.

Therefore, we primed CD4$^+$ Tn cells with NCD- or WTD-serum with or without the addition of the LXR agonist T0901317 and assessed the proliferation and Treg/Th1 differentiation after antigen-stimulation. After 18 and 96 hours of antigen-induced CD4$^+$ T cell activation, no difference in the percentage of IL-2 producing cells was observed between NCD- and WTD-serum primed CD4$^+$ T cells (fig. 3A,B). Interestingly, activation of LXR with T0901317 during priming slightly decreased the percentage of proliferated CD4$^+$ T cells, which is in line with previous reports describing LXR-agonists to suppress T cell proliferation [6]. In the same experiment, we also assessed the effect of serum-priming on the differentiation skewing of CD4$^+$ T cells. Again, no difference in the percentage of Treg cells was observed after priming with WTD-serum as compared to NCD-serum (fig. 3C). Interestingly, 48 hours of priming with T0901317 did increase the percentage of Treg cells (as defined by CD44$^+$FoxP3$^+$ cells in the CD4$^+$ population) in the presence of both NCD- and WTD-serum. T0901317 also increased the percentage of Treg cells when OT-II CD4$^+$ T cells were incubated with unloaded BMDCs, suggesting that part of the Treg cell simulating effect of T0901317 was OVA323 independent. Furthermore, T0901317 treatment specifically during the priming of CD4$^+$ Tn cells with WTD-serum reduced the percentage of Th1 cells (as defined by CD44$^+$Tbet$^+$ cells in the CD4$^+$ population) as compared to CD4$^+$ which were primed with only WTD-serum (fig. 3D). Altogether, these results indicated that priming of CD4$^+$ Tn cells with WTD-serum did not affect T cell proliferation or differentiation but T0901317 treatment under dyslipidemia-like conditions inhibited proliferation and skewed CD4$^+$ T cell differentiation to Treg cells.

WTD-induced dyslipidemia does not induce autophagy in CD4$^+$ T cells

As acetylated-LDL induces lipophagy in foam cells [25] we aimed to examine whether dyslipidemia induced lipophagy in CD4$^+$ T cells as well. This is relevant as this would affect the outcome of stimulation or inhibition of autophagy on lipid accumulation during the priming of CD4$^+$ Tn cells under dyslipidemia-like conditions. To this end, we fed *Ldlr*$^{-/-}$ mice a WTD for 4 weeks, isolated CD4$^+$ T cells and compared the level of autophagy to CD4$^+$ T cells from NCD-fed mice. An immunoblot for LC3 showed that the amount of lipidated LC3 (LC3-II, indicating active autophagy) was equal between CD4$^+$ T cells from NCD- and WTD-fed mice (fig. 4A). Another method to quantify active autophagy is by measuring the amount of LC3 puncta through confocal microscopy as LC3 puncta reflect matured autophagosomes [34]. The percentage of cells which contained LC3 puncta

did not differ between $CD4^+$ T cells isolated from NCD- and WTD-fed mice (fig. 4B). This suggested that diet-induced dyslipidemia prompted lipid accumulation in $CD4^+$ T cells but not severe enough to induce autophagy. In addition, as autophagy is not induced by dyslipidemia, lysosomal inhibition during the priming of $CD4^+$ Tn cells mostly inhibits the endolysosomal uptake of lipoproteins and not lipophagy-mediated LD degradation.

Chloroquine inhibits serum-induced lipid accumulation

Chloroquine inhibits LAL, thereby impairing the degradation of cholesteryl esters and triglycerides and formation of LD as well as autophagy-mediated degradation of LD (i.e. lipophagy). To assess whether chloroquine would inhibit the endolysosomal uptake of lipoproteins during dyslipidemia-like priming conditions, we isolated $CD4^+$ Tn cells and incubated them with chloroquine 2 hours prior to and during priming with NCD- and WTD-serum to induce lipid accumulation as compared to a medium control. To quantify whether chloroquine induces lipid accumulation in lysosomes, we performed a proof-of-principle experiment where we stained neutral lipids (esterified lipids and choles-

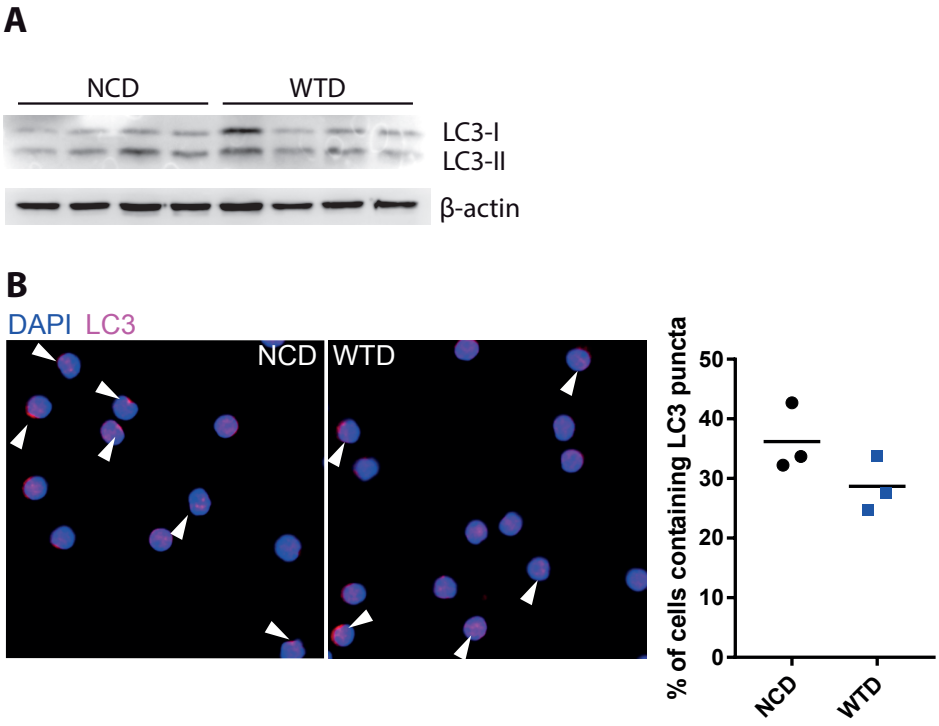


Figure 4 Diet-induced dyslipidemia did not induce autophagy in $CD4^+$ T cells. (A) Immunoblot showing LC3-I and LC3-II (the lipidated LC3 indicating active autophagy) in CD4+ T cells derived from NCD or WTD fed mice (n=4/group). (B) Assessment of lipophagy by quantification of the percentage of $CD4^+$ T cells with LC3 puncta as a measure for active autophagy. White arrows indicate cells with clear LC3 puncta.

terol) and lysosomes using Bodipy and Lysotracker, respectively, and used automated segmentation of neutral lipid- and lysosome foci in <150 $CD4^+$ Tn cells per condition. $CD4^+$ Tn cells incubated with NCD- and WTD-serum for 24 hours showed an increase in the number of neutral lipid foci per cell which was inhibited by incubating the cells with chloroquine 2 hours prior to and during the 24 hours priming (fig. 5A). As chloroquine also inhibits the fusion between endosomes and lysosomes [35], chloroquine treatment likely decreased the level of lipoprotein handling through several mechanisms in $CD4^+$ Tn cells and thus decreased the formation of LD. The fraction of neutral lipid foci which colocalize with lysosomes, after normalization to the number of lysosomes and cells, was increased in the chloroquine treated condition (fig. 5B). This was especially apparent in the WTD-serum primed $CD4^+$ Tn cells (fig. 5C). Since dyslipidemia did not result in the induction of autophagy, increased colocalization between neutral lipid foci and lysotracker foci is likely due to lipid accumulation in lysosomes. Naturally, it cannot be ruled out that chloroquine treatment impaired the autophagic degradation of LD which would also result in enhanced colocalization between neutral lipid- and lysosome foci. Automated segmentation of neutral lipid foci (fig. 5D) and lysosomes (fig. 5E) resulted in a slight overestimation of the number of foci, as indicated by segments of lysosomes in the absence of a clear lysosome signal. Nevertheless, a proof-of-principle experiment showed that automated segmentation is suitable to identify neutral lipid and lysosome foci in confocal images of $CD4^+$ Tn cells which suggested that chloroquine impaired the endolysosomal uptake and degradation of lipids and formation of lipid droplets under dyslipidemia-like priming conditions.

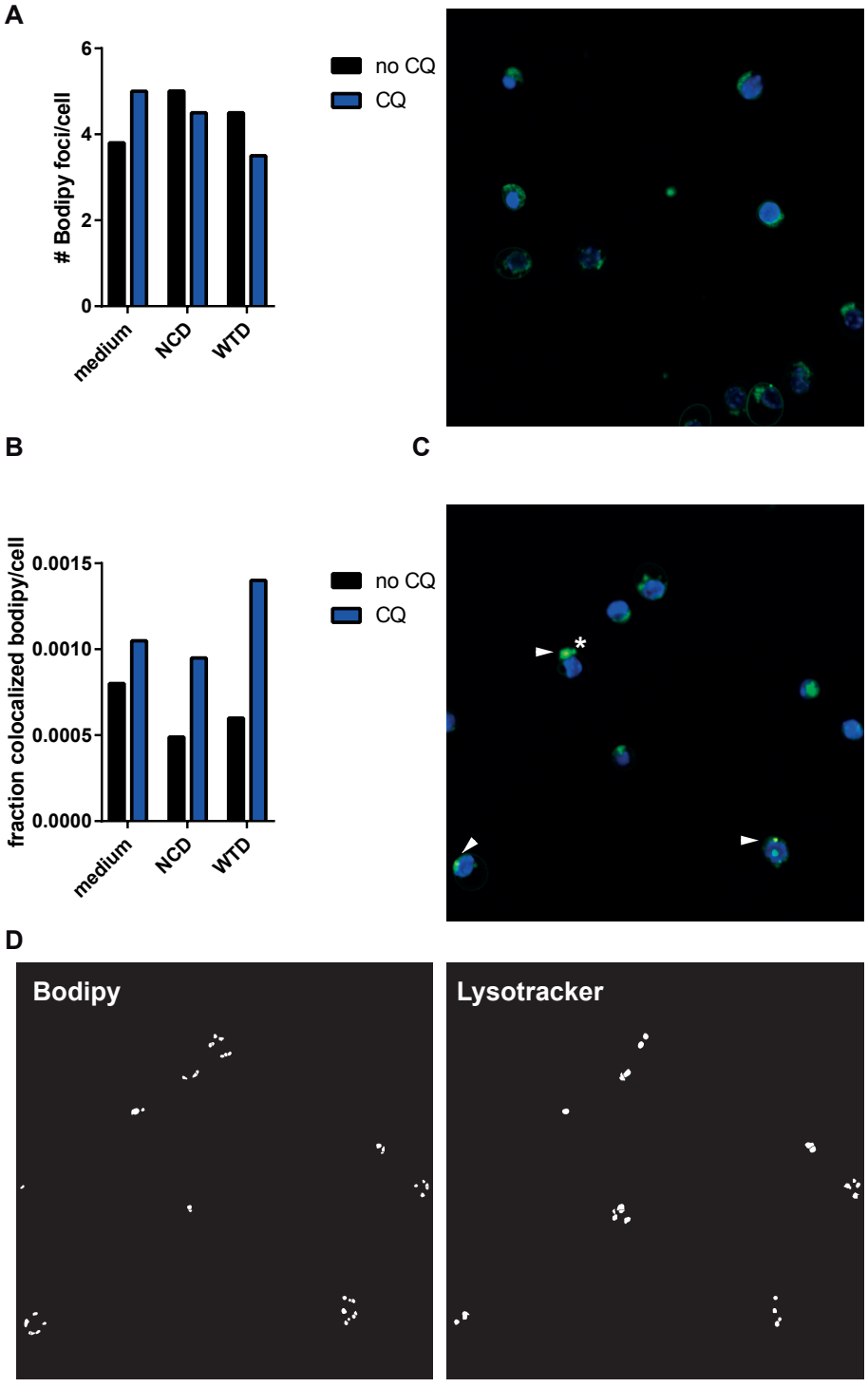
Lysosomal inhibition while priming naïve T cells with serum inhibits T cell activation

Priming of $CD4^+$ Tn cells under dyslipidemia-like conditions had minor effects on the effector phenotype of $CD4^+$ T cells as shown in figure 2 and 3. Interestingly, promoting cholesterol efflux with an LXR-agonist during priming did have minor anti-inflammatory effects as it slightly inhibited proliferation of $CD4^+$ T cells and promoted Treg cell differentiation. To further explore whether inhibiting the availability of free cholesterol through lysosomal inhibition would have similar anti-inflammatory effects we treated $CD4^+$ Tn cells during priming with chloroquine. In $CD4^+$ Tn cells primed with WTD serum, chloroquine treatment significantly inhibited T cell activation as measured by the percentage of IL-2 producing cells after overnight activation with antibody-induced TCR stimulation (fig. 6A). Perhaps this is because fewer lipids can be taken up through the endolysosomal pathway as well as decreased LD degradation via lipophagy. In line, proliferation showed a strong trend ($p=0.0506$) towards being decreased in chloroquine treated WTD-serum primed T cells activated for 72 hours (fib. 6B). To assess the effect of autophagy inhibition on the differentiation status of T cells, we measured the percentage of Treg and Th1 cells

after 72 hours activation. No effects were observed on the percentage of Treg cells, while Th1 cells were decreased in Tn cells primed with WTD-serum and chloroquine as compared to WTD-serum primed Tn cells (fig. 6C). These *in vitro* results suggested that the decrease in cholesterol availability after lysosomal inhibition, which limits the uptake of lipoproteins from the environment as well as the degradation of LD through autophagy, in the initial phase of T cell activation might have anti-inflammatory effects. Proliferation and Th1 differentiation was severely impaired after chloroquine treatment during WTD-serum priming, which is in line with literature describing pharmacological inhibition of autophagy to inhibit proliferation of Th1 cells [36]. To explore the therapeutic potential of chloroquine to inhibit T cell-mediated inflammation we fed mice a NCD or a WTD for 4 weeks and treated them with chloroquine for three consecutive days and assessed the percentage of Treg- and Th1 cells in the spleen. Surprisingly, chloroquine actually increased the percentage of Treg cells in WTD-fed mice but not in NCD-fed mice (fig. 6D) while we did not observe any effect of chloroquine treatment on the percentage of Th1 cells (fig. 6E). Moreover, we did not observe an anti-proliferative effect of chloroquine on T cells as the percentage of proliferating cells was elevated in chloroquine-treated mice fed a WTD (fig. 6F).
In conclusion, these data suggest that lysosomal inhibition with chloroquine might have anti-inflammatory effects on T cells specifically during dyslipidemia and dyslipidemia-like conditions.

DISCUSSION

Dyslipidemia has been shown to have lipid-mediated antigen-independent effects on T cell effector function in atherosclerosis. Moreover, autophagy-induced degradation of LD is an essential metabolic process in foam cells which facilitates cholesterol efflux. However, it is unknown whether atherogenic lipoproteins prime $CD4^+$ Tn cells to have an altered effector phenotype, and whether lipid-associated metabolic processes contribute to the priming effect. Through *in vitro* and *in vivo* studies we showed that dyslipidemia and dyslipidemia-like conditions affect lipid metabolism in $CD4^+$ T cells but that pharmacological modulation of lipid metabolism in $CD4^+$ Tn cells had the largest immunomodulatory effect specifically under dyslipidemic conditions.
First we showed that $CD4^+$ T cells accumulate lipids in *Ldlr*$^{-/-}$ mice fed a WTD as compared to NCD-fed controls. This was reflected by an increase in the mRNA expression of ABCA1 and ABCG1 in T cells in an LXR-dependent manner and increased *Plin2* expression. Further characterization of the content of LD and how dietary lipids affect their content should shed more light on the exact functional implications of LD accumulation and expansion in T cells. In macrophages and dendritic cells, different types of LD exist within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no CQ
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Bodipy
Lysotracker

Figure 5 Chloroquine inhibits endolysosomal processing of lipoproteins and impairs serum-induced lipid accumulation. (A) Quantification of Bodipy-stained lipid droplets as detected by automated segmentation in indicated treatments. An example of Bodipy stained lipid droplets is shown. (B) Colocalization between lipid droplets and lysosomes (stained with Lysotracker-RED DND-99) by automated segmentation in indicated treatments. (C) Example of immunofluorescent image of lipid droplets and lysosomes in WTD-serum and chloroquine primed $CD4^+$ Tn cells. White arrows indicate colocalization between lipid droplet and lysosome in yellow. An asterisk indicates a lipid droplet (D) Corresponding images showing neutral lipid foci and lysosome segments in white. CQ indicates chloroquine.

individual cells [37]. Moreover, it has been shown that different types of LD can affect various intracellular processes involved in inflammation, including IFN-signaling, cross presentation and bioenergetic metabolism [38]. LD are also involved in the metabolism of arachidonic acid and its metabolites, eicosanoids [38,39]. Some eicosanoids are potent immunological mediators and are potent ligands for peroxisome proliferator activated receptor delta, which mediates altered cellular metabolism in Treg cells during dyslipidemia (J. Amersfoort et al., unpublished). Therefore, although we did not observe any major effects of priming of $CD4^+$ Tn cells using human serum-derived lipoproteins or WTD-serum, a different method of priming could induce distinct types of LD which could modulate the inflammatory phenotype of effector $CD4^+$ T cells. A recent report described that mitochondria-associated LD have unique bioenergetics properties, dynamics and composition [40]. These reports suggest that LD have the potential to modulate T cell metabolism and therefore effector function and call for the characterization of the different types of LD present in T cells from patients with dyslipidemia and cardiovascular disease. Lipid accumulation in $CD4^+$ T cells was not associated with increased autophagy, as opposed to foam cells [25]. This was surprising as cholesterol accumulation in Treg cells is associated with decreased mTORC1 activity during diet-induced dyslipidemia [11] and mTORC1 inhibits autophagy by phosphorylating Ulk1 [41]. Given that scavenger receptors mediate the uptake of (modified) lipoproteins [42,43], perhaps scavenger receptor expression or cytoplasm volume in $CD4^+$ Tn cells is too low to induce lipid accumulation to an extent which requires autophagy induction to facilitate reverse cholesterol transport and prevent lipotoxicity. As Treg cells appear to have higher lipid scavenging capacity than conventional T cells (J. Amersfoort et al., unpublished), dyslipidemia might induce autophagy in Treg cells. Importantly, this does not mean that lipophagy is not involved in the inflammatory effects of lipid-mediated priming of Tn cells. Lipophagy-mediated degradation of LD could enhance the availability of cholesterol and other lipids in the initial stage of T cell activation and affect their effector phenotype under inflammatory conditions which we did not examine. Of note, chloroquine inhibits atherosclerosis in apolipoprotein-E deficient mice in a p53-dependent manner [44]. p53 is a tumor suppressor protein which induces apoptosis and has been described to inhibit cholesterol synthesis [45], suggesting that chloroquine could impair T cell-mediated inflammation by

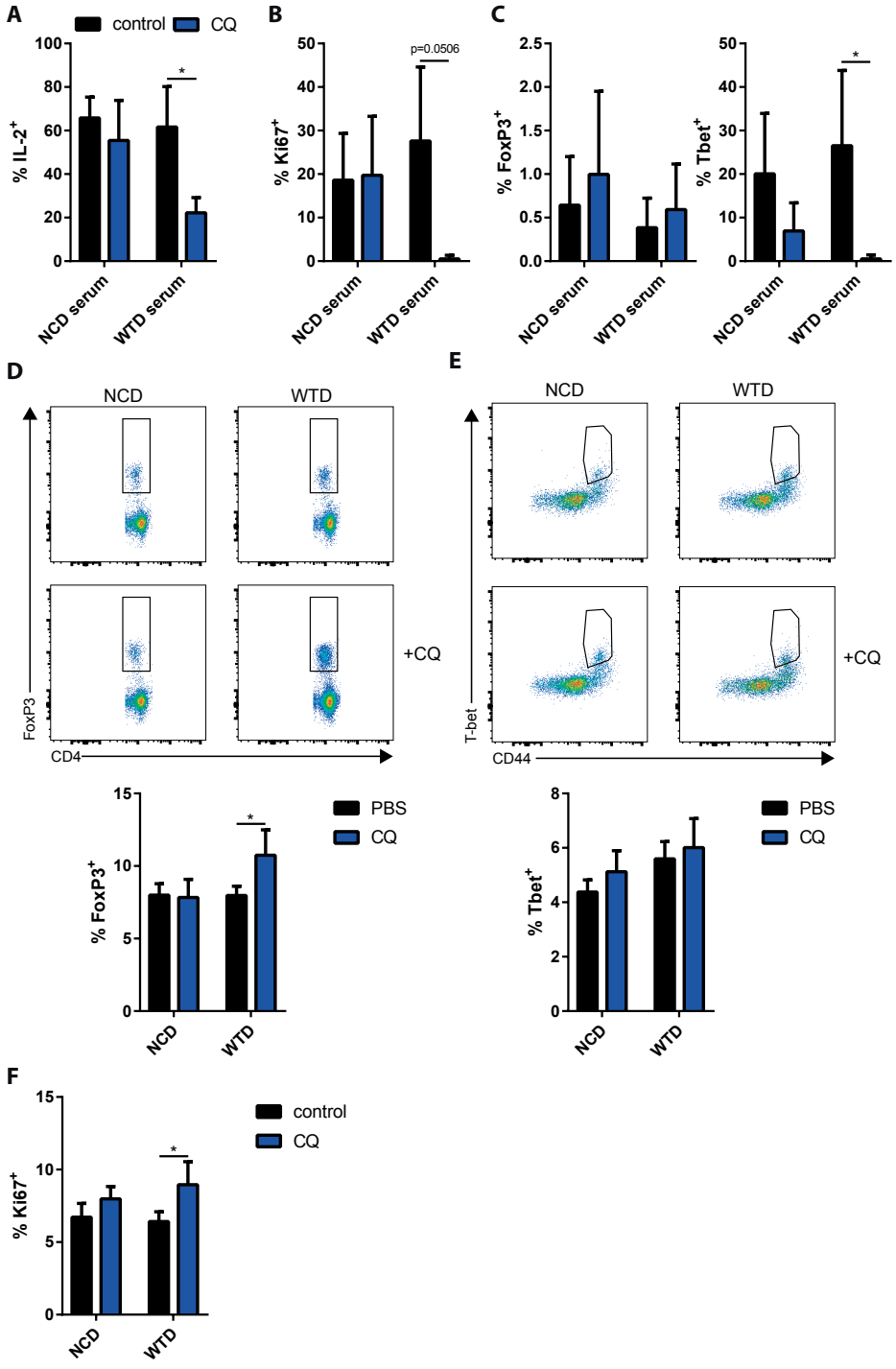
A
control
CQ
% IL-2+
NCD serum
WTD serum
B
% Ki67+
p=0.0506
C
% FoxP3+
% Tbet+
D
NCD
WTD
+CQ
FoxP3
CD4
% FoxP3+
PBS
CQ
E
T-bet
CD44
% Tbet+
F
% Ki67+

Figure 6 Lysosomal inhibition during WTD serum priming inhibits activation and proliferation. (A) IL-2 production after ON activation of $CD4^+$ T cells primed as indicated. (B) $CD4^+$ T cell proliferation of primed $CD4^+$ T cells after 72h of activation. (C) Percentage of Treg- and Th1 cells in same experiment as in (B). (D) Percentage of Treg cells in splenic $CD4^+$ population in *Ldlr*$^{-/-}$ mice on a NCD or WTD treated with control (PBS) or chloroquine. (E) Percentage of Th1 cells in same experiment as in D. (F) Percentage of proliferating $CD4^+$ T cells in same experiment as in D. CQ indicates chloroquine.

decreasing cholesterol-synthesis. These data underline the therapeutic possibility of using chloroquine or chloroquine-diphosphate to inhibit T cell-mediated inflammation in atherosclerosis.

Priming of $CD4^+$ Tn cells with atherogenic lipoproteins induced lipid accumulation in $CD4^+$ Tn cells *in vitro* but did not increase cell growth after overnight antibody-induced activation. However, both priming of naïve T cells with lipoproteins as activating unprimed (vehicle control) $CD4^+$ Tn cells in the presence of lipoproteins enhanced their proliferation in response to prolonged stimulation. Activation of $CD4^+$ T cells in the presence of oxLDL and VLDL decreased the percentage of IL-2 producing $CD4^+$ T cells. This reduction of T cell blast and IL-2 production is in line with a report describing human T cells proliferate less when activation occurs in the presence of 10 ug/mL oxLDL [46]. Interestingly, priming with lipoproteins did not enhance the antigen-specific proliferation after 96 hours incubation of OT-II T cells with OVA323 loaded BMDCs, suggesting the priming of $CD4^+$ Tn cells with lipoproteins has stimulation-dependent effects on proliferation. Another explanation is that antigen-specific proliferation was not enhanced in primed OT-II T cells but was enhanced in *Ldlr*$^{-/-}$ T cells which were activated using antibody-stimulation, is the presence of a functional LDL receptor. In $CD4^+$ T cells isolated from transgenic OT-II mice, LDL receptor-mediated uptake of native lipoproteins could have overruled the priming effect.

Priming $CD4^+$ Tn cells with normolipidemic or dyslipidemic serum did not affect antigen-specific proliferation either. This could be because the affinity of the OVA323-peptide for the TCR of OT-II T cells is higher than the affinity of physiological antigens, such as ApoB100 derived-peptides. Hence, priming of $CD4^+$ Tn cells might be overruled by strong TCR signals which enhance the transcriptional program facilitating cell growth and proliferation. Priming might only affect proliferation during suboptimal stimulation, as was the case in the antibody-stimulation which we used.

Enhanced cholesterol content in T cells is generally associated with elevated proliferation by enhancing the availability of cholesterol for membrane synthesis and fine-tuning the TCR [47]. However, the modulatory effect of cholesterol on TCRs is not as straightforward as suggested by reports describing cholesterol to enhance the nanoclustering of TCRs [48] and free cholesterol to enhance TCR signaling in $CD8^+$ T cells [20]. The activity of Sult2b, which inactivates oxysterols as LXR ligands through sulfation, might negatively modulate cholesterol-mediated enhancement of TCR signaling. Moreover, cholesterol

binding to the TCRβ subunit has been shown to both drive TCR dimerization and avidity to MHC-peptide complex as inhibit spontaneous TCR signaling [49]. Promoting cholesterol efflux with an LXR agonist during dyslipidemia-like priming slightly decreased antigen-specific proliferation. Upon TCR stimulation, T cells acquire a transcriptional program which drives cholesterol synthesis, in part by increasing the expression of Sult2b1 [6]. Moreover, during the blasting phase, but not during the cell division phase, lipid synthesis is increased in a SREBP-dependent manner [7]. Presumably, LXR agonism during priming of $CD4^+$ Tn cells did not inhibit their antigen-induced proliferation in a major way because cholesterol synthesis is induced to such an extent that the effects of LXR agonism on proliferation are overruled.

Inducing cholesterol efflux during priming by treating $CD4^+$ Tn cells with T0901317 enhanced the generation of Treg cells and decreased Th1 differentiation after antigen-induced activation. The former is in line with a report describing LXR activation with GW3965 to promote differentiation of $CD4^+$ T cells towards Treg cells [50]. Interestingly, another report by Takeuchi et al. suggested that pharmacological activation of retinoid-X-receptor, the protein with which LXR forms a heterodimer [51], promotes the differentiation of $CD4^+CD25^-$ to Treg cells but LXR agonism alone using T0901317 does not [52]. The Treg cells in those reports were mainly generated *in vitro* using antibodies and transforming growth factor β1, which is a common method to generate Treg cells from murine $CD4^+$ T cells. In our T cell-BMDC co-culture, Treg cell differentiation was likely induced by the BMDC-derived cytokines after T0901317 was washed away after the preceding priming step. Therefore, these results support the notion that modulation of cholesterol metabolism only during the priming of $CD4^+$ Tn cells with dyslipidemic serum can induce T cell skewing. Whereas LXR agonism enhanced Treg cell differentiation under normolipidemia- and dyslipidemia-like conditions, it inhibited Th1 cell differentiation only under dyslipidemia-like conditions. LXR agonists have been described to inhibit IFNγ secretion in murine [50] and human T cells [53]. Again, in our experimental setup, LXR activation was mainly exerted in the priming phase as it was washed away prior to the activation of OT-II T cells. Altogether, the presented study suggests that promoting the efflux of cholesterol in $CD4^+$ Tn cells under dyslipidemia-like conditions affects T cell skewing in the effector phase.

Finally, we assessed whether modulation of lipid metabolism through chloroquine-induced lysosomal inhibition under dyslipidemia-like priming conditions affects T cell proliferation and skewing in a manner similar to LXR activation. Chloroquine treatment decreased T cell activation specifically under dyslipidemia-like conditions as shown by a decrease in the percentage of IL-2 producing T cells after overnight antibody-induced activation. Moreover, chloroquine treatment reduced the percentage of proliferating T cells after 72 hours of stimulation, specifically under dyslipidemia-like priming conditions. Treg cell differentiation was not altered as adequate Treg cell differentiation *in*

vitro requires addition of TGFβ [54] or weak TCR stimulation [55]. However, in line with LXR activation, which reduces intracellular cholesterol content and Th1 differentiation, chloroquine pre-treatment decreased the percentage of Th1 cells after 72 hours of stimulation. This suggests that lysosomal inhibition could prove useful to alter the effector T cell phenotype by altering lipid metabolism of Tn cells. In line, lysosomal inhibition using chloroquine inhibits the activity of LAL, which decreases ABCA1 expression in fibroblasts [56], suggesting that lysosomal inhibition decreases the intracellular oxysterol abundance. Accordingly, fibroblasts isolated from patients with cholesteryl ester storage disease, which have impaired LAL activity, have impaired upregulation of *Abca1* expression in response to LDL loading [56]. *In vivo*, chloroquine did enhance the percentage of Treg cells in WTD-diet fed mice but had no effect on Th1 cell differentiation and actually increased the percentage of proliferation T cells. These contrasting effects of chloroquine *in vivo*, as compared to *in vitro,* are most likely explained by the effects of chloroquine on APCs as it has been shown to induce tolerogenic DCs in a STAT1-dependent manner [57] and can inhibit experimental autoimmune encephalomyelitis (EAE) by modulating DCs [58]. Tolerogenic DCs are potent inducers of Treg cells [59]. In the report by Thome et al., systemic treatment with chloroquine enhanced the Treg cell population [58]. Systemic chloroquine treatment does not reduce Th1 differentiation which was surprising as chloroquine has been reported to decrease the secretion of IL-12p40 by DCs, which is a major Th1-inducing cytokine [60]. Moreover, Th1 differentiation is inhibited by chloroquine [61] and proliferation of *in vitro* generated Th1 cells is dampened by the inhibition of autophagy [36]. In our *in vivo* experimental setup, chloroquine might have affected various other immune cell types and non-immune cells which may compensate for the DC-dependent and T cell-specific effects of chloroquine on Th1 cells. Surprisingly, chloroquine treatment in mice with WTD-induced dyslipidemia enhanced the percentage of proliferating $CD4^+$ T cells which is counterintuitive based on reports describing pharmacological and genetic blockade of autophagy to inhibit proliferation [36,62].

Moreover, chloroquine inhibits the antigen processing in DCs and thereby antigen presentation on MHC-I [63] and MHC-II molecules [64]. In line, genetic blockade of autophagy through the knockout of autophagy related protein 7 in DCs inhibits EAE in a T cell expansion-dependent manner [65]. However, chloroquine treatment of DCs during cross presentation actually enhances $CD8^+$ T cell responses suggesting that chloroquine does not have exclusively anti-inflammatory effects on DC function [66]. Therefore, the T cell aspecific effects of *in vivo* chloroquine may explain the observed increase in Ki-67^+ T cells.

Altogether, modulation of lipid metabolism by pharmacological LXR activation or lysosomal inhibition during the priming of $CD4^+$ Tn cells under dyslipidemia-like conditions affects the effector phenotype after antibody- and antigen-induced activation. This suggests that antigen-independent modulation of lipid metabolism in $CD4^+$ Tn cells may

have immunomodulatory effects already prior to activation, which is especially relevant to lymphoid tissue residing T cells. As the effects of priming which we observed were limited, further research is required to fully examine the relevance of the presented findings. Moreover, further mechanistic research is required to further elucidate how specific lipids affect the effector phenotype when modulating lipid metabolism under dyslipidemia-like conditions. Additionally, the relevance of the presented findings to humans with dyslipidemia where physiological antigens stimulate primed $CD4^+$ Tn cells in the presence of excess lipoproteins, should be investigated.

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SUPPLEMENTARY TABLES

Table 1 Antibodies/dyes used for flow cytometry

antigen	label	clone	manufacturer
CD25	FITC	PC61.5	eBioscience
CD4	PE	GK1.5	eBioscience
CD4	eFluor 405	GK1.5	eBioscience
CD4	PerCP	RM4-5	BD Biosciences
CD44	eFluor 405	IM7	eBioscience
CD44	PE-Cy7	IM7	eBioscience
CD62L	PerCP-Cy5.5	MEL-14	eBioscience
T-bet	Alexe Fluor 647	4B10	eBioscience
FoxP3	eFluor 405	FJK-16s	eBioscience
FoxP3	APC	FJK-16s	eBioscience
Ki-67	FITC	SolA15	eBioscience
IL-2	APC	JES6-5H4	Biolegend
IL-2	APC	JES6-5H4	eBioscience
Thy1.2	PE-Cy7	53-2.1	eBioscience
eFluor 780 viability dye	APC-Cy7	-	eBioscience

Table 2 List of primers used for qRT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (3′-5′)
Abca1	agagcaaaaagcgactccacatagaa	cggccacatccacaactgtct
Abcg1	ttgacaccatcccagcctac	cagtgcaggtcttctcggt
Plin2	gcacagtgccaaccagaaaattcagg	cagtctggcatgtagtctggagctg
CD36	atggtagagatggccttacttggg	agatgtagccagtgtatatgtaggctc
Rpl27	cgccaagcgatccaagatcaagtcc	agctgggtccctgaacacatccttg
Rpl37	agagacgaaacactaccgggactgg	cttgggtttcggcgttgttccctc
36B4	ctgagtacaccttcccacttactga	cgactcttcctttgcttcagcttt

CHAPTER 5

Defective autophagy in T cells impairs the development of diet-induced hepatic steatosis and atherosclerosis

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ABSTRACT

Macroautophagy (or autophagy) is a conserved cellular process in which cytoplasmic cargo is targeted for lysosomal degradation. Autophagy is crucial for the functional integrity of different subsets of T cells in various developmental stages. Since atherosclerosis is an inflammatory disease of the vessel wall which is partly characterized by T cell mediated autoimmunity, we investigated how advanced atherosclerotic lesions develop in mice with T cells that lack autophagy related protein 7 (Atg7), a protein required for functional autophagy.

Mice with a T cell specific knock-out of Atg7 (Lck-Cre $Atg7^{f/f}$) had a diminished naïve $CD4^+$ and $CD8^+$ T cell compartment in the spleen and mediastinal lymph node as compared to littermate controls ($Atg7^{f/f}$). Lck-Cre $Atg7^{f/f}$ and $Atg7^{f/f}$ mice were injected intravenously with rAAV2/8-D377Y-mPCSK9 and fed a Western-type diet to induce atherosclerosis. While Lck-Cre $Atg7^{f/f}$ mice had equal serum Proprotein Convertase Subtilisin/Kexin type 9 levels as compared to $Atg7^{f/f}$ mice, serum cholesterol levels were significantly diminished in Lck-Cre $Atg7^{f/f}$ mice. Histological analysis of the liver revealed less steatosis, and liver gene expression profiling showed decreased expression of genes associated with hepatic steatosis in Lck-Cre $Atg7^{f/f}$ mice as compared to $Atg7^{f/f}$ mice. The level of hepatic $CD4^+$ and $CD8^+$ T cells was greatly diminished but both $CD4^+$ and $CD8^+$ T cells showed a relative increase in their IFNγ and IL-17 production upon Atg7 deficiency. Atg7 deficiency furthermore reduced the hepatic NKT cell population which was decreased to less than 0.1% of the lymphocyte population. Interestingly, T cell specific knock-out of Atg7 decreased the mean atherosclerotic lesion size in the tri-valve area by over 50%. Taken together, T cell specific deficiency of Atg7 resulted in a decrease in hepatic steatosis and limited inflammatory potency in the (naïve) T cell compartment in peripheral lymphoid tissues, which was associated with a strong reduction in experimental atherosclerosis.

KEYWORDS

Autophagy, Atg7, T cells, steatosis, atherosclerosis

INTRODUCTION

Atherosclerosis is an autoimmune-like disease of the vessel wall in which local accumulation of (modified) lipoproteins elicits an inflammatory response, which is among others T cell-mediated [1]. The progression and stability of an atherosclerotic lesion largely depends on the subtype of the T cell, as $CD4^+$ T helper cells are generally considered atherogenic, while T regulatory (Treg) cells predominantly act atheroprotective [1]. T helper 1 (Th1) cells represent a major fraction of the T cells which drive local inflammation through the secretion of inflammatory cytokines such as interferon-gamma (IFNy) [2,3]. In contrast, Treg cells are immunosuppressive T cells, which can inhibit effector T cells and other immune cells in lymphoid tissues and atherosclerotic lesions. Treg cells act via direct cell-cell interactions and via secretion of cytokines such as interleukin (IL)-10 and transforming growth factor β [4,5]. Other T helper cell subsets besides Th1 and Treg cells have a less dichotomous contribution to atherosclerosis. Th17 cells represent another subset of T helper cells, which are functionally characterized by the secretion of the interleukin IL-17. Th17 cells are involved in mucosal immunity where these cells help clearance of extracellular pathogens. Interestingly, their contribution to the ongoing inflammatory response in atherosclerotic lesions is context-dependent as Th17 cells have been described to have both atheroprotective as atherogenic functions [6]. Cytotoxic $CD8^+$ T cells exert their inflammatory function by secreting cytokines, by performing cell-lysis via perforin or granzyme-B and by inducing cell-death of their target cells through Fas-FasL interactions [7]. $CD8^+$ T cells might diminish the development of atherosclerotic lesions in early stages of the disease by killing macrophages and other antigen-presenting cells but they actually might promote lesion development by secreting pro-inflammatory cytokines [8]. Thus, the contribution of $CD8^+$ T cells to atherosclerosis seems context dependent and remains to be elucidated. One particular process which has gained interest in T cells but has not been studied extensively yet in the context of atherosclerosis is macroautophagy.

Macroautophagy (from henceforth called autophagy) is a well-conserved cellular process in which cytoplasmic cargo is (non-)selectively isolated in double-membrane vesicles called autophagosomes and subsequently transported to lysosomes for lysosomal degradation. Various autophagy-related proteins (Atg) contribute to consecutive phases of the autophagic process [9].

The engulfment of cytoplasmic cargo by autophagosomes is mediated by two ubiquitin-like conjugation systems which are involved in the expansion and closure of the autophagosomal membranes. In the Atg12 conjugation system, Atg12 is activated by the E1 enzyme Atg7 after which Atg12 forms a conjugate with Atg5 and forms a complex with Atg16L [10,11]. In mammalian cells, the Atg12-Atg5-Atg16L is bound to the isolation membrane from which it dissociates after its maturation to an autophagosome [12]. The

other conjugation system involves microtubule-associated protein 1 light chain 3 (LC3) which is activated by Atg7 and subsequently transferred to Atg3 [13]. The Atg12-Atg5-Atg16L complex is required for the adequate conjugation of LC3 to the phospholipid phosphatidylethanolamine (PE) by Atg3 [9,13]. Finally, the LC3-PE conjugate subsequently facilitates the tethering and fusion of the autophagosome membrane [14], thus closing the autophagosome.

Under homeostatic conditions, autophagy is important for the quality control of key organelles for example by degrading and recycling damaged or dysfunctional mitochondria [9]. Accordingly, genetic blockade of the Atg5 and Atg7 proteins impact the (functional) stability of $CD8^+$ T cells [15], Th1 [16] and regulatory T cells [17].

In $CD8^+$ T cells for example, deficiency of Atg5 or Atg7 does not affect clonal expansion, but does impair memory formation and survival, which was also associated with an altered metabolic phenotype in Atg7 deficient T cells [15]. Genetic blockade of Atg7 decreases the proliferation of activated naïve $CD4^+$ T cells whereas pharmacological blockade of autophagy in differentiated Th1 cells blocks proliferation and IFNγ secretion [16]. Treg cell-specific genetic blockade of Atg7 severely disrupts their immunosuppressive phenotype. Atg7 deficient Treg cells are apoptotic, lose expression of FoxP3 and gain an inflammatory phenotype characterized by high levels of glycolysis and IFNγ secretion [17]. Thus, genetic or pharmacological inhibition of autophagy modulates the inflammatory phenotype of various $CD4^+$ and $CD8^+$ T cell subsets albeit in different stages and through different mechanisms. Given the contribution of the aforementioned T cell subsets to the development of atherosclerosis, their reliance on functional autophagy and the therapeutic implication of certain autophagy inhibitors (such as chloroquine) to treat cardiovascular disease [18], we aimed in this study to determine how atherosclerosis is affected by T cell specific deletion of Atg7.

Here, we show that atherosclerosis development is severely hampered in mice with T cell specific Atg7 deficiency and that this is associated with decreased hepatic steatosis and by decreased frequencies of $CD4^+$,$CD8^+$ T cells and natural killer T (NKT) cells.

MATERIAL & METHODS

Mice

All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and the experimental work was approved by the Animal Ethics committee of Leiden University. *B6.Cg-Atg7<tm1Tchi>* ($Atg7^{f/f}$) and *B6.Cg-Tg(Lck-cre)1Jtak* (Lck-Cre) mice were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. To generate mice with T cell-specific deficiency of Atg7, $Atg7^{f/f}$ mice were crossed with mice expressing Cre recombinase under control of the

Lck promotor (Lck-Cre), thus creating Lck-Cre Atg7$^{f/f}$ mice. Atg7$^{f/f}$ littermates served as controls. 18 week old Lck-Cre Atg7$^{f/f}$ mice and their littermates were used to examine the effects of Atg7 deficiency on the T cell populations in the blood, spleen and mediastinal lymph nodes (medLN) under normolipidemic conditions.

Flow cytometry

Spleens and mediastinal lymph nodes (medLN) were isolated and mashed through a 70 µm cell strainer. Erythrocytes were subsequently eliminated from the blood and spleen by incubating the cells with ACK erythrocyte lysis buffer to generate a single-cell suspension prior to staining of surface markers. To isolate hepatic lymphocytes, non-parenchymal cells from the liver were first separated from parenchymal cells by centrifugation at low speed. Subsequently, the non-parenchymal cells were put on a Lympholyte gradient (Cedarlane) to isolate hepatic lymphocytes prior to staining of surface markers. For analysis of surface markers identifying CD4$^+$, CD8$^+$ and NKT cells, splenocytes or lymphocytes were stained at 4°C for 30 min. in staining buffer (phosphate buffered saline with 2% (vol/vol) fetal bovine serum (FBS)). All antibodies used for staining of surface markers or transcription factors were from Thermo Fischer and BD Biosciences (supplementary table 1). To identify NKT cells, an allophycocyanin labeled α-GalCer/CD1d tetramer kindly provided by the NIH tetramer core facility (Atlanta, GA) was used.

For staining of intracellular cytokines, splenocytes or liver-derived lymphocytes were incubated for 4h with 50 ng/mL phorbol myristate acetate (PMA) (Sigma), 500 ng/mL ionomycin (Sigma) and Brefeldin A (ThermoFisher). Extracellular staining was then performed with subsequent fixation and permeabilisation with Cytofix/Perm and Perm Wash buffer (both from BD Biosciences). Staining for intracellular cytokines was performed in Perm Wash Buffer after which the cells were washed with staining buffer prior to flow cytometric analysis.

Flow cytometric analysis was performed on a FACSCantoII (BD Biosciences) and data was analyzed using Flowjo software (TreeStar).

T cell proliferation

Splenocytes were isolated from Lck-Cre Atg7$^{f/f}$ or Atg7$^{f/f}$ mice and activated with anti-CD3e (1 µg/mL) and anti-CD28 (0.5 µg/mL) (both from ThermoFischer) for 72h and incubated with 0.5 µCi/well ^{3}H-thymidine (Perkin Elmer, The Netherlands) for the last 16h. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as the mean disintegrations per minute (dpm). The stimulation index (s.i.) was defined by dividing the dpm under activated conditions by the dpm under non-activated conditions per mouse.

Atherosclerosis

To investigate atherosclerosis in Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice , 18-20 week old female mice were administered rAAV2/8-D377Y-mPCSK9 (5x10 ^{11}genome copies/mouse) by i.v. injection [19], which results in overexpression of PCSK9 and subsequent development of atherosclerosis. After 1 day, mice were switched from a normal chow diet to a Western-type Diet (WTD, Special Diet Services) containing 0.25% cholesterol and 15% cocoa butter. The weight of the mice was monitored regularly. After 22 weeks, the mice were anesthetized by subcutaneous injections with ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL) after which their vascular system was perfused with PBS at a continuous low flow via heart puncture in the left ventricle. Next, the spleen, liver and inguinal white adipose tissue (iWAT) were collected for further processing. The hearts were collected, embedded in O.C.T. compound (Sakura) and then snap-frozen using dry-ice and stored at -80°C until further use.

Histology

To examine atherosclerotic lesions in the aortic root, the hearts were sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, 10 µm sections were collected. In order to visualize hepatic steatosis, a small piece of liver was dissected upon sacrifice and fixed using Zinc Formal-Fixx solution. Subsequently, the livers were embedded in O.C.T. compound after which 8 µm sections were prepared. After fixation with Zinc Formal-Fixx solution (Thermo Fischer) the neutral lipids in both aortic root and liver were stained using Oil-red-O (Sigma). Collagen content of the plaques was stained using a Mason's Trichrome staining kit (Sigma). Monocytes and macrophages were detected using a Moma2 primary antibody (Serotec) and biotinylated secondary antibody and visualized using the VECTASTAIN® Avidin-Biotin Complex Staining Kit (Vector Labs). After dissection of the iWAT, the tissue was fixed, dehydrated and subsequently embedded in paraffine and sectioned in 8 µm sections. After this, iWAT sections were deparaffinized and rehydrated prior to staining with Gill No. 3 hematoxylin solution (Sigma). Adipocyte size was quantified using the Adiposoft plugin in Fiji software [20]. The liver sections were examined visually to assess the degree of Oil-red-O staining as a measure for hepatic steatosis. For morphologic and morphometric analysis of the aortic root, the slides were analyzed using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems). Mean plaque size (in µm^2) was calculated from five sequential sections, displaying the highest plaque content. (Immuno)histochemical stainings were expressed as the percentage of positive stained area of the total lesion area. All morphometric analyses were performed by blinded independent operators.

Real-time quantitative PCR

RNA was extracted from mechanically disrupted livers by using Trizol reagent per the manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase according to the manufacturer's protocol (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to housekeeping genes (supplementary table 2).

Immunoblot

Immunoblot was performed as described previously [21] with minor modifications. Briefly, CD4$^+$ T cells were isolated using MACS microbeads (Miltenyi Biotec). For protein isolation, cells were lysed with 1xRIPA (Cell Signaling Technology) supplemented with cOmplete™ Protease Inhibitor Cocktail (Sigma) and 0.1% SDS for 30 minutes on ice. Proteins were detected using rabbit-anti-mouse Atg7 (Abcam) and rabbit-anti-mouse β-actin (Novus Biologicals) antibodies and visualized using chemiluminescence.

Serum analysis

The serum PCSK9 concentrations were determined using the Mouse Proprotein Convertase 9 DuoSet ELISA kit (R&D Systems) per the manufacturer's instructions. Concentration of total cholesterol in the serum was determined by an enzymatic colorimetric assay (Roche Dagnostics). Precipath (standardized serum, Roche Diagnostics) was used as an internal standard in the measurements for cholesterol.

Statistical analysis

For statistical analysis, a two-tailed Student's T-test was used to compare individual groups with Gaussian distributed data. Non-parametric data was analyzed using a Mann-Whitney U-test. Data from three or more groups were analyzed using a one-way ANOVA whereas data from three groups with more than one variable were analyzed by a two-way ANOVA, both with a subsequent Sidak multiple comparison test. Correlation was assessed using Pearson's correlation coefficient. A p-value below 0.05 was considered significant. Throughout the manuscript a * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ and **** indicates $p<0.0001$.

RESULTS

Atg7 deficiency affects T cell populations in medLN and spleen

To confirm that Cre recombinase expression in the Lck-Cre Atg7$^{f/f}$ mice was sufficiently high to induce Atg7 deficiency in T cells, we isolated splenic CD4$^+$ T cells from Lck-Cre Atg7$^{f/f}$ mice and Atg7$^{f/f}$ littermates, and analyzed Atg7 expression on a protein level by immunoblot. Atg7 was successfully knocked out as Atg7 could not be detected by immunoblot in CD4$^+$ T cells from Lck-Cre Atg7$^{f/f}$ mice whereas the control showed a clear Atg7 signal (fig. 1A). As described previously, T cell specific deficiency of Atg7 compromises single positive T cell generation in the thymus and induces peripheral lymphopenia [22]. We identified CD4$^+$ and CD8$^+$ T cells from peripheral tissues by flow cytometry using the gating strategy described in fig. S1A. The spleens of Lck-Cre Atg7$^{f/f}$ mice indeed contained significantly fewer CD4$^+$ and CD8$^+$ cells both in percentage and numbers (fig. 1B). We assessed the naïve CD4$^+$ T cell compartment in blood, spleen and mediastinal lymph node (medLN, the lymph node draining from the trivalve area) as CD4$^+$ T cells can be activated by lipoprotein-derived antigens during atherosclerosis[3]. The percentage of CD4$^+$ naïve T (Tn) cells as defined by CD4$^+$CD44$^-$CD62L$^+$ was significantly decreased in the spleen and medLN of Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (fig. 1C). Accordingly, the number of CD4$^+$ Tn cells was decreased in the spleen of Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (fig. S1B). Similar to the percentage of CD4$^+$ Tn cells, the percentage of CD8$^+$ Tn cells was decreased in the same tissues in Lck-Cre Atg7$^{f/f}$ mice (fig. 1D) and the number of CD8$^+$ Tn cells in the spleen was decreased as well in Lck-Cre Atg7$^{f/f}$ mice (fig. S1C). Next, we assessed whether the proliferative capacity of Atg7 deficient T cells is impaired which was confirmed using a proliferation assay measuring ^{3}H-thymidine incorporation with/without anti-CD3 and anti-CD28 stimulation (fig. 1D). The stimulation index was calculated and this parameter showed the proliferation of Atg7 deficient T cells was lower than Atg7 competent T cells (fig. 1F). The percentage of IFNγ, IL-17 and IL-10 was measured in splenic CD4$^+$ T cells using flow cytometry to assess the inflammatory capacity of the diminished T cell population in Lck-Cre Atg7$^{f/f}$ mice. Atg7 deficiency significantly increased the percentage of IFNγ-producing CD4$^+$ T cells (fig. 1G), causing the absolute number of IFNγ producing CD4$^+$ T cells to be unaltered between both genotypes (fig. S1D). The percentages of IL-17 and IL-10 producing T cells were unaltered.

Together, these results indicate that the generation of mice with Atg7 deficient T cells was successful from a genotypic and phenotypic perspective.

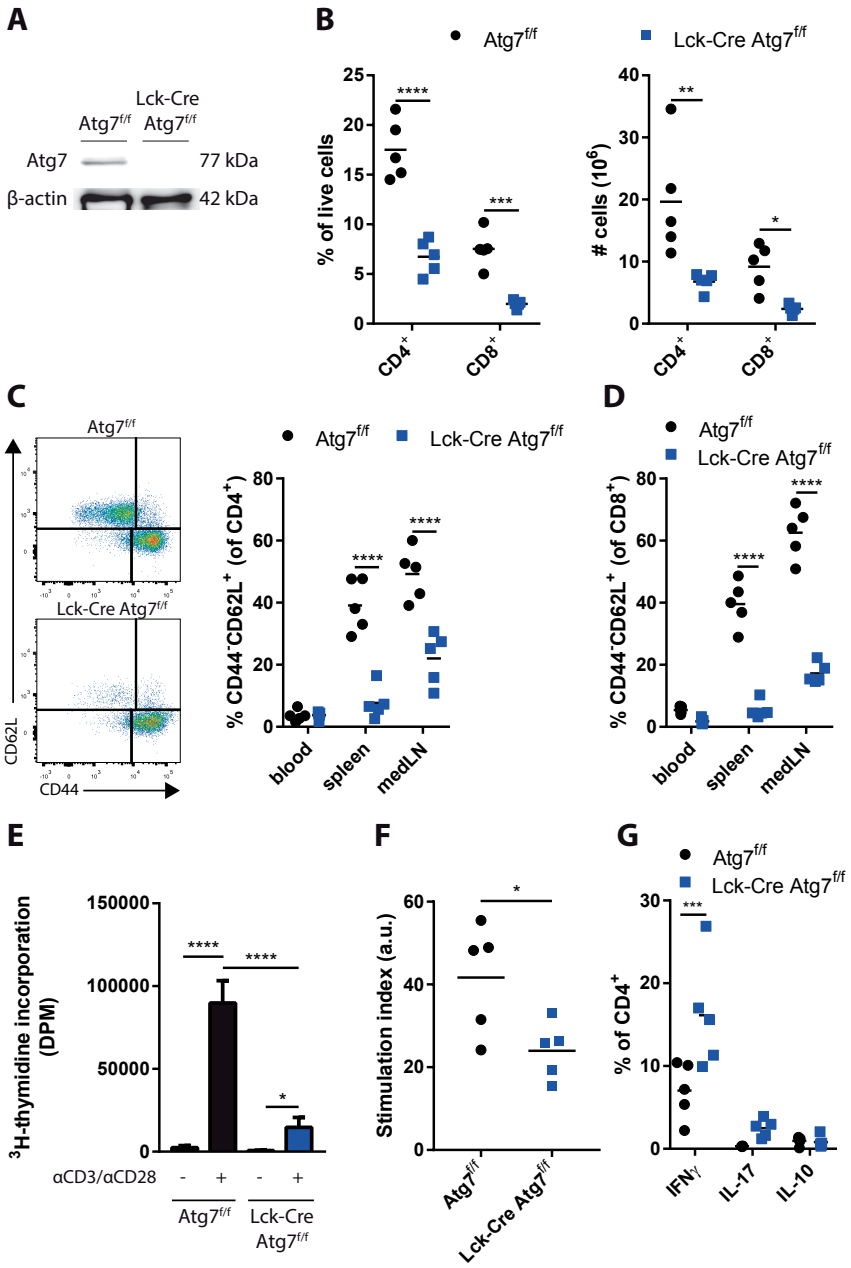

Figure 1 Effect of Atg7 deficiency on CD4$^+$ and CD8$^+$ T cells. (A) Immunoblot of Atg7 in CD4$^+$ T cells. β-actin was used as a loading control. (B) Percentage and numbers of CD4$^+$ and CD8$^+$ cells in the live lymphocyte fraction of spleens of indicated genotypes. (C) Gating and percentages of naïve T cells in the CD4$^+$ compartment. (D) Percentages of naïve T cells in the CD8$^+$ compartment. (E) T cell proliferation in stimulated or unstimulated splenocyte cultures. Expressed as mean ± standard deviation. (F) Stimulation index as calculated by dividing DPM of anti-CD3/anti-CD28 stimulated splenocytes by the DPM of non-stimulated splenocytes for each genotype. (G) Quantification of cytokine producing CD4$^+$ T cells.

Atg7 deficiency in T cells decreased hepatic steatosis and dyslipidemia during WTD-induced atherosclerosis

As we were interested in the impact of Atg7 deficiency in T cells on the development of diet-induced experimental atherosclerosis, we injected Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice with an adenoassociated virus encoding an active variant of PCSK9 (rAAV2/8-D377Y-mPCSK9) and fed them a WTD for 22 weeks to induce advanced atherosclerosis. As a result of overexpression of murine PCSK9 in the liver, the LDL receptor is targeted for lysosomal degradation and upon WTD-feeding circulating cholesterol levels are significantly elevated, to a level which is comparable to WTD-fed LDL receptor deficient mice [19]. After 4 weeks of WTD, the levels of mPCSK9 and cholesterol in the serum were measured to confirm that the viral transduction was successful. In general, the levels of serum PCSK9 exceeded 10,000 ng/mL which is sufficiently high to induce atherosclerosis upon prolonged WTD feeding [19]. Moreover, the levels of serum PCSK9 did not differ between both genotypes (fig. 2A). Interestingly, despite the serum PCSK9 levels being equal between both genotypes, serum cholesterol was significantly lower after 4 weeks of WTD in Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (fig. 2B). The lowest serum PCSK9 levels in Lck-Cre Atg7$^{f/f}$ (~8000 ng/mL) in this study were sufficiently high to be associated with serum cholesterol levels comparable to what we observed in Atg7$^{f/f}$ mice [19], suggesting that Atg7 deficiency results in decreased serum cholesterol levels after WTD feeding. Furthermore, after prolonged WTD feeding, the weight of Lck-Cre Atg7$^{f/f}$ mice was lower compared to Atg7$^{f/f}$ mice (fig. 2C) and in line, the weight of the inguinal white adipose tissue was lower in Lck-Cre Atg7$^{f/f}$ mice (fig. 2D) also when this was corrected for body weight at sacrifice (fig. 2E). Accordingly, the mean adipocyte size was decreased in Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice, although this did not reach significance (p=0.06, fig. 2F and 2G). As compared to Atg7$^{f/f}$ mice, the livers of Lck-Cre Atg7$^{f/f}$ mice appeared less steatotic (fig. 2H), which is consistent with a decrease in total serum cholesterol levels. Next, we measured the expression of genes that are associated with hepatic steatosis [23] and the expression of a number of additional genes involved in lipid metabolism was decreased in livers of Lck-Cre Atg7$^{f/f}$ mice. The expression of CD36, for example, a scavenger receptor known to mediate the uptake of native and modified lipoproteins, was decreased in Lck-Cre Atg7$^{f/f}$ mice (fig. 2I). Furthermore, the expression of the transcription factor peroxisome proliferator activated receptor gamma (*Pparg*), which is known to be associated with hepatic steatosis [24], was decreased while the expression of sterol regulatory element binding protein 2 (*Srebp2*) was increased in the liver of Lck-Cre Atg7$^{f/f}$ mice (fig. 2J). In line with the latter, the expression of *Fdft1*, which is involved in cholesterol synthesis, was elevated in Lck-Cre Atg7$^{f/f}$ mice (fig. 2K). On the other hand, the mRNA expression of genes involved in fatty acid synthesis in the liver was decreased, including *Acaa2*, *Scd1* and *Fas* (fig. 2L). The macrophage content in

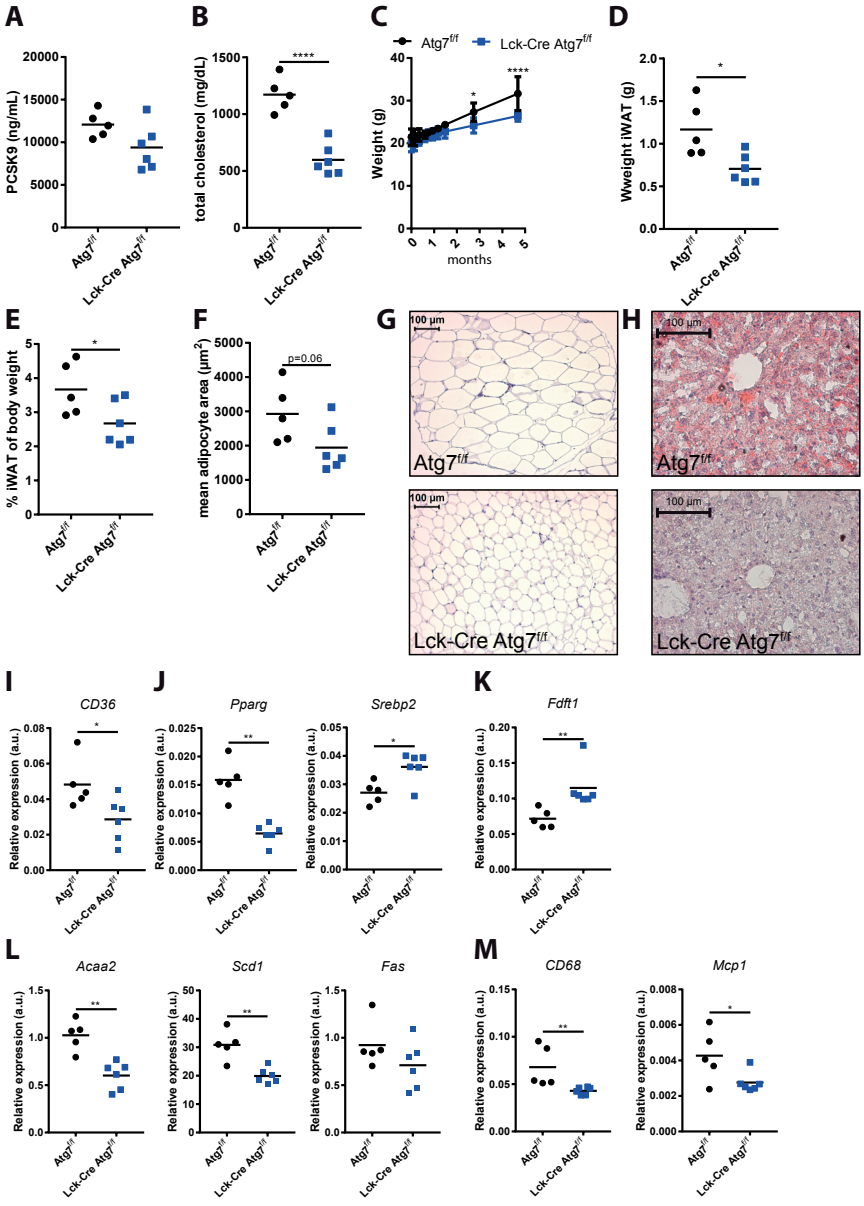

Figure 2 Atg7 deficiency in T cells decreased hepatic steatosis and dyslipidemia. (A) PCSK9 levels in serum. (B) Total cholesterol levels in serum. (C) Weight of the mice over the course of the experiment. (D) Weight inguinal white adipose tissue. (iWAT). (E) Weight iWAT as a percentage of body weight at sacrifice. (F) Quantification of adipocyte area in iWAT. (G) Representative sections of iWAT used for adipocyte size quantification in F (H) Representative Oil-Red-O stained sections of liver. (I) Gene expression in liver of scavenger receptor *CD36*. (J) Gene expression in liver of transcription factors *Pparg* and *Srebp2* (K) Gene expression in liver of enzyme involved in cholesterol synthesis, *Fdft1*. (L) Gene expression in liver of fatty acid synthesis genes *Acaa2, Scd1* and *Fas*. (M) Gene expression in liver of macrophage lineage marker *CD68* and monocyte chemoattractant *Mcp1*.

livers of Lck-Cre Atg7$^{f/f}$ mice was also decreased as suggested by the decreased mRNA expression of the macrophage lineage marker *CD68* and the chemokine *Mcp1* (fig. 2M). Altogether, T cell specific Atg7 deficiency hampered WTD-induced hepatic steatosis and dyslipidemia despite successful viral transduction with rAAV2/8-D377Y-mPCSK9.

Atg7 deficiency in T cells decreases T cell abundance in the liver but increases inflammatory cytokine production

As inflammation is one of the drivers of hepatic steatosis [25], we postulated that Lck-Cre Atg7$^{f/f}$ mice developed less severe hepatic steatosis during our experiments as the inflammatory capacity of the hepatic T cell population was impaired. Therefore, we characterized the CD4$^+$ and CD8$^+$ T cell populations in the liver. In line with the observations in the spleen, the percentage of CD4$^+$ T cells in the lymphocyte fraction of the liver was decreased as a result of Atg7 deficiency (fig. 3A). Additionally, the percentage of CD8$^+$ T cells was decreased as well, albeit to a lesser extent (fig. 3A). We measured the inflammatory capacity of CD4$^+$ and CD8$^+$ T cells through flow cytometry by measuring the percentage of IFNγ and IL-17 producing cells in both genotypes (fig. 3B). The percentage of IFNγ$^+$ cells in the hepatic CD4$^+$ T cells showed a two-fold increase in Atg7 deficient CD4$^+$ T cells whereas the percentage of IL-17 producing cells was also increased by Atg7 deficiency going from ~0.4% in Atg7 wildtype CD4$^+$ T cells to ~6.3% in Atg7 deficient CD4$^+$ T cells (Fig. 3C). Similarly, the IFNγ and IL-17 production was increased in Atg7 deficient CD8$^+$ T cell compartment as compared to Atg7 wildtype CD8$^+$ T cells (fig. 3D). As this suggested Atg7 deficiency induced the skewing of the diminished T cell population towards an inflammatory phenotype and IL-10 is an anti-inflammatory cytokine, we measured *Il10* expression in the livers of Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice. In line with an inflammatory phenotype and Atg7 deficiency disrupting Treg cell function and stability, the expression of *Il10* was decreased in livers of Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7f/f control mice (fig. 3E). Whether this decrease in *Il10* expression is truly T cell dependent or whether it is due to the less advanced stage of hepatic steatosis in Lck-Cre Atg7$^{f/f}$ mice remains to be determined.

In conclusion, although Atg7 deficiency resulted in a relative increase in T cells which produce inflammatory cytokines, the decrease in CD4$^+$ and CD8$^+$ T cells in the liver likely impairs the development of hepatic steatosis in Lck-Cre Atg7$^{f/f}$ mice.

Lack of NKT cells in mice with T cell-specific Atg7 deficiency

In mice, natural killer T (NKT) cells represent a relatively large fraction of hepatic lymphocytes (up to 35%). NKT cells recognize lipid-derived antigens when presented on the major-histocompatibility complex-resembling protein CD1d. The most common type of NKT cells in mice is the type I NKT cells, also called invariant NKT cells. Upon stimulation, NKT cells secrete a plethora of cytokines, including Th1-like (IFNγ, TNFα) cytokines and

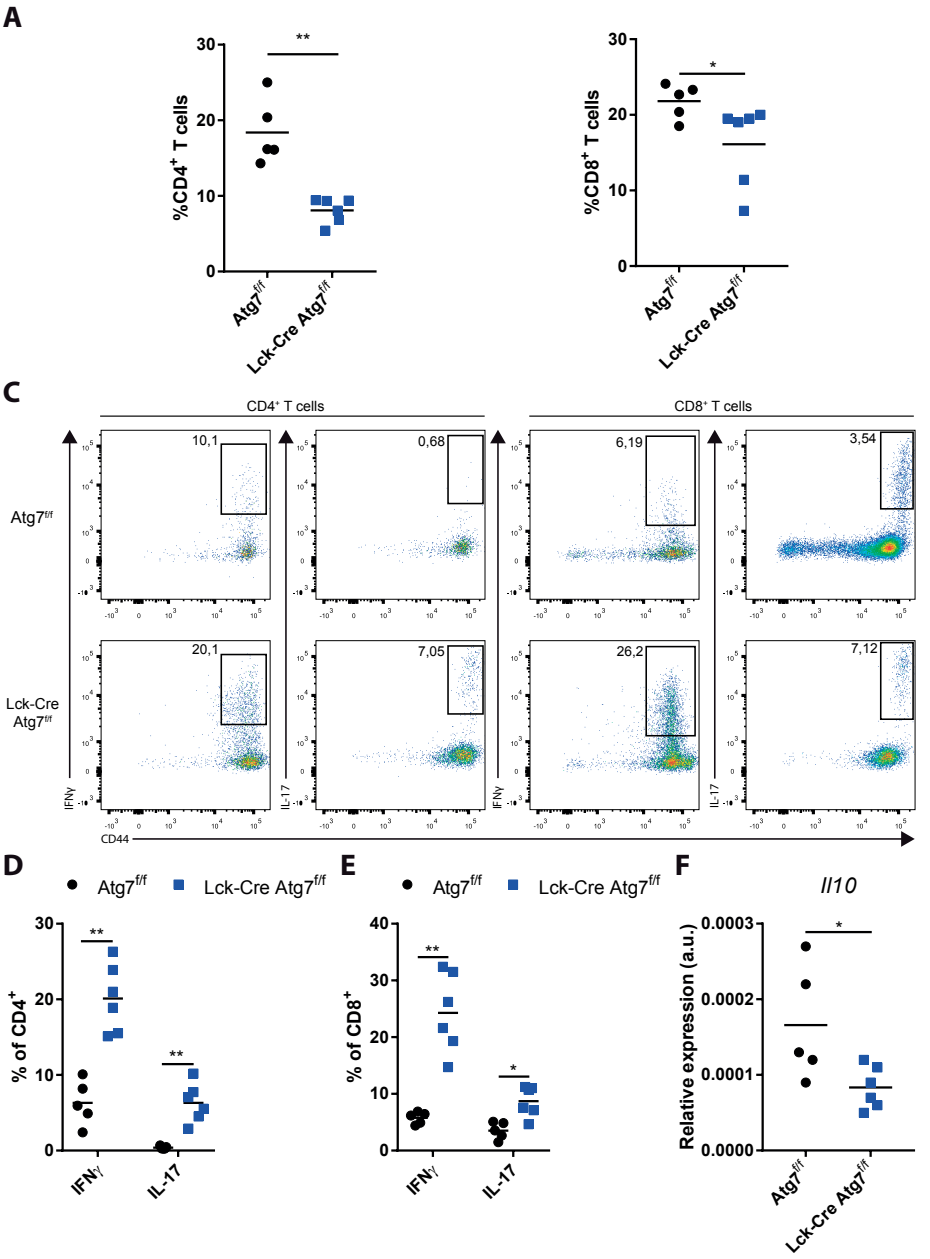

Figure 3 Atg7 deficiency in T cells diminishes T cells in liver but increases relative inflammatory cytokine production. (A) Percentage of CD4$^+$ and CD8$^+$ T cells in live hepatic lymphocyte fraction. (B) Gating strategy to identify IFNγ and IL-17 producing T cells in liver. (C) Percentage of IFNγ and IL-17 producing hepatic CD4$^+$ T cells. (D) Percentage of IFNγ and IL-17 producing hepatic CD8$^+$ T cells. (E) *Il10* expression in liver.

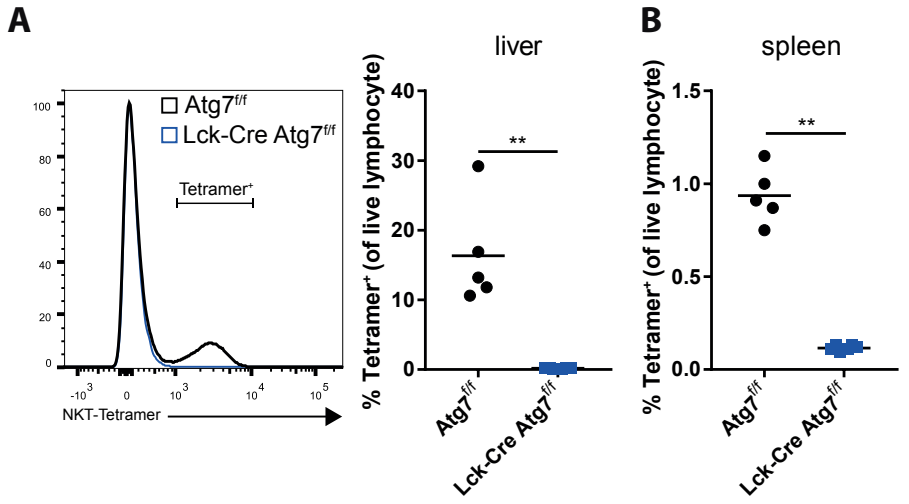

Figure 4 Atg7 deficiency in T cells reduces the amount of NKT cells in liver and spleen. (A) NKT cells as detected by an α-GalCer/CD1d tetramer staining and presented as a percentage of the lymphocyte fraction in the liver. (B) Percentage of NKT cells in spleen.

IL-10. As NKT cells have a functional TCRαβ and express one of its proximal signaling kinases Lck [26], we hypothesized that Atg7 deficiency disrupted NKT cell function. Using flow cytometry, we observed that the percentage of NKT cells was severely diminished in the hepatic lymphocyte fractions of Lck-Cre Atg7$^{f/f}$ mice from ~18% to ~0.1%(fig. 4A). Similarly, in the spleens of Lck-Cre Atg7$^{f/f}$, only ~0.1% of the lymphocyte population consisted of NKT cells compared to ~1% in the control (fig. 4B).
Thus, T cell specific Atg7 deficiency not only diminished the CD4^{+} and CD8^{+} T cell populations but also severely reduced the percentage of hepatic NKT cells, which may have contributed to impaired hepatic steatosis development as suggested by literature [25].

T cell specific Atg7 deficiency decreases atherosclerosis

Since we were interested in the effect of T cell specific Atg7 deficiency on the development of diet-induced advanced atherosclerosis we quantified atherosclerotic lesion size in the aortic root after 22 weeks of WTD. Lck-Cre Atg7$^{f/f}$ mice developed 50% smaller lesions than Atg7$^{f/f}$ control mice (fig. 5A, $p<0.01$). Interestingly, the correlation between lesion size and serum total cholesterol levels was stronger in Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (fig. S2A and fig. S2B), suggesting that the decrease in T cell mediated autoimmunity renders serum cholesterol to be a stronger driver of atherogenesis in Lck-Cre Atg7$^{f/f}$ mice. Additionally, T cell specific Atg7 deficiency reduced the collagen content by approximately 50% (fig. 5B). Lastly, the relative amount of monocytes and macrophages in the lesions was quantified using a MOMA-2 staining. No differences

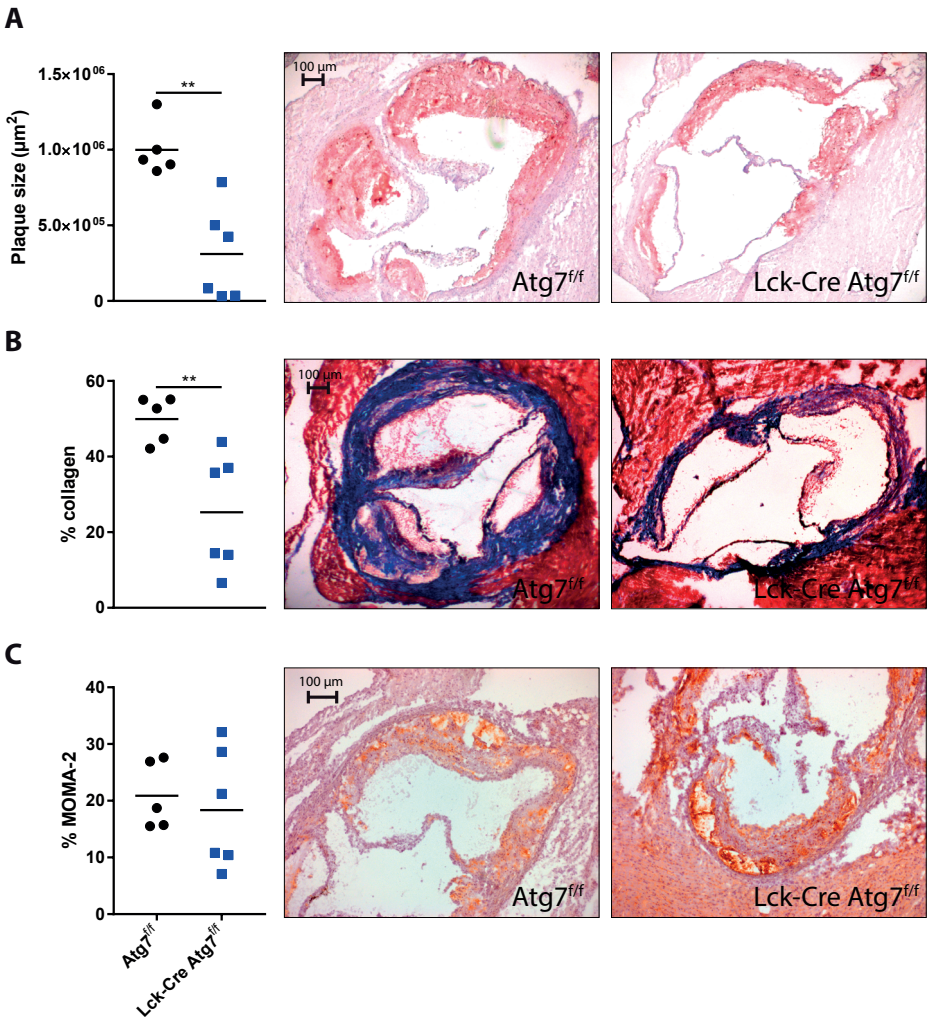

Figure 5 Histological analysis of atherosclerosis in the aortic root. (A) Quantification of mean plaque size using Oil-red-O staining. (B) Quantification of collagen content using a Masson's Trichrome staining. Collagen fibers are indicated in blue. (C) Quantification of monocyte-macrophage content using a MOMA-2 antibody.

were observed in terms of macrophage content between Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice (fig. 5C).

DISCUSSION

Different T cell subsets which are crucially involved in the development of atherosclerosis depend on autophagy for their functional integrity and survival. Systemic administration of autophagy inhibitors such as chloroquine or hydroxychloroquine has therapeutic potential to treat atherosclerosis as it shows anti-inflammatory effects in other autoimmune diseases such as rheumatoid arthritis. In mice, systemic low-dose administration of chloroquine inhibits diet-induced atherosclerosis in ApoE-deficient mice [27]. To gain more insight in the T cell specific contribution of the anti-inflammatory effect of autophagy blockade, we genetically blocked autophagy in T cells and studied the impact on diet-induced atherosclerosis in experimental models of disease.

In this model, knock-out of Atg7 in T cells significantly decreased the percentage and numbers of $CD4^+$ and $CD8^+$ T cells. The percentage of naïve T cells was also decreased in lymphoid tissues, the mediastinal lymph nodes and spleen, in which naïve T cells respond to lesion and lipoprotein-derived antigens. These findings are in line with data that naïve T cells go into apoptosis without functional autophagy [28], which is highly relevant for atherosclerosis research as this would result in relatively fewer T cells to respond to atherosclerosis derived antigens and thus to the ongoing inflammation in atherosclerotic lesions. Atg7 deficiency in T cells impaired their proliferative capacity in a splenocyte culture under anti-CD3 and anti-CD28 antibody induced stimulation, which is in line with a previous report describing that Atg7 deficient naïve $CD4^+$ T cells proliferate less after antibody-mediated TCR stimulation [16]. TCR stimulation also activated $CD8^+$ T cells in the splenocyte culture but as autophagy is not induced upon activation of $CD8^+$ T cells [15] and their proliferative capacity is not affected by Atg5 or Atg7 deficiency [15] it is unlikely that the decrease in T cell proliferation we observed in our experiments was $CD8^+$ T cell-mediated. Under normolipidemic conditions, the spleens of Lck-Cre $Atg7^{f/f}$ mice contained fewer $CD4^+$ T cells although a relatively higher percentage of these $CD4^+$ cells secreted IFNγ. Although we did not observe a difference in total numbers of $CD4^+$ $IFN\gamma^+$ T cells between Lck-Cre $Atg7^{f/f}$ and $Atg7^{f/f}$ mice, Atg7 deficient T cells have been described to secrete lower amounts of IFNγ and IL-10 and also lower amounts of other T helper cell cytokines including IL-4 and IL-17 [29]. These results suggested that Atg7 deficiency severely compromised the inflammatory capacity of all the T helper cell populations in the medLN and spleen in our studies. Therefore, the fact that Atg7 deficient $CD4^+$ and $CD8^+$ T cells had a higher percentage of IFNγ producing cells than their Atg7 competent counterparts is negated by the diminished number of $CD4^+$ and $CD8^+$ T cells and their capacity to not only produce but also secrete cytokines in Lck-Cre $Atg7^{f/f}$ mice as compared to $Atg7^{f/f}$ mice.

As LDL receptor competent mice barely develop atherosclerosis we injected $Atg7^{f/f}$ and Lck-Cre $Atg7^{f/f}$ mice with a single injection of rAAV2/8-D377Y-mPCSK9. Compared to

Atg7$^{f/f}$ mice, Lck-Cre Atg7$^{f/f}$ mice had lower serum cholesterol levels and less hepatic steatosis under dyslipidemic conditions based on histological evaluation and gene expression of genes associated with hepatic steatosis. As Lck-Cre Atg7$^{f/f}$ mice gained less weight, and had a lower iWAT weight , the decreased extent of hepatic steatosis in these mice had effects beyond just the decrease in circulating cholesterol levels. However, given the role of T cells in the development of obesity-associated inflammation of white adipose tissue [30–32], the impaired weight gain could also be explained by impairments in T cell-mediated inflammation of white adipose tissues. This indicates that, under dyslipidemia conditions, Atg7 deficiency has impact on the immunometabolic phenotype of mice on a systemic level which is relevant for further research examining autophagy blockade in models of dyslipidemia *in vivo*.

Since hepatic steatosis was decreased upon T cell specific deletion of Atg7, we hypothesized that this was due to a reduction in the numbers and profile of cytokine secretion of CD4$^+$ and CD8$^+$ T cells in the livers of Lck-Cre Atg7$^{f/f}$ mice. Similar to the spleens and medLNs during normolipidemia, the livers of mice with Atg7 deficient T cells contained fewer CD4$^+$ and CD8$^+$ T cells. Also in the hepatic T cell populations, the percentages of IFNγ and IL-17 secretion were increased but since the reduction in the percentages of CD4$^+$ and CD8$^+$ T cells is considerable, the total number of IFNγ and IL-17 secreting T cells would be lower. It is unclear why the relatively few CD4$^+$ and CD8$^+$ T cells in the liver had a higher level of inflammatory cytokine secretion. Inhibition of autophagy in Th1 cells using 3-methyladenine or NH_4Cl and leupeptin impairs IFNγ secretion [16], suggesting that it is unlikely that Atg7 deficiency increases Th1 differentiation resulting in enhanced IFNγ secretion. Interestingly, Treg cell specific Atg7 deficiency induces a loss of FoxP3 and enhances their production of IFNγ and IL-17 [17]. Likewise, knock out of Atg16L, another essential protein in autophagy, mimics the effects of Atg7 deficiency in Treg cells as Atg16L1 deficient Treg cells have increased IFNγ and IL-17 production [33]. Though Atg7 deficiency in Treg cells impairs their survival and immunosuppressive capacity it also increases their homeostatic proliferation [17], suggesting that Atg7 deficient Treg cells are more resilient to defective autophagy as compared to Atg7 deficient conventional T cells. Therefore, it is most likely that the increase in IFNγ and IL-17 producing CD4$^+$ T cells in the liver and spleen of Lck-Cre Atg7$^{f/f}$ mice is partly due to Atg7 deficiency in Treg cells which contribute to the increase in the percentage of IFNγ and IL-17 producing Treg cells under specific inflammatory conditions [17]. In line, Treg cells with a Th1- or Th17-like phenotype have been described before in (models for) cardiovascular disease [34,35]. Furthermore, as Atg7 deficient Treg cells have impaired immunosuppressive capacity, other IFNγ and IL-17 expressing CD4$^+$ T cells are improperly inhibited by Treg cells. Further research is however required to examine what the functional effects of autophagy deficiency in Th17 cells are.

Hepatic steatosis (or fatty liver) can develop when hepatocytes accumulate dietary lipids, potentially resulting in lipotoxicity. When this persists, immune cells such as Kupffer cells are activated and monocytes can be recruited when damaged or dead cells release danger signals such as damage-associated molecular patterns, leading to the development of non-alcoholic steatohepatitis (NASH) [25]. *Ldlr*$^{-/-}$ mice which are fed a high fat, high cholesterol diet are a suitable model to study the onset of inflammation in hepatic steatosis [32], suggesting the development of hepatic steatosis in virus-induced LDL receptor deficient mice is physiologically relevant. In the hepatic lymphocyte population, it is mainly Th17 cells which drive the development of steatosis [36]. Through the secretion of IL-17, Th17 cells directly drive sinusoidal cells such as fat storing cells to produce type 1 collagen and activate macrophages to secrete inflammatory cytokines [37]. Patients with hepatic steatosis have increased intrahepatic IL-17 expressing CD4$^+$ T cells while in the blood, more IFNy secreting CD4$^+$ T cells were detected as compared to healthy controls [36]. In line, morbidly obese patients with NASH have higher intrahepatic gene expression of Th1-associated genes and a decreased ratio of IL-10/IFNy as compared to patients with non-alcoholic fatty liver disease [38]. Whereas Th17 and Th1 cells appear to drive NASH, Treg cells presumably inhibit its development as Treg cell deficient mice with WTD-induced atherosclerosis have more severe hypercholesterolemia due to impaired clearance of chylomicron remnants and very low density lipoproteins [39]. Taken together, in our study we deem it most likely that the diminishment in the amount of hepatic T cells impaired the development of hepatic steatosis.

The contribution of NKT cells to the development of hepatic steatosis remains to be elucidated. In high-fat diet induced obesity, hepatic NKT cell numbers diminish, possibly contributing to the development of hepatic steatosis as the cytotoxicity-mediated killing of hepatocytes, which are under lipotoxicity-induced stress, is impaired [40,41]. The expansion of NKT cells during diet-induced steatosis development using probiotics actually protects against hepatic steatosis and insulin resistance [42]. In contrast, expansion of hepatic NKT cells through the Hedgehog-pathway contributes to hepatic fibrosis [43], suggesting that the contribution of NKT cells to the pathogenesis of steatosis and NASH depends on the dynamics and inflammatory phenotype of hepatic NKT cells.

Studies describing the abundance of NKT cells in different organs during experimental atherosclerosis show contradictory results [44]. Both an increase and a decrease in NKT cell number in atherosclerosis has been reported. In our experiments, the abundance of NKT cells was severely diminished in the livers of WTD-fed Lck-Cre Atg7$^{f/f}$ mice, which is relevant as the liver contains the highest number of NKT cells in mice. This effect of Atg7 deficiency on hepatic NKT cell abundancy is due to reduced thymic NKT cell output as Atg7 deficiency inhibits the progression of NKT cells through the cell cycle and increases NKT cell apoptosis [45]. Interestingly, Atg5 deficiency primarily hampers the secretion of IFNy by Th1-like NKT cells and Atg7 deficiency presumably has the same

effect [45]. Given their modulatory role in steatosis development, the lack of inflammation competent NKT cells diminished the development of hepatic steatosis. As LDLr$^{-/-}$CD1d$^{-/-}$ mice, which lack NKT cells, have similar cholesterol levels when fed a WTD as compared to LDLr$^{-/-}$ mice [46] it is likely that in Lck-Cre Atg7$^{f/f}$ mice, the combined effect of the lower numbers of inflammatory CD4^{+}, CD8^{+} and of NKT cells inhibited the development of WTD-induced hepatic steatosis.

Atherosclerosis development was severely impaired in mice with T cell specific Atg7 deficiency which can be explained by low levels of serum cholesterol and low numbers of CD4^{+} T cells, CD8^{+} T cells and NKT cells. Additionally, the lesions of Lck-Cre Atg7$^{f/f}$ mice contained less collagen as compared to lesions from Atg7$^{f/f}$ mice which is most likely due to more advanced and stabilized lesions in the latter group. The lack of a proper NKT cell population in Lck-Cre Atg7$^{f/f}$ mice likely contributed to decreased lesion growth as NKT cells can drive atherogenesis through various mechanisms, including through perforin and granzyme-B mediated cytotoxicity and cytokine secretion [47–49]. The fact that activated iNKT cells can decrease lesion stability by reducing collagen content [47] was likely overruled by their low abundancy in our study.

In this study we determined the effect of genetic blockade of autophagy in T cells on late stages of atherosclerosis in a mouse model where T cells lack functional autophagy from an early developmental stage and subsequently induced atherosclerosis. For a translation into a clinical setting in which cardiovascular patients could be treated with pharmacological autophagy inhibitors, it would be interesting to knock-out Atg7 using an inducible Cre mouse model in mice with pre-developed atherosclerosis. In addition it would have been highly interesting to dissect the effect of Atg7 deficiency in specific T cell subsets using mice with Cre recombinase under control of the promotor of T-bet, RORγt and FoxP3 to induce Atg7 deficiency in Th1-, Th17- and Treg cells, respectively.

In conclusion, T cell specific Atg7 deficiency decreased the degree of diet-induced hepatic steatosis and atherosclerosis due to a decrease in numbers of CD4^{+}, CD8^{+} and NKT cells. These results suggest that autophagy inhibition in T cells is feasible to diminish atherosclerosis. Further research focusing on the effect systemic administration of pharmaceuticals such as chloroquine has on non-T cells could contribute to its applicability to inhibit inflammation and potentially prevent cardiovascular disease.

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SUPPLEMENTARY FIGURES

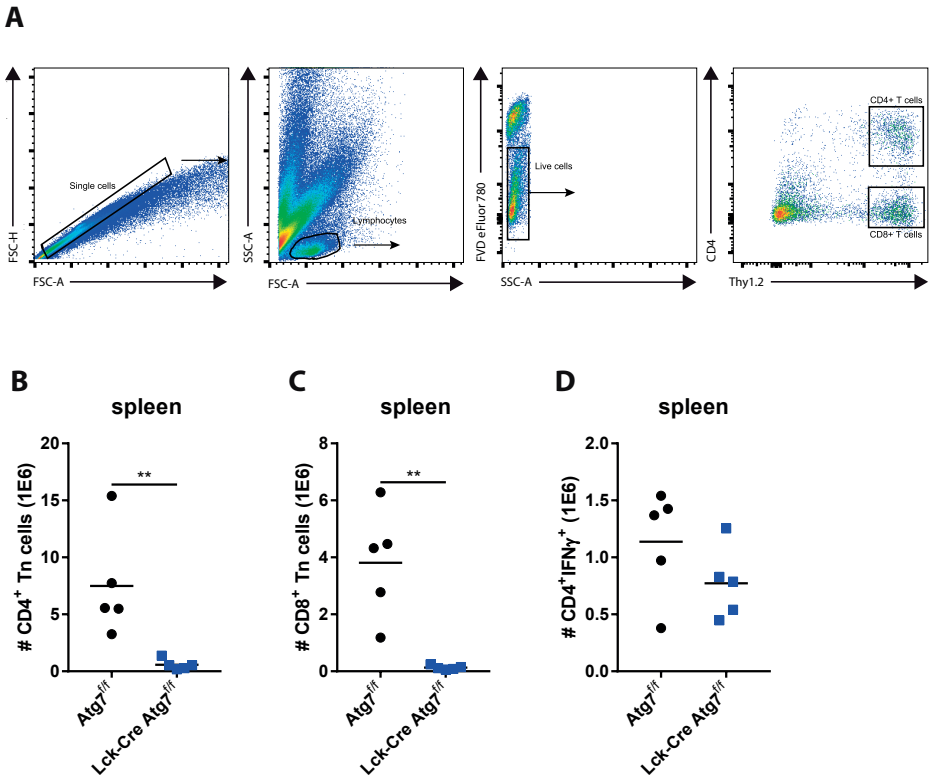

Figure S1 Gating strategy for flow cytometry analysis of T cells and quantification of T cells. (A) Gating strategy to identify live CD4$^+$ and CD8$^+$ T cells. (B) Number of CD4$^+$ Tn cells in spleen of indicated genotypes. (C) Number of CD8$^+$ Tn cells in spleen of indicated genotypes. (D) Number of CD4$^+$IFNγ$^+$ cells in spleen of indicated genotypes.

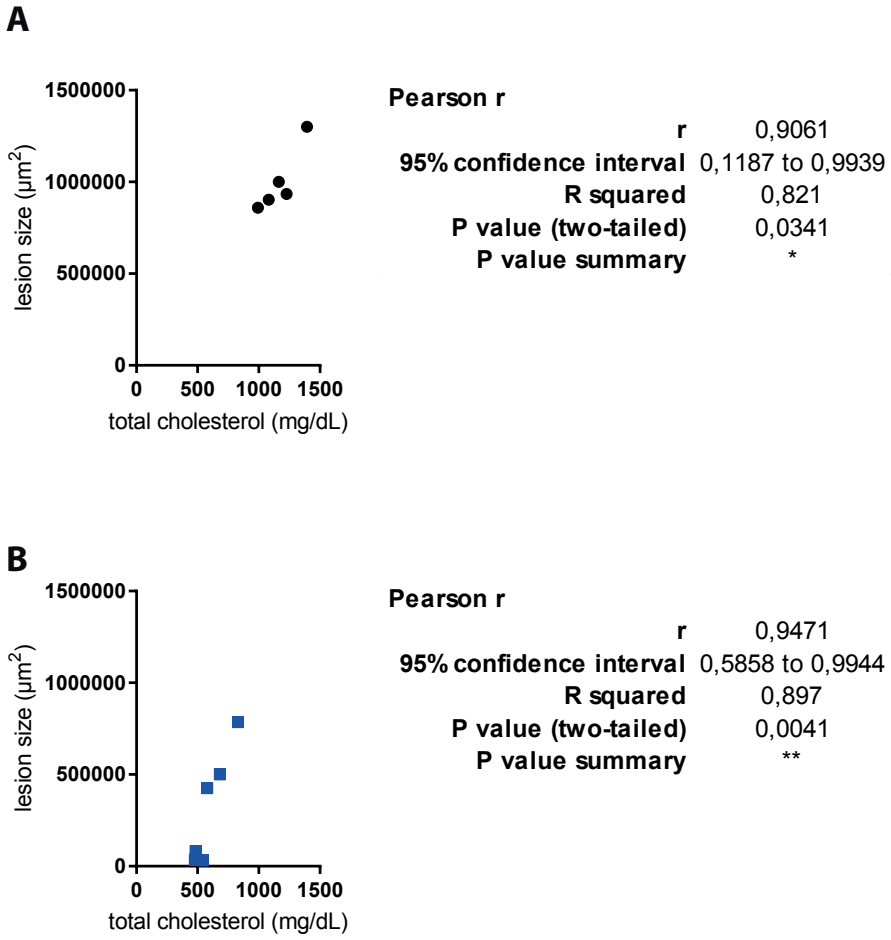

Figure S2 Correlation between lesion size and serum cholesterol levels. (A) Correlation between lesion size and total serum cholesterol levels in Atg7$^{f/f}$ mice. (B) Correlation between lesion size and total serum cholesterol levels in Lck-Cre Atg7$^{f/f}$ mice.

SUPPLEMENTARY TABLES

Table 1 Antibodies used for flow cytometry

Antigen	Label	Clone	Manufacterer
fixable viability dye	eFluor 780	n/a	ThermoFischer
Thy1.2	PE-Cy7	53-2.1	ThermoFischer
CD8	FITC	53-6.7	ThermoFischer
IFNy	Alexa fluor 488	XMG1.2	ThermoFischer
IL-10	APC	JES-16E3	ThermoFischer
CD44	APC	IM7	ThermoFischer
CD44	eFluor 450	IM7	ThermoFischer
CD62L	eFluor 450	MEL-14	ThermoFischer
CD62L	PerCP-Cy5.5	MEL-14	ThermoFischer
IL17	PE	TC11-18H10	BD Biosciences
CD4	PerCP	RM4-5	BD Biosciences

Table 2 List of primers used for qPCR expression analysis. Expression of genes were normalized to housekeeping genes *Eef2* and *36B4*.

Gene	Forward primer (5′-3′)	Reverse primer (3′-5′)
CD36	atggtagagatggccttacttggg	agatgtagccagtgtatatgtaggctc
Pparg	aagccctttggtgactttatggagcc	tgcagcaggttgtcttggatgtcc
Srebp2	ccagctcctgggtgagacctac	caggcgcacagtggcttcat
Scd1	ggaaagtgaggcgagcaactgacta	caggacggatgtcttcttccaggtg
Fas	gctgttttcccttgctgcagacatg	aacccgcctcctcagctttaaactc
Il10	gggtgagaagctgaagaccctc	tggccttgtagacaccttggtc
CD68	tgcctgacaagggacacttcggg	gcgggtgatgcagaaggcgatg
Fdft1	aacatgcctgccgtcaaagctatca	gcttgatgatgggtctgagttgggg
Acaa2	cttgaccccagcaaaaccaatgtgag	gatcccactgcgtactttccacctc
Mcp1	ctgaagccagctctctcttcctc	ggtgaatgagtagcagcaggtga
Eef2	gaacaggaagcgtggccatgtgttt	ggctgctgttgtcaaaaggatcccc
36B4	ctgagtacaccttcccacttactga	cgactcttcctttgcttcagcttt

CHAPTER 6

Lipocalin-2 contributes to experimental atherosclerosis in a stage-dependent manner

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ABSTRACT

Background

Lipocalin-2 (Lcn2) is a glycoprotein which can be secreted by immune cells. Several studies in humans have suggested Lcn2 can be used as a biomarker for the detection of unstable atherosclerotic lesions, partly as it is known to interact with MMP-9.

Methods

In this study we generated *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice to study the functional role of Lcn2 in different stages of atherosclerosis. Atherosclerotic lesions were characterized through histological analysis and myeloid cell populations were examined using flow cytometry.

Results

We show that *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice developed larger atherosclerotic lesions during earlier stages of atherosclerosis and had increased circulating Ly6C^{hi} inflammatory monocytes compared to *Ldlr*$^{-/-}$ mice. Advanced atherosclerotic lesions from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice had decreased necrotic core area suggesting Lcn2 deficiency may affect lesion stability. Furthermore, MMP-9 activity was diminished in plaques from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice.

Conclusion

Altogether, these findings suggest that Lcn2 deficiency promotes lesion growth in earlier stages of the disease while it decreases MMP-9 activity and necrotic core size in advanced atherosclerosis.

KEYWORDS

Atherosclerosis, lipocalin-2, necrotic core, MMP-9, monocytes

INTRODUCTION

The development of atherosclerosis and subsequent atherosclerotic plaque destabilization are the main underlying pathology of (ischemic) heart disease. Therefore, early detection of unstable atherosclerotic plaques using biomarkers could prove useful to reduce the incidence of acute cardiovascular syndromes. A potential candidate to use as a biomarker for unstable atherosclerosis is lipocalin-2 (Lcn2), which is also known as 24p3 in mice and in humans as neutrophil-gelatinase associated lipocalin (NGAL).

Lcn2 is a secreted glycoprotein which was originally identified as a product of human neutrophils [1, 2]. Upon bacterial infection, Lcn2 functions as a bacteriostatic agent by sequestering iron from bacterial siderophores, such as enterobactin [3]. During inflammation, Lcn2 can act as an inflammatory mediator by binding N-formylmethionyl-leucyl-phenylalanine and leukotriene B4 (LTB4) [4]. Interestingly, Lcn2 itself can also act as a chemoattractant for neutrophils during infection [5]. Furthermore, Lcn2 can form a complex with matrix metalloproteinase (MMP)-9, thereby preventing it from being inhibited by tissue inhibitor of metalloproteinases-1 [6]. Active MMP-9 inside atherosclerotic lesions can degrade extracellular matrix and may thus contribute to advanced plaque instability [7, 8]. By stabilizing active MMP-9, Lcn2 may contribute to the degradation of the fibrous cap and destabilization of atherosclerotic plaques in general [9]. In line, several reports suggest that serum NGAL levels can be used to predict the incidence of cardiac events. Serum NGAL levels were for example shown to correlate with levels of C-reactive protein and to predict major adverse cardiac event (MACE) as well as all-cause mortality in patients with a history of CVD [10-12]. Furthermore, serum NGAL/MMP-9 complex levels are associated with MACE in patients 1 year after coronary angiography [13]. Serum NGAL levels were higher in patients with angiographically confirmed coronary artery disease compared to patients without. Additionally, serum NGAL levels were associated with the number of diseased vessels, suggesting serum NGAL levels might be indicative of the severity of disease [14]. Furthermore, patients with symptomatic atherosclerosis in carotid arteries were shown to have higher levels of serum NGAL as compared to asymptomatic patients [15]. mRNA expression of NGAL was also elevated in atherosclerotic plaques of patients with symptomatic carotid atherosclerosis compared to asymptomatic patients [16]. Local NGAL protein levels were also demonstrated to be elevated in unstable versus stable plaques and NGAL content correlated specifically with MMP-9 activity [17]. In mouse models for atherosclerosis, Lcn2 was shown to colocalize with MMP-9 in atherosclerotic plaques [18].

Lcn2 is actually a very pleiotropic protein, as it is also associated with the development of metabolic diseases, which can subsequently contribute to cardiovascular disease [19]. Serum NGAL levels were elevated in obese individuals compared to lean controls and correlated with insulin resistance [20]. Furthermore, NGAL expression was shown to be

elevated in visceral adipose tissue of obese individuals compared to non-obese controls. In adipose tissue, enzymatic activity of NGAL/MMP-9 complexes was increased in obese individuals compared to lean controls [21]. Despite the evidence from literature which suggests that Lcn2 is associated with the development of atherosclerosis and obesity, there are still many questions regarding the exact pathophysiological role for Lcn2 in atherogenesis. Mechanistic insights gained from experimental models studying Lcn2 in multiple stages of atherosclerosis could contribute to the applicability of Lcn2 as a biomarker for early detection of unstable atherosclerosis and coronary artery disease. In this study, we thus aimed to investigate the contribution of Lcn2 to different stages of diet-induced atherosclerosis. We show here that Lcn2 has a stage-dependent contribution to experimental atherosclerosis as it seems to limit lesion development, whereas it potentially contributes to plaque instability in more advanced stages of atherosclerosis.

MATERIALS AND METHODS

Mice

All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and the experimental work was approved by the Animal Ethics committee of Leiden University. *Ldlr* deficient (*Ldlr*$^{-/-}$) mice were originally purchased from Jackson Laboratory and further bred in the Gorlaeus Laboratory in Leiden, The Netherlands. Lcn2 deficient (*Lcn2*$^{-/-}$) mice were kindly provided by Dr. Mak [22] and were backcrossed to *Ldlr*$^{-/-}$ mice to generate *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice. The animals were kept under standard laboratory conditions and were fed a normal chow diet and water *ad libitum*, unless otherwise stated.

Microarray on non-constrictive collar-induced carotid atherosclerosis

To determine *Lcn2* gene expression levels during atherosclerotic lesion development, RNA was extracted from atherosclerotic lesions as previously described [23]. In short, *Ldlr*$^{-/-}$ mice were fed a Western-type diet (WTD) (Special Diet Services) two weeks before surgery and throughout the experiment. To determine the gene expression levels in plaques, atherosclerotic carotid artery lesions were induced by perivascular collar placement as described previously [24]. Both common carotid arteries were excised, snap-frozen in liquid nitrogen and stored at −80°C until further use. Three carotid artery segments carrying carotid plaques from 2, 4, 6, 8 or 10 weeks after collar placement (t=2 until t=10) were pooled for each sample and homogenized by grounding in liquid nitrogen with a pestle [25, 26]. Carotid arteries without atherosclerosis from mice which were only fed a WTD for two weeks served as a control (t=0). Per time point we performed a microarray on three pooled samples.

An additional collar-induced atherosclerosis experiment with a similar design was performed in parallel to generate RNA samples for real-time quantitative PCR analysis as described below. In this setup we again pooled three carotids into one sample and generated the following pooled samples per time point: for t=0 (n=4), for t=2,4 or 6 (n=5) for t=8 (n=4) and for t=10 (n=3). In addition we also stored carotids from *Ldlr*$^{-/-}$ mice that had not been fed a Western type diet and these carotids were used to compare with the t=0 time point, which reflects carotids that were in a hyperlipidemic environment but did not contain atherosclerotic plaques. Total RNA was extracted from the tissue homogenates using Trizol reagent according to manufacturer's instructions (Invitrogen). Gene expression profiles were generated using the Illumina Bead-Chip Whole Genome Microarray platform (ServiceXS).

Atherosclerosis

Diet-induced atherosclerosis was established by feeding female *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice from 9-12 weeks of age a WTD containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services). Atherosclerosis was induced for either 6 or 12 weeks to study atherosclerotic lesions in an earlier or more advanced stage. At the end-point of the study, the mice were anesthetized by subcutaneous injections with ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL) after which their vascular system was perfused with PBS at a continuous low flow via heart puncture in the left ventricle. The hearts were collected to examine the atherosclerotic lesions in the aortic root through histological and morphometric analysis.

Histological and morphometric analysis of atherosclerotic lesions

All hearts were embedded in O.C.T. compound (Sakura) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, 10 µm sections were collected. Mean plaque size (in μm^2) was calculated from five sequential sections, displaying the highest plaque content, using an Oil-red-O staining (Sigma), which stains neutral lipids. Monocytes and macrophages were visualized using a Moma2 antibody (1:1000, Serotec) and an alkaline phosphatase conjugated secondary antibody (1:100, Sigma). To quantify smooth muscle cell content, smooth muscle cells were visualized by an α-smooth muscle cell actin (αSMA) staining (1:1000, Abcam) and a horseradish peroxidase conjugated secondary antibody. Collagen content inside the plaques was quantified using a Mason's Trichrome staining (Sigma). In the same sections, necrotic areas were identified as intimal a-cellular, debris-like areas. Neutrophils were stained for using the naphtol AS-D chloroacetate esterase staining kit (Sigma). Image quantification was performed blinded for genotype using the Leica Image analysis system (Leica Ltd). Apoptotic cells were stained using the In Situ Cell Death Detection Kit (Sigma) per the manufacturer's protocol. On the same sections,

macrophages were stained using a rat-anti-mouse F4/80 antibody (1:100, Biorad) and an Alexa Fluor 647 conjugated goat-anti-rat IgG as a secondary antibody (1:100, Thermo Fisher Scientific). Nuclei were visualized using Fluoroshield mounting medium with DAPI (Sigma). To quantify apoptosis, TUNEL positive nuclei were quantified inside atherosclerotic lesions. To quantify apoptotic macrophages, TUNEL positive nuclei which were colocalized with F4/80 staining were quantified. Quantification of apoptosis was performed using a Nikon TiE 2000 confocal microscope.

In situ zymography

Non-fixed hearts were snapfrozen on dry ice and stored until further use. 10 µm cryosections were acquired from the three-valve area and air dried for 1-2 hours before storage at -80°C. Cryosections were washed in reaction buffer (150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH=7.6) and incubated in reaction buffer containing 30 µg/mL DQ-Gelatin (Molecular probes) and 20 mM MMP2-inhibitor OA-Hycis-9-Octadeconoyl-N-hydroxylamide (Merck Millipore) overnight at 37°C. 1 mM 1,10-phenantrolin is an MMP inhibitor and thus served as a control. After incubation, the sections were washed in reaction buffer and subsequently incubated in 0.5% Chicago Sky Blue (Sigma) for 5 min. to dampen autofluorescence. Finally, the slides were mounted in Fluoroshield medium with DAPI (Sigma) and imaged using the Leica Image analysis system (Leica Ltd).

Real-time quantitative PCR

RNA was extracted from mechanically disrupted common carotid arteries or aortic arches by using Trizol reagent according to manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase per the instructions of the manufacturer (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to housekeeping genes (supplementary table 1).

Immunoblot

Atherosclerotic aortic arches were snapfrozen in liquid nitrogen and stored at -80°C until further use. For protein isolation, tissue samples were homogenized by mechanical disruption and lysed with 1xRIPA (Cell Signaling Technology) supplemented with cOmplete™ Protease Inhibitor Cocktail (Sigma) and 0.1% SDS for 1h on ice. Subsequently, the samples were centrifuged for 15 min. at 15,000 rpm at 4°C and the supernatant was harvested. The samples were diluted in NuPAGE™ LDS Sample Buffer (Thermo Fisher Scientific) prior to SDS-PAGE and subsequent transfer to a nitrocellulose membrane. Proteins were detected using rabbit-anti-mouse MMP-9 (1:1000, Abcam) and rabbit-anti-mouse β-actin (1:1000, Novus Biologicals) antibodies.

Flow cytometry

Blood samples and spleens were collected upon sacrifice. Erythrocytes were subsequently lysed using ACK lysis buffer to prepare the leukocyte fraction for staining of surface markers. For analysis of surface markers, cells were stained at 4°C for 30 min. in PBS containing 2% (vol/vol) fetal bovine serum (FBS) with antibodies from eBioscience and BD Biosciences (supplementary table 2). Flow cytometric analysis was performed on a FACSCantoII (BD Biosciences) and data was analyzed using Flowjo software (TreeStar).

Serum analysis

For measurement of blood glucose levels, mice were fasted for 4 hours prior to blood collection. Blood samples were taken from the tail vein and directly applied to an Accu-Check glucometer (Roche Diagnostics). The levels of serum CCL2, insulin and mouse Amyloid A were determined by ELISA according to manufacturer's protocol (CCL2; BD Biosciences, insulin and mouse Amyloid A; Thermo Fisher Scientific). Concentration of total cholesterol in the serum of unfasted mice was determined by an enzymatic colorimetric assay (Roche Dagnostics). Concentration of triglycerides in the serum was determined by an enzymatic colorimetric assay. Precipath (standardized serum, Roche Diagnostics) was used as an internal standard in the measurements for cholesterol and triglycerides.

Statistical analysis

Data are expressed as mean ± SEM. Datasets were examined for outliers using the ROUT method in GraphPad Prism version 7.00. A two-tailed Student's T-test was used to compare individual groups with Gaussian distributed data. Non-parametric data was analyzed using a Mann-Whitney U-test. Data from three or more groups were analyzed using a one-way ANOVA whereas data from three groups with more than one variable were analyzed by a two-way ANOVA, both with a subsequent Sidak multiple comparison test. A p-value below 0.05 was considered significant.

RESULTS

Lcn2 mRNA expression during collar-induced atherosclerosis

To confirm that Lcn2 expression is increased in atherosclerotic carotid arteries as compared to healthy non-atherosclerotic carotid arteries, we assessed *Lcn2* expression levels by performing a microarray on collar-induced atherosclerotic lesions from Western type diet (WTD, 0.25% cholesterol and 15% cacao butter) fed *Ldlr*$^{-/-}$ mice at different time points after collar placement. Herein, carotid arteries from mice which were fed a WTD for 2 weeks but without collar-induced atherosclerosis served as a control (t=0). *Lcn2*

expression is sharply increased two weeks (t2) after collar placement when compared to unaffected carotid arteries (figure 1A). Although not as pronounced, *Lcn2* expression remained elevated as plaque development progresses four to ten weeks after collar placement (figure 1A). In the same experiment, microarray analysis revealed that the expression of the gene encoding the Lcn2 receptor *24p3r* was unaltered during atherosclerosis (figure 1B). RT-qPCR analysis of *Lcn2* expression in atherosclerotic carotid arteries from an additional collar-induced atherosclerosis experiment in *Ldlr*$^{-/-}$ mice confirmed that Lcn2 expression was elevated during collar-induced atherosclerosis especially after 6, 8 and 10 weeks (figure 1C).

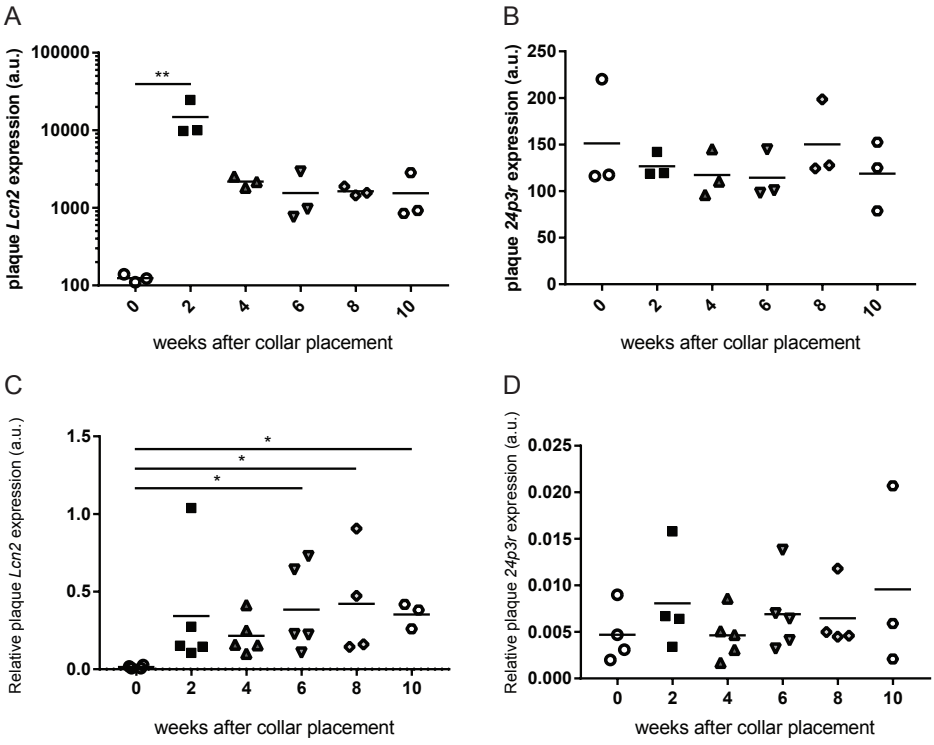

Figure 1 *Lcn2*, but not *24p3r*, mRNA expression was increased in collar-induced atherosclerosis in *Ldlr*$^{-/-}$ mice. (A) A microarray on carotid arteries of *Ldlr*$^{-/-}$ mice without (t=0) or with collar-induced atherosclerosis showed *Lcn2* expression is significantly upregulated 2 weeks after collar placement. Hereafter, it remained elevated from 4 until 10 weeks after collar placement, albeit less pronounced. (B) In the same microarray as in (A), expression of the Lcn2 receptor *24p3r* was unaltered during plaque development. (C) In a parallel collar-induced atherosclerosis experiment, we performed RT-qPCR analysis of *Lcn2* expression in the carotid arteries of mice and *Lcn2* expression was confirmed to be elevated during atherosclerosis. (D) In accordance to (B), RT-qPCR analysis revealed that *24p3r* expression was unaltered during plaque development. n=3 per time point for A-B and n=3-5 for C-D. (*p<0.05, **p<0.01).

Importantly, RT-qPCR analysis of *Lcn2* expression in carotid arteries from *Ldlr*$^{-/-}$ mice which were fed a normal chow diet or a WTD showed that a WTD alone did not induce *Lcn2* expression (median±interquartile range; NCD: 0.0067±0.009 vs WTD: 0.013±0.019, p=0.35). *24p3r* expression was also measured using RT-qPCR and confirmed to be unaltered during atherosclerosis (figure 1D). Thus, atherosclerotic carotid arteries displayed higher levels of *Lcn2* expression as compared to non-atherosclerotic carotid arteries.

Metabolic health status of *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice

To examine the effects of Lcn2 deficiency on atherosclerosis, *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were fed a WTD for 6 or 12 weeks. As Lcn2 is associated with the development of metabolic diseases which can contribute to atherosclerosis (e.g. obesity) various parameters of obesity and insulin resistance were examined. During the experiment, Lcn2 deficiency resulted in a lower body weight after 7 weeks of WTD but otherwise did not result in differences in body weight (figure 2A). Accordingly, inguinal white adipose tissue (iWAT) weight did not differ significantly when comparing *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice to *Ldlr*$^{-/-}$ mice after 6 or 12 weeks of feeding WTD (figure 2B). Total cholesterol levels in serum after 3, 6 or 12 weeks of WTD showed no differences between *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice (figure 2C). Triglyceride levels in serum after 3, 6 or 12 weeks were also equal between *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice (figure S1A). Fasting glucose levels were also similar between the groups at these time points (figure 2D). In line, insulin levels were similar between both groups after both 6 and 12 weeks of WTD feeding (figure S1B).

Atherosclerotic plaque formation in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice

Lcn2 expression in atherosclerotic plaques of aortic arches was increased after 12 weeks WTD compared to 6 weeks WTD in the *Ldlr*$^{-/-}$ group (figure 3A). After 6 weeks of WTD feeding, *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice developed ~20% larger plaques (figure 3B). The intraplaque macrophage content as measured by MOMA2 staining was not significantly altered (figure 3C). Total neutrophil numbers in the plaque and adventitia were not significantly different between both groups (figure 3D). As compared to *Ldlr*$^{-/-}$ mice, *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice had higher intraplaque smooth muscle cell content as measured by αSMA staining (figure 3E). The collagen content in the plaques was equal between the two groups after 6 weeks of WTD (figure 3F). Interestingly, as opposed to the effect on plaque size after 6 weeks of diet feeding, plaque size after prolonged diet feeding was similar in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ and *Ldlr*$^{-/-}$ mice after 12 weeks of WTD (figure 3G). Plaques of *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ did not differ significantly from *Ldlr*$^{-/-}$ mice in terms of MOMA2 staining (figure S2A), total neutrophil numbers (figure S2B), or intraplaque smooth muscle cell percentage (figure S2C) after 12 weeks of WTD. However, a reduction in acellular, necrotic area was observed in advanced atherosclerotic lesions from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice (figure 3G), which was not the case for atherosclerotic plaques after 6 weeks of WTD feeding (figure S2D). The collagen

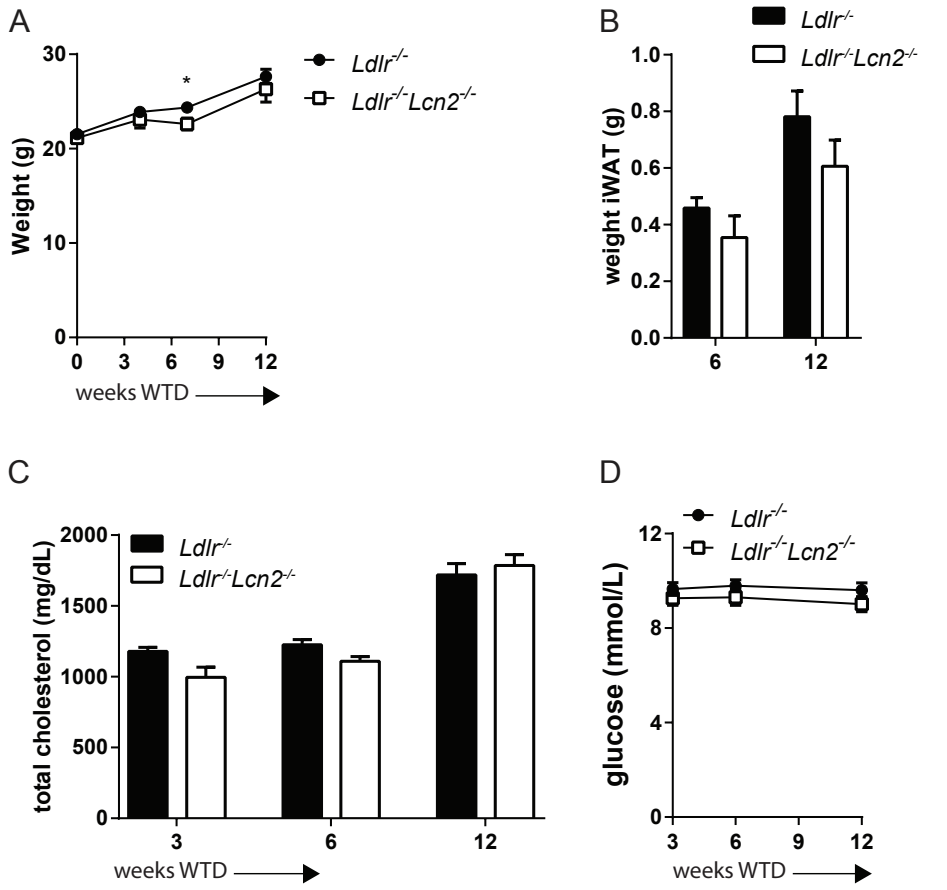

Figure 2 *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice were metabolically similar to *Ldlr*$^{-/-}$ mice (A) *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice showed a significantly higher body weight as compared to *Ldlr*$^{-/-}$ mice after seven weeks of WTD. Otherwise, no differences in weight were observed at different time points (B) In line with this, inguinal white adipose tissue (iWAT) was equal between both groups. (C) Total cholesterol levels in the serum were unaltered between both groups. (D) Fasting glucose was measured over time. No significant changes were observed between the *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ group and its *Ldlr*$^{-/-}$ controls. n=10-12 per group. All values are depicted as mean±SEM. (*p<0.05).

content in advanced lesions was unaltered when comparing lesions from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ to *Ldlr*$^{-/-}$ mice (figure 3H). The percentage of plaque (plaque area/lumen area*100%) was not significantly higher in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice although it did show a trend towards being higher after 6 (figure S2E) and 12 weeks of WTD (figure S2F), suggesting outward remodeling occurred as a response to atherosclerosis formation.

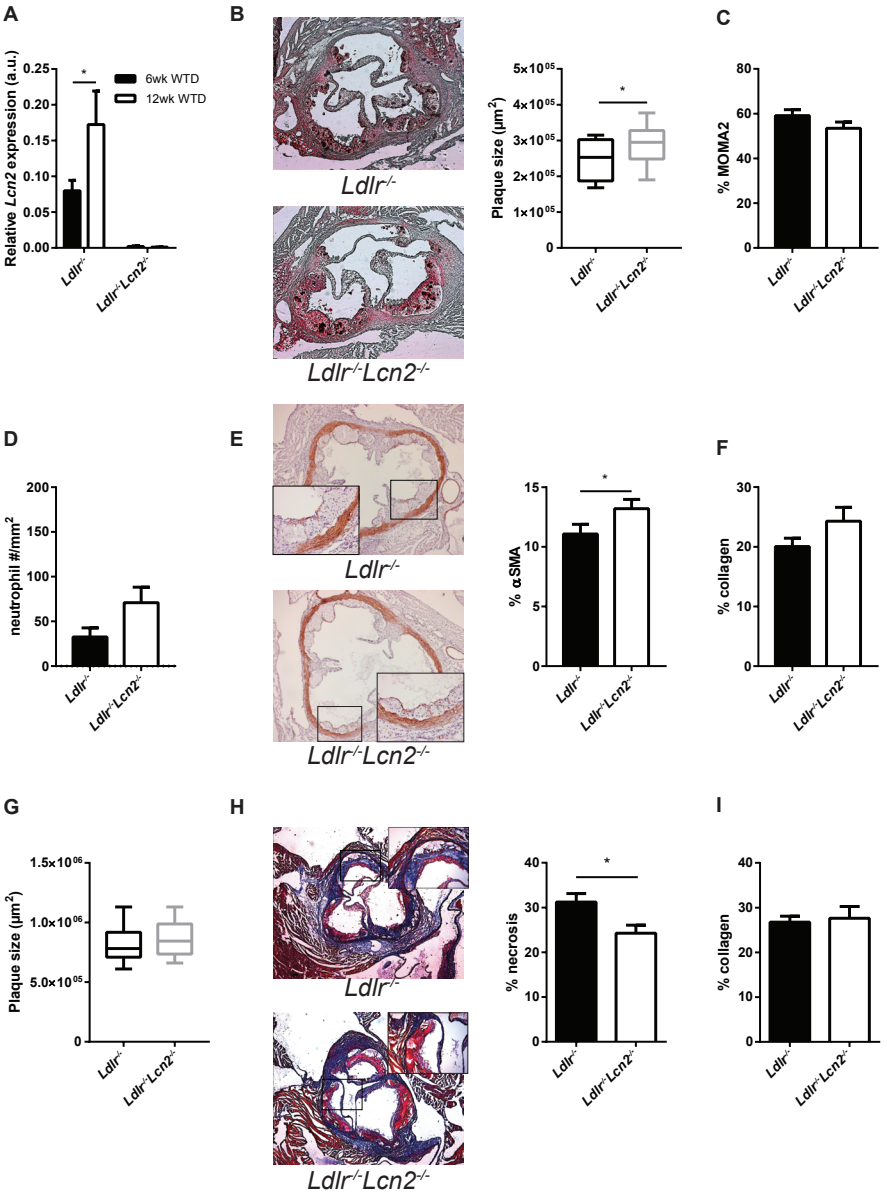

Figure 3 Lcn2 deficiency affected atherosclerosis. (A) *Lcn2* expression in atherosclerotic plaques of aortic arches was increased after 12 weeks WTD compared to 6 weeks WTD in the *Ldlr*$^{-/-}$ group. (B) Lcn2 deficiency resulted in a significant increase in plaque size after 6 weeks WTD. (C) Macrophage content as a percentage of total plaque area measured by MOMA2 staining was unaltered. (D) Neutrophil numbers in the plaque and adventitia showed no difference between both groups. (E) Smooth muscle cell percentage within the lesions was higher in the *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ group as compared to the controls. (F) Collagen content was equal between both groups. (G) Plaque size of advanced lesions was not significantly different. (H) Necrotic core size was significantly decreased in advanced lesions. (I) Collagen content in advanced lesions was equal between both groups. n=6 per group for (A). n=12 for (B-H). All values are depicted as mean±SEM (*p<0.05).

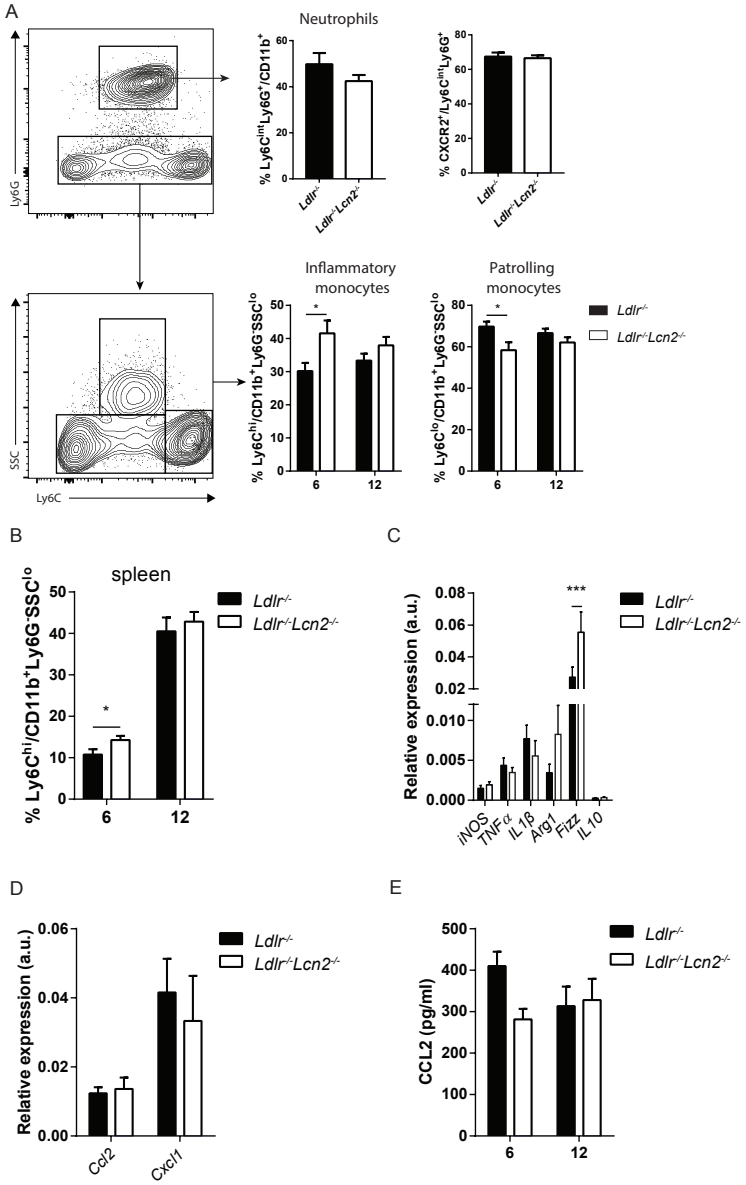

Figure 4 Analysis of myeloid cell populations. (A) The gating strategies for neutrophils, eosinophils and monocytes are depicted. Circulating neutrophil and eosinophil abundance was unaltered. Neutrophils had similar expression levels of the homing receptor CXCR2. Inflammatory monocytes were increased in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice after 6 weeks of WTD with concomitant decreases in patrolling monocytes. (B) The splenic inflammatory monocyte population showed a similar increase in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice. (C) mRNA expression of the M2 macrophage marker Fizz was elevated in the atherosclerotic arch of *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice as compared to *Ldlr*$^{-/-}$ mice (D) mRNA expression of CCL2 and CXCL1 were unaltered in the atherosclerotic aortic arch. (E) No significant differences were observed in serum CCL2 levels between both groups. n=6 per group. All values are depicted as mean±SEM (*p<0.05, **p<0.01, ***p<0.001).

Effects of Lcn2 deficiency on the myeloid cell populations during atherosclerosis

To establish whether Lcn2 deficiency has any effects on the myeloid cell populations during atherosclerosis, flow cytometry on blood and spleen was performed. In line with our observation that neutrophil numbers were unaltered in the lesions of *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice, no significant changes were observed in circulating neutrophils after 6 weeks of WTD, neither in percentages nor in the C-X-C chemokine receptor type 2 (CXCR2)$^{+}$ subset (figure 4A). No significant changes in percentage of eosinophils, as defined as CD11b^{+}Ly6G^{-}Ly6C^{int}SSC^{hi} cells, between both groups was observed (figure S3A). The same results were obtained after 12 weeks of WTD (figure S3B). Interestingly, a higher percentage of circulating Ly6C^{hi} inflammatory monocytes was measured after 6 weeks of WTD. Inflammatory monocytes followed a similar trend after 12 weeks of WTD. Ly6C^{lo} patrolling monocytes were decreased accordingly after 6 weeks, although this did not reach significance after 12 weeks of WTD (figure 4A). No significant changes in myeloid cell or lymphocyte populations were observed in the bone marrow compartment or draining lymph node of the aortic root (data not shown). Together, these data indicate that the circulating monocytes are skewed towards a pro-inflammatory phenotype in the *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice. Although not as pronounced as in the blood, splenic monocytes were significantly elevated as well after 6 weeks WTD (figure 4B). Interestingly, serum amyloid A levels in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice were higher as compared to *Ldlr*$^{-/-}$ mice after 6 and 12 weeks of WTD (figure S3C) indicating that Lcn2 deficiency alters the inflammatory status during atherogenesis. To examine whether a relative increase in inflammatory monocytes resulted in an increase in M1-like macrophages in the plaque the mRNA expression of M1 markers (*iNOS, TNFα, IL1β*) and M2 markers (*Arg1, Fizz, IL10*) was measured. Lcn2 deficiency had no effect on the expression of *iNOS*, *TNFα* and *IL1β*. Of the M2 markers, only *Fizz* was elevated in the atherosclerotic aortic arch after 12 weeks of WTD (figure 4C). Similarly, after 6 weeks of WTD, Lcn2 deficiency resulted in increased *Fizz* expression in the aortic arch while other M1 and M2 macrophage markers were unaltered (figure S3D). Expression of *CCL2*, a well-recognized chemoattractant for inflammatory monocytes which is also known as MCP-1, was measured in atherosclerotic aortic arches to examine whether increased monocyte recruitment was induced by local expression of CCL2. The expression of CXCL1 (the mouse homologue of IL-8), an important chemoattractant for CXCR2^{+} neutrophils, was measured to confirm that neutrophil recruitment to atherosclerotic lesions was unaltered in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice. mRNA expression of *CCL2* and *CXCL1* were unaltered in the atherosclerotic aortic arch (figure 4D). No significant changes were observed in CCL2 levels in the serum when comparing *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice to *Ldlr*$^{-/-}$ at the 6 or 12 week time points (figure 4E).

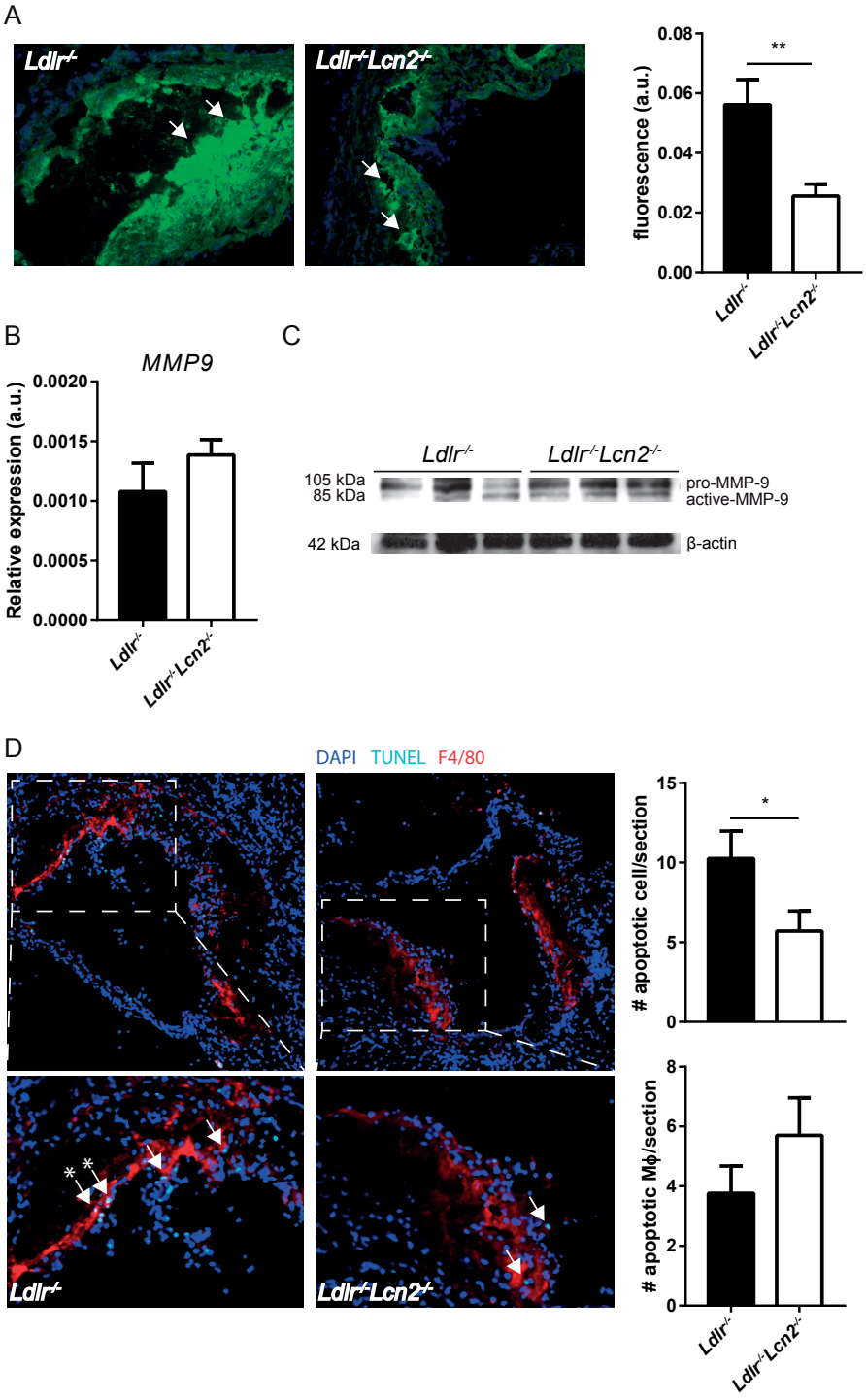
A
Ldlr-/-
Ldlr-/-Lcn2-/-
fluorescence (a.u.)
0.08
0.06
0.04
0.02
0.00
**
Ldlr-/-
Ldlr-/-Lcn2-/-
B
MMP9
Relative expression (a.u.)
0.0020
0.0015
0.0010
0.0005
0.0000
Ldlr-/-
Ldlr-/-Lcn2-/-
C
Ldlr-/-
Ldlr-/-Lcn2-/-
105 kDa
85 kDa
pro-MMP-9
active-MMP-9
42 kDa
β-actin
D
DAPI TUNEL F4/80
Ldlr-/-
Ldlr-/-Lcn2-/-
apoptotic cell/section
15
10
5
0
*
apoptotic Mφ/section
8
6
4
2
0
Ldlr-/-
Ldlr-/-Lcn2-/-

Figure 5 Assessment of MMP-9 activity in atherosclerotic plaques. (A) MMP-9 activity in the aortic root as measured by an in situ zymography was decreased in mice deficient for Lcn2. (B) MMP-9 gene expression in atherosclerotic lesions of the aortic arch was equal between both groups. (C) Similarly, protein expression of MMP-9 was similar between both groups. (D) Lesions from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice contained fewer apoptotic cells per section but not fewer apoptotic macrophages (MΦ) as compared to lesions from *Ldlr*$^{-/-}$ mice. In (A), gelatinolytic activity is visualized in green and nuclei are stained in dark blue. The white arrows indicate regions with high enzymatic activity. In (D), TUNEL positive nuclei of apoptotic cells are depicted in light blue (indicated with a white arrow) and TUNEL positive nuclei of apoptotic macrophages are depicted in white (indicated with a white arrow and asterisk). Macrophage positive area is depicted in red and nuclei in dark blue. The data in (A) is representative of two experiments. n=10 for figure 5A and 5C. n=6 for figure 5B. All values are depicted as mean±SEM (*p<0.05, **p<0.01).

MMP-9 activity in the aortic root

As a decreased necrotic core size in advanced atherosclerosis could be due to decreased intraplaque proteolytic activity an *in situ* zymography was performed to measure MMP-9 activity in atherosclerotic lesions in the aortic root. An MMP-2 inhibitor was added in the reaction mix to ensure that DQ-gelatin degradation was mainly MMP-9 mediated although some residual signal from other MMPs with gelatinolytic activity may be present. A significant decrease in gelatinolytic activity, a measure for MMP activity, was observed in the *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ group compared to the control group (Fig 5A). *MMP-9* mRNA expression in the atherosclerotic aortic arch was not significantly different in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice compared to controls, suggesting that the effects are primarily caused by MMP-9 protein stabilization in the *Ldlr*$^{-/-}$ mice (figure 5B). Immunoblot analysis also showed that MMP-9 protein expression was equal between *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice (figure 5C) and quantification of the blot confirmed this (figure S4A). Furthermore, the ratio between the pro- and active form of the MMP-9 protein was unaltered between both genotypes (figure S4B). Interestingly, no differences in *MMP-2* mRNA expression (figure S4C) or *MMP-14* mRNA expression (figure S4D) in the atherosclerotic aortic arches were observed when comparing *Ldlr*$^{-/-}$ mice to *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$. In line with a decreased necrotic core size, lesions from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice contained fewer apoptotic cells. The number of apoptotic macrophages was equal between both genotypes (figure 5D).

DISCUSSION

Several studies reported that the detection of unstable atherosclerotic plaques using Lcn2 could prove useful in the future to reduce the incidence of acute cardiovascular syndromes, however the direct contribution of Lcn2 to atherosclerotic plaque progression remained up to date uninvestigated.

Microarray analysis of non-constrictive collar-induced atherosclerosis showed that local *Lcn2* expression increased during early stages of atherosclerotic lesion development, and remained elevated during plaque progression. As expression of the Lcn2-receptor *24p3r* was unaltered, local Lcn2 effects in atherosclerotic plaques were mainly regulated by regulating the expression of the protein itself or by regulating the influx of myeloid cells expressing Lcn2, such as neutrophils and monocytes. Based on these findings we studied the effect of Lcn2 deficiency on different stages of atherosclerosis and related this to changes in myeloid cell populations. After 6 weeks of WTD, lesions from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ were somewhat larger and had a higher smooth muscle cell percentage as compared to those of *Ldlr*$^{-/-}$ control mice, but plaque composition remained otherwise unaltered. Interestingly, Lcn2 deficiency did not affect advanced lesion size but did display a decrease in necrotic area.

The effects observed on plaque size could not be explained by increased hepatic steatosis and resulting increases in circulating cholesterol levels. A study by Ye et al. described Lcn2 to exert pro-inflammatory effects in a high fat, high cholesterol diet induced model of non-alcoholic steatohepatitis (NASH) [27]. Lcn2 deficiency in mice attenuated the transition of hepatic steatosis to NASH whereas administration of recombinant Lcn2 exacerbated disease in a neutrophil-dependent manner. In the present study, *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice showed no differences in total serum cholesterol or circulating neutrophils which makes it unlikely that hepatic steatosis contributed to the observed lesion effects. *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice were fed a WTD to study atherosclerosis, while a high fat high cholesterol diet was used to study NASH. Presumably, the fact that this diet contains 40% fat and was fed to the mice for 12 to 20 weeks to induce NASH explains why Lcn2 deficiency did not affect the development of hepatic steatosis in the present study.

In this study, Lcn2 deficiency had no effects on body weight or iWAT weight. Feeding a high fat diet to C57BL/6 mice has previously been shown to increase Lcn2 expression by adipocytes and to increase its abundance in serum [28]. These increases are even more pronounced in iWAT compared to epididymal WAT [29]. Furthermore, Lcn2 expression by adipocytes is induced by various pro-inflammatory cytokines which are known to play a role in obesity development, including TNFα, IL1-β and IL-6 [30]. Interestingly, Lcn2 deficient mice which are fed a high fat diet gain more weight compared to wildtype mice [29]. These effects were not observed in our study, possibly due to the fact that our study used female mice whereas most studies on the role of Lcn2 in obesity were performed

in male mice. Additionally, feeding a WTD for 6 or 12 weeks might not have induced a severe enough obese phenotype to observe differences between Lcn2 wildtype or Lcn2 deficient mice. LDLr deficient mice develop hyperglycemia and insulin resistance after approximately 20 weeks of feeding them a WTD [31] and Lcn2 has been shown to affect insulin resistance [19]. The fact that fasting glucose levels were equal between *Ldlr*$^{-/-}$and *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice suggest that in the present study the time frame was too limited to induce obesity and insulin resistance. As the metabolic parameters we examined did not differ between both genotypes it is improbable that atherosclerosis was affected by altered metabolic disease development in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice.

After 6 weeks of WTD, lesions in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice were slightly larger compared to *Ldlr*$^{-/-}$ control mice. This finding was rather counterintuitive as Lcn2 is generally considered to be secreted under and promote inflammatory conditions [19]. Interestingly, there are actually also multiple studies describing Lcn2 to be protective in a number of inflammatory diseases. Lcn2 deficient mice have for example increased liver damage in various models for liver injury with concomitant increases in expression of pro-inflammatory factors such as *CCL2, TNFα* and a decrease in *IL-10* expression [32]. Additionally, Lcn2 appears to play a protective role in experimental autoimmune encephalomyelitis pathogenesis as Lcn2 deficient mice have higher lesion burden and increased expression of *IFNγ* and *TNFα* inside lesions [33]. In our study, Lcn2 deficiency did not affect the expression of *TNFα*, *CCL2* in the atherosclerotic aortic arches, suggesting Lcn2 exerts a protective function in atherogenesis which is distinct from other diseases. Upon Lcn2 deficiency, circulating monocytes were skewed towards a more inflammatory phenotype, which may have driven lesion progression, considering the atherogenic effects inflammatory monocytes have on early lesion development [34]. No differences were however observed in macrophage content of the plaques. When plaques from the aortic arch were analyzed for different M1 and M2 macrophage marker expression, it was shown that only the M2 marker *Fizz* was elevated in Lcn2 deficient mice. The phenotypic effects that Lcn2 has on macrophages appear to be condition-specific as different studies have reported treatment of bone marrow-derived macrophages (BMDM) with Lcn2 to increase expression of M1 markers [35] but also IL-10 production [36]. Furthermore, BMDM from *Lcn2*$^{-/-}$ mice showed a more profound upregulation of *IL-1β* and *iNOS* after lipopolysaccharide stimulation compared to wildtype controls [37] whereas infection of Lcn2 deficient peritoneal macrophages with *Salmonella Typhimurium* was reported to result in increased *IL-10* expression as compared to wildtype controls [38]. In the presented study, as Lcn2 can induce foam cell formation in BMDM by inducing the expression of scavenger receptors [35], the absence of Lcn2 might have compensated for increased monocyte homing towards atherosclerotic lesions. It is unclear what causes the increase in monocyte activation. Lcn2 is known to interact with LTB4, which acts as a chemokine for monocytes during development of atherosclerosis [39], but it is unknown what functional consequences this interaction has [4].

The absence of Lcn2 may have increased the potency of LTB4 to activate monocytes, however future studies should shed more light on exact mechanisms involved in Lcn2 mediated monocyte differentiation and migration.

Based on the results, Lcn2 did not function as a chemoattractant for neutrophils to home towards atherosclerotic lesions in this study as neither the number of neutrophils inside the plaques nor the circulating neutrophil percentages were changed. Lcn2 has been shown to serve as a chemoattractant for neutrophils during acute inflammation [5] and during development of NASH by increasing CXCR2-dependent homing towards the liver [27]. Lcn2 deficiency had no effect on *CXCL1* expression in atherosclerotic aortic arches further suggesting Lcn2 deficiency did not alter neutrophil recruitment to atherosclerotic plaques in this study. As compared to Lcn2 levels during acute inflammation, the levels of circulating and local Lcn2 during atherogenesis might not be high enough for Lcn2 deficiency to alter neutrophil recruitment, possibly explaining why Lcn2 deficiency during experimental atherosclerosis had no effect on neutrophil migration.

In advanced atherosclerotic plaques, *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice did show a decreased necrotic core area inside lesions of the aortic root, suggesting that Lcn2 deficiency affected matrix degradation. This was most likely due to the decreased local MMP-9 activity in the Lcn2 deficient mice as measured by zymography. Lcn2 binds to MMP-9 to prevent its inhibition by tissue inhibitor of metalloproteinases [6], leading to more active MMP-9. Proteolytic activity has been shown to induce the apoptosis of macrophages and smooth muscle cells and inhibition of proteolytic activity can decrease necrotic core size accordingly [40-42]. As lesions from Lcn2 deficient mice contained fewer apoptotic cells, a decreased necrotic core size in lesions from Lcn2 deficient mice could be due to a decrease in MMP activity and subsequent proteolysis-induced apoptosis. Previously, Lcn2 and MMP-9 have been shown to colocalize in atherosclerotic lesions [18]. As MMP9 expression inside atherosclerotic lesions of the aortic arch was equal between the *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ and control groups, the decrease in MMP-9 activity in the Lcn2 deficient mice was unlikely due to decreased MMP-9 abundance, but mainly caused by decreased MMP-9 activity. These findings are in line with literature describing unstable plaques to have higher levels of NGAL than stable plaques [17] and support serum NGAL/MMP-9 complex levels to predict MACE 1 year after follow up [13]. The present study provides evidence that local Lcn2 in advanced stages of atherosclerosis might indeed decrease lesion stability. Whether Lcn2 actually affects lesion stability in advanced atherosclerosis or it only modulates MMP-9 activity and necrotic core size remains to be determined. Nevertheless, our findings suggest that lowering of NGAL levels through therapeutic intervention might be feasible to increase plaque stability in patients with unstable atherosclerosis as the presence of NGAL in advanced atherosclerosis appears to contribute to plaque instability. Interestingly, in patients with carotid artery atherosclerosis, serum NGAL levels were lower in patients on statins as compared to patients without. Also in patients with vulnerable lesions, statin-

treated patients had lower levels of serum NGAL compared to patients without statins [15]. Another study in patients which underwent carotid endarterectomy as a treatment for advanced atherosclerosis showed that serum NGAL levels were equal between patients with or without statin treatment [43]. This suggests that statin treatment can decrease circulating NGAL levels but that this may depend on the patient group which is targeted. There are some limitations to this study. For example, plaque size was determined in the aortic root but not at other sites of lesion development. Another limitation is that we did not perform an oral glucose tolerance test to examine the effects Lcn2 deficiency has on the metabolic health status in these experiments. As this is a highly relevant parameter in assessing the metabolic syndrome-like phenotype diet-induced dyslipidemia can induce, it is not possible to definitely conclude that Lcn2 deficiency had no effects on the metabolic status in our experiments. However, as the total body weight, iWAT weight, fasting glucose and insulin levels in the serum were unaltered between groups it is highly unlikely that Lcn2 deficiency had any effect on the metabolic health status in these experiments. Lastly, in our zymography, other MMPs besides MMP-9 which can degrade gelatin might have caused some background signal. However, areas with high gelatinolytic activity have been shown to colocalize with MMP-9 [18], indicating the gelatinolytic activity which we measured was mainly MMP-9 derived.

In conclusion, Lcn2 deficiency was shown to increase plaque size during earlier stages of lesion development, possibly due to an increase in inflammatory monocytes and without affecting neutrophil recruitment. In addition, this study showed that Lcn2 deficiency decreased the local degree of MMP-9 activity thereby possibly contributing to more stable atherosclerotic plaques. Further studies are required to further examine the effects of Lcn2 on myeloid cell populations during atherogenesis as this might further improve the interpretation of changes in circulating Lcn2 levels.

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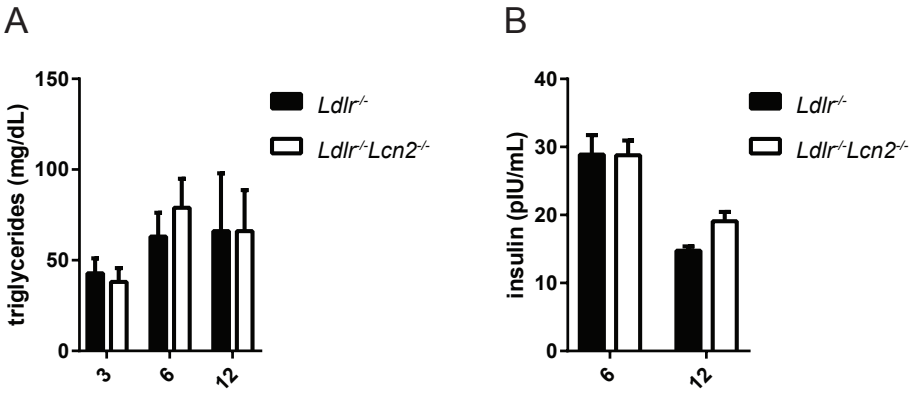
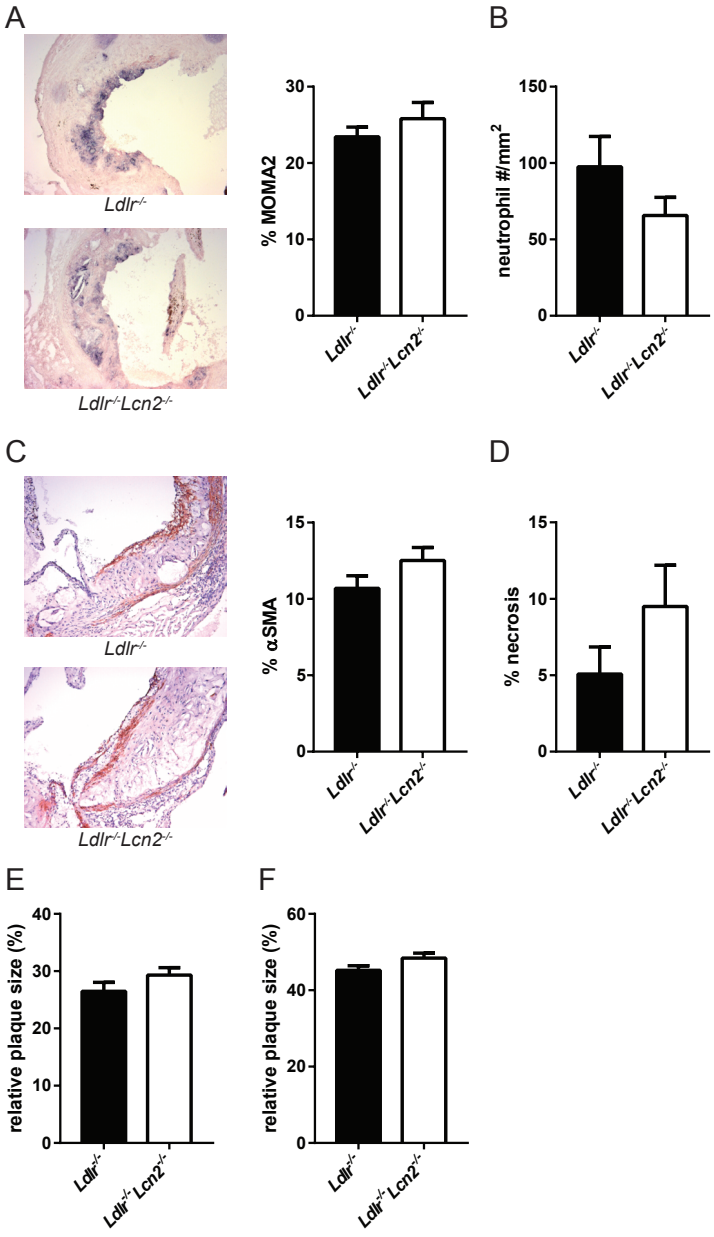
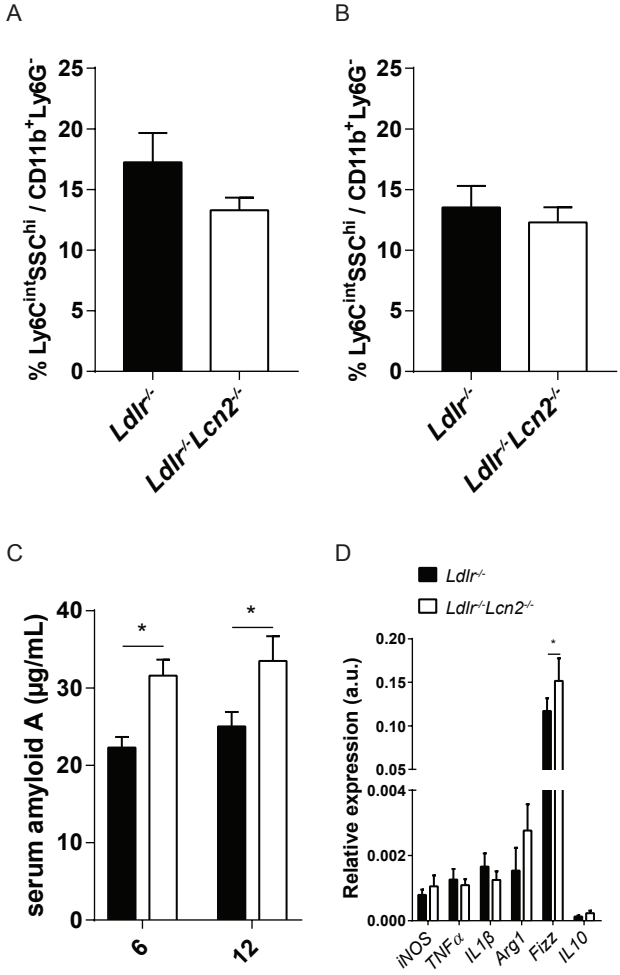
SUPPLEMENTARY FIGURES



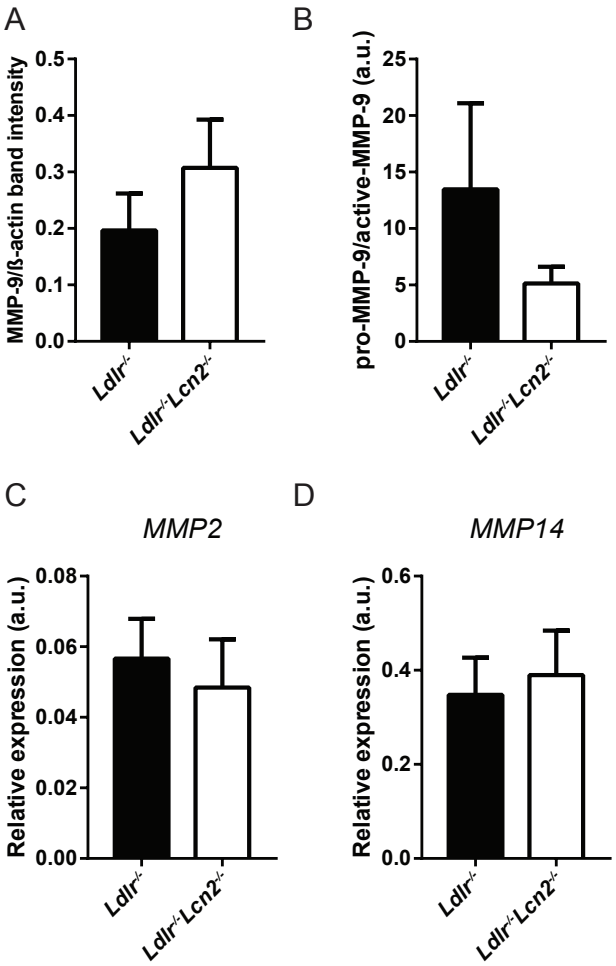
Supplementary figure 1 Triglyceride and insulin concentrations in serum. (A) The levels of triglycerides did not differ between both genotypes after 3, 6 or 12 weeks of WTD. (B) The insulin levels were equal in *Ldlr*$^{-/-}$ mice as compared to *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice after 6 and 12 weeks of WTD. n=10-12 per group.



Supplementary figure 2 Plaque composition in 6 weeks and 12 weeks studies (A) Macrophage content as measured by a MOMA2 staining did not differ between groups in advanced lesions. (B) Similarly, total neutrophil numbers was equal between both groups. (C) Also the percentage of smooth muscle cell area inside advanced lesions of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ was equal between groups. (D) After 6 weeks of WTD, the necrotic core area inside the lesions was equal between both groups. (E) Relative plaque size after 6 weeks WTD was unaltered between both groups. (F) Similarly, after 12 weeks of WTD, relative plaque size in *Ldlr*$^{-/-}$ *Lcn2*$^{-/-}$ mice was equal to *Ldlr*$^{-/-}$ mice. n=12 per group.



Supplementary figure 3 Assessment of the inflammatory status. (A) No significant changes after 6 weeks of WTD feeding in percentage of eosinophils, as defined as $CD11b^{+}Ly6G^{-}Ly6C^{int}SSC^{hi}$ cells, between both groups was observed. (B) Also after 12 weeks of WTD, no changes were observed in the percentages of eosinophils in the blood. (C) The levels of mouse amyloid A in the serum was higher in *dlr*$^{-/-}$*Lcn2*$^{-/-}$ mice as compared to *Ldlr*$^{-/-}$ mice after both 6 and 12 weeks of WTD. (D) After 6 weeks of WTD, analysis of the expression of M1 and M2 markers in atherosclerotic aortic arches revealed that *Fizz* expression was elevated in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice. n=6 per group for A, B and D. n=12 for C.



Supplementary Figure 4 MMP expression in atherosclerotic aortic arches. (A) After normalization of MMP-9 protein expression to β-actin protein expression, MMP-9 expression in atherosclerotic aortic arches was equal between *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. (B) The ratio between the pro- and active form of the MMP-9 protein was unaltered between *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice. (C) Gene expression of MMP2 in atherosclerotic aortic arches was unaltered in *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice as compared to *Ldlr*$^{-/-}$ mice. (D) Likewise, MMP14 gene expression was equal between both genotypes. n=3 for A-B. n=6 for C and D.

SUPPLEMENTARY TABLES

Supplementary table 1 List of primers used for qPCR expression analysis. Expression of genes were normalized to housekeeping genes *Rpl37* and *Rpl27*.

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
Lcn2	GAACGTTTCACCCGCTTTGCCAAG	GGCAACAGGAAAGATGGAGTGGCAG
24p3r	ctcaatgactctcacggggattgca	aggagaagaggccaaggacagagaa
Arg1	TGGCAGAGGTCCAGAAGAATGG	GTGAGCATCCACCCAAATGACAC
iNOS	TCTGCAGCACTTGGATCAGGAACCT	AGAAACTTCGGAAGGGAGCAATGCC
Fizz	GCCAATCCAGCTAACTATCCCTCCA	CAAGATCCACAGGCAAAGCCACAA
TNFα	GCCTCTTCTCATTCCTGCTTGTG	ATGATCTGAGTGTGAGGGTCTGG
CCL2	CTGAAGCCAGCTCTCTCTTCCTC	GGTGAATGAGTAGCAGCAGGTGA
IL1β	AACGACAAAATACCTGTGGCCTTG	CCGTTTTTCCATCTTCTTCTTTGGGT
MMP2	GATAACCTGGATGCCGTCGTGGA	ACTTCACGCTCTTGAGACTTTGGTTCT
MMP9	CCCTGGAACTCACACGACATCTTC	CTCATTTTGGAAACTCACACGCCAG
MMP14	GGGGTCATTCATGGGCAGTGATGA	GATGATCACCTCCGTCTCCTCCTCA
Rpl37	AGAGACGAAACACTACCGGGACTGG	CTTGGGTTTCGGCGTTGTTCCCTC
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG

Supplementary table 2 List of antibodies used for flow cytometry analysis.

Antigen	Fluorochrome	Clone	Manufacterer
CD11b	eFluor 450	M1/70	Ebioscience
Ly6G	FITC	1A8	BD Biosciences
Ly6C	APC	HK1.4	Ebioscience
CXCR2	PE	242216	R&D systems

CHAPTER 7

General discussion and perspectives

BACKGROUND

Atherosclerosis is the main underlying pathology of cardiovascular disease (CVD), which is the most common cause of death worldwide [1]. Atherosclerosis is a lipid-driven autoimmune-like disease in which stenotic lesions develop as a result of local accumulation of native and modified lipoproteins. These lipoproteins elicit a chronic inflammatory response which is largely T cell-mediated [2–5]. Hence, dyslipidemia, in the form of hypercholesterolemia and/or hypertriglyceridemia, is a major risk factor of developing atherosclerosis and subsequent acute cardiovascular events such as a myocardial infarction or stroke. Surgical treatments to restore blood flow after such an ischemic episode include bypass surgery, endarterectomy or percutaneous coronary intervention to circumvent or remove stenotic lesions. However, surgical treatment of perfusion defects to prevent mortality is invasive and often associated with recurrent CVD. Patients at risk of developing CVD, such as familial hypercholesterolemia (FH) patients, or with a history of CVD are often treated with lipid lowering therapies. The current standard of lipid lowering therapy is based on statins which have proven to effectively lower the amount of circulating low density lipoprotein (LDL) cholesterol and thereby reduce the risk of developing CVD [6]. Unfortunately, statins only lower the risk of CVD by 25-30% and patients with a history of acute coronary events have a 20% higher chance of a recurrent cardiovascular event, despite treatment [7]. Novel therapeutic approaches are required to treat atherosclerosis and prevent acute cardiovascular events. Given the contribution of immune cells to the pathophysiology of atherosclerosis [8], experimental research has focused on developing immunomodulatory therapies to treat atherosclerosis. Immunological research in atherosclerosis has shown that dyslipidemia drives T cell-mediated immunity in atherosclerosis by increasing the abundance of antigens derived from native and modified lipoproteins [9]. Long-lasting immunomodulation to treat atherosclerosis through vaccination is successful in mice using peptide sequences from ApoB100, the core-protein of LDL and very low density lipoprotein [10,11]. Thus, the antigen-dependent contribution of dyslipidemia to T cell mediated-autoimmunity in atherosclerosis and the immunomodulatory potential thereof have been explored extensively. The antigen-independent immunomodulatory effects of dyslipidemia on T cells is relatively unexplored but has recently gained more interest [12]. In part, this is due to recent advances in the field of immunometabolism and its link with autophagy which have shown that bioenergetic and biosynthetic processes in T cells are crucial in the different phases that a T cell undergoes during an immune response, including cell growth, clonal expansion, differentiation and migration [13]. Whether lipid-associated metabolic processes in specific subsets of T cells are altered by dyslipidemia and whether these metabolic processes contribute to the pathophysiology of atherosclerosis or can be targeted for anti-atherosclerotic therapies is unknown.

THIS THESIS

In this thesis, we used an experimental approach to identify antigen-independent immunomodulatory effects that diet-induced dyslipidemia can have on the cellular metabolism and autophagy in $CD4^+$ T cells. By using this approach we aimed to identify novel and confirm known pathogenic mechanisms involving cellular metabolism and autophagy in T cell-mediated inflammation during atherosclerosis development (fig. 1). Moreover, we aimed to contribute to knowledge on the immunomodulatory effects of clinically available modulators of cellular metabolism and autophagy and their feasibility to dampen T cell-mediated inflammation and atherosclerosis.
We aimed to elucidate the antigen-independent effect of dyslipidemia on T cells by:

1) conceptualizing how metabolic disease affects T cell metabolism,
2) examining how dyslipidemia-induced metabolic adaptations in Treg cells are linked to their impaired phenotypic integrity in atherosclerosis,
3) examining how dyslipidemia-induced priming of $CD4^+$ naïve T (Tn) cells alters their effector phenotype,
4) examining the link between dyslipidemia and autophagy in T cells,
5) examining the effect of lipocalin-2 (Lcn2) deficiency on different metabolic parameters associated with atherosclerosis which could affect T cell metabolism.

The first study was literature-based and in **chapter 2** we described the main metabolic pathways in T cells, discussed the most important modulators of these metabolic pathways and summarized the role of autophagy in T cells and its connection with cellular metabolism. Also, we described different therapeutic approaches based on T cell metabolism and autophagy to ameliorate diseases hallmarked by both metabolic disorder and T cell-mediated autoimmunity. We postulated different mechanisms through which a metabolic disease-associated aberrant microenvironment can affect T cell-mediated immunity and progress disease. As described in chapter 2, we proposed five ways through which this could occur: 1) through increased substrate abundance, 2) through increases in intracellular substrate reservoirs, 3) through skewing of substrate dependence, which could alter the activity of bifunctional enzymes or, 4) skew differentiation of Tn cells into T helper cells, Treg cells and affect their differentiation into memory T cells and lastly, 5) through selective metabolic restriction.
We discuss the five mechanisms in type 1 diabetes mellitus (DM) and hyperglycemia as an example but these are also applicable to atherosclerosis and dyslipidemia as was illustrated by our results reported in chapter 3 and 4. Below, we contemplate where the possibilities and challenges lie in the modulation of T cell metabolism for immunomodulatory therapy atherosclerosis-induced CVD.

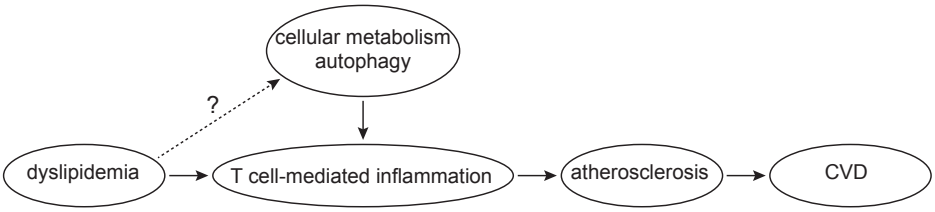


Figure 1 Simplified scheme of the aim of this thesis. Current knowledge of T cell-mediated inflammation in atherosclerosis, and in the development of cardiovascular disease (CVD), is mainly focused on the antigen-dependent effects of dyslipidemia on T cells. Cellular metabolism and autophagy are crucial determining factors in T cell-mediated inflammation. This thesis aimed to examine the antigen-independent effects of dyslipidemia on cellular metabolism and autophagy in T cells (dotted arrow) and how this may affect T cell-mediated inflammation.

One of the translational challenges, which arises from research on cellular metabolism in T cells is the difficulty of targeting T cells in an antigen-independent manner. This can be overcome by applying *ex vivo* cell therapy in which peripheral blood T cells are isolated, treated *ex vivo* to modulate metabolism and then adoptively transferred into the same individual. An *ex vivo* cellular engineering approach is used in T cell-based immunotherapy to successfully treat some forms of cancer [14]. In patients at risk of developing CVD, *ex vivo* modulation of the metabolism of peripheral blood T cells to expand Treg cells may be feasible as their immunosuppressive capacity can be exploited in an antigen-independent manner. *In vivo,* therapeutics which modulate metabolism, such as rapamycin, can be easily and specifically delivered to antigen presenting cells using nanoparticles such as liposomes [15]. Interestingly, antibody-liposome conjugates might be used to target T cells in a similar manner as suggested by a report describing that Burkitt's lymphoma cells can be targeted using anionic liposomes which contain rapamycin and were conjugated to anti-CD19 antibodies [16].

Targeting specific T cells to modulate their cellular metabolism is feasible only when it has been identified which unique membrane proteins correspond to which subset of T cells. In humans this identification has been performed to a certain extent in the Human Immuno Phenotyping project [17]. For example, naïve Treg cells are identified using flow cytometry as $CD3^{+}CD4^{+}CCR4^{+}CD25^{+}CD127^{low}CD45RO^{-}$ cells [17]. Naturally, targeting cells with such a complex 'membrane signature' is difficult and future research is required to assess the feasibility of targeting the metabolism of specific subsets of human T cells *in vivo* using antibodies. In chapter 2, the theoretical usage of drug-cytokine conjugates to deliver specific compounds to T cells with high expression of cytokine receptors (such as CD127) was discussed. A prerequisite for the feasibility of this approach is that drug-cytokine-cytokine receptor complexes are internalized through clathrin-mediated or clathrin-independent endocytosis [18] for the conjugated drug to enter T cells and have the anticipated effect. This approach remains highly speculative but, given the thera-

peutic potential of metabolism modulation in T cells, warrants additional research to the feasibility of antigen-independent T cell targeting.

Another approach to modulate metabolism of specific T cell populations *in vivo* is by targeting the metabolic pathway, which distinguishes the population of interest from other cell types. For example, murine Treg cells and memory T cells have been described to rely more on fatty acid (FA) oxidation fueled oxidative phosphorylation than on glycolysis for their bioenergetic demand as compared to effector T cells [19–21]. Of note, a recent publication has suggested that some of the reported FA oxidation-dependent processes in T cell differentiation were actually caused by off-target effects of the FA oxidation inhibitor etomoxir on mitochondrial complex 1 [22]. T helper (Th) cells have higher membrane expression of glucose transporter 1 (Glut1) than Tn cells and Treg cells [23] suggesting that $CD4^+$ T cell-specific inhibition of Glut1 to inhibit effector function in atherosclerosis predominantly impacts Th cells. Further stratification of the different metabolic signatures of subsets of T cell populations is required to predict the impact of this proposed therapeutic approach. In support of this, even subsets of Treg cells, e.g. thymus-derived and induced Treg cells, have distinct metabolic pathways which function to meet the required bioenergetic and biosynthetic demand [24]. Elaborate characterization of the metabolic signature on a single cell level of atherosclerotic lesion-derived immune cells is required to assess the feasibility of metabolism-based T cell targeting. Naturally, potential side effects of the long term use of systemically administered metabolism modulating compounds, such as rapamycin, need to be considered [25,26]. Nevertheless, research in the field of angiogenesis has proven that at optimal dosage, a small molecule inhibitor of PFKFB3, a glycolytic enzyme, can be used for therapeutic purposes to induce vessel normalization whereas at high dosage the inhibitor causes toxicity [27–29]. This suggests that systemic low dosage administration of metabolism modulators can fine-tune dyslipidemia-induced alterations in the metabolism of T cells for therapeutic purposes with minimal side effects.

Targeting of T cell metabolism for therapeutic purposes is particularly interesting as it can be exploited to modulate the adaptive arm of immunity in diseases of affluence without knowing the disease-associated antigen. Moreover, the problem of antigenic diversity in these diseases of affluence due to varying human leukocyte antigen molecules [30] in the patient population of interest is irrelevant.

An appealing therapeutic approach to inhibit T cell mediated inflammation in atherosclerosis can be based on the modulation of T cell metabolism during vaccination. Vaccination against atherosclerosis with oxidized LDL [31], heat-shock protein 60 [32] and specific ApoB100 peptides [10] reduces atherosclerosis by inducing Treg cells. As both rapamycin and metformin have been shown to expand Treg cells [19,33,34], and are clinically available, short-term treatment with these Treg inducing compounds during vaccination can boost the induction of tolerance. As vaccination can induce immunological memory

and memory Treg cells exert antigen-specific immunosuppressive function upon secondary exposure [35], memory Treg cells represent an interesting subset of Treg cells in the context of vaccination against atherosclerosis. Moreover, rapamycin also induces tolerogenic dendritic cells (DC) which also promote Treg cell expansion [36]. However, Treg cell induction has been shown to depend on mammalian target of rapamycin complex 1 activity [37] which suggests that systemic administration of rapamycin to drive the expansion of Treg cells during vaccination against atherosclerosis could be a double-edged sword. A final consideration in the targeting of T cell metabolism for immunotherapy is the proposedly limited permeability of atherosclerotic lesions, which will cause some systemically administered compounds to affect T cells in the blood, lymphatic vessels and lymphoid organs but not T cells inside the lesions.

Our second approach, as described in **chapter 3**, to study the antigen-independent effects of dyslipidemia on T cell-mediated autoimmunity was based on examining how dyslipidemia-induced metabolic adaptations in Treg cells is linked to their impaired phenotypic integrity in advanced atherosclerosis. We showed that feeding LDL-receptor deficient mice (*Ldlr*$^{-/-}$) a Western-type diet to induce dyslipidemia and advanced atherosclerosis skewed the cellular metabolism of Treg cells to less glycolysis and more FA oxidation. We showed that these diet-induced metabolic alterations in Treg cells were mediated by cholesterol-induced mTORC1 inhibition and the activation of peroxisome proliferator activated receptor delta (PPARδ) by various polyunsaturated FA (PUFA), eicosanoids and eicosanoid-metabolites. Moreover, we showed that Treg cell migration was skewed away from lymph node homing in an mTORC2-dependent manner and that the metabolic phenotype induced by dyslipidemia actually enhanced their capacity to migrate towards sites of inflammation. Despite this effect of dyslipidemia on the metabolic and migratory phenotype of Treg cells, advanced atherosclerotic lesions in humans and mice contain low numbers of Treg cells and is associated with diminished immunosuppression [38]. In mice, the low abundance has been suggested to be caused by impaired migration of Treg cells towards atherosclerotic lesions [39] but our data suggests this is not the case. Dyslipidemia has been linked to Treg cell apoptosis inside atherosclerotic lesions [39,40] and may also induce the loss of FoxP3 expression and differentiation into T helper cell subsets, including Th1 cells [41] and T follicular helper (Tfh) cells inside atherosclerotic lesions [42]. Apparently, the unique microenvironment within atherosclerotic lesions impairs the functional integrity of Treg cells. Part of what makes the microenvironment in atherosclerotic lesions unique is the vast amounts of cholesterol in the form of native LDL and oxLDL particles. Interestingly, intracellular cholesterol content is a decisive factor in the conversion of Treg cells to Tfh cells inside atherosclerotic lesions as the administration of ApoA1, a protein which extracts cholesterol from cells via cholesterol efflux transporters, prevents their conversion inside lesions [42]. Another factor which makes the atherosclerotic microenvironment aberrant from the

blood and lymphoid tissues is that atherosclerotic plaques contain hypoxic regions [43] and macrophage-rich regions which can be low in glucose in humans and mice [44,45]. Sufficient FoxP3 expression is a prerequisite for Treg cells to adapt to a metabolically challenging environment with low-glucose levels [46]. Human carotid atherosclerotic lesions with high-risk histological characteristics have a distinct metabolic profile, which is characterized by increased glycolysis and amino acid utilization, as compared to low-risk lesions [47] suggesting that the local metabolic environment in atherosclerotic lesions is a key factor in the stability of atherosclerosis.

An important question with respect to the data reported in chapter 3 is whether dyslipidemia in humans also induces metabolic and migratory changes in Treg cells and whether the human atherosclerotic lesions impair the functional and phenotypic integrity of Treg cells. LDL-cholesterol levels in CVD patients and FH patients are controlled with lipid-lowering therapies. Consequently, LDL-cholesterol levels >400 mg/dL are rare in the clinic although FH homozygotes can have cholesterol levels of 650-1000 mg/dL when untreated [48]. In fact, dyslipidemia in mice is quite distinct from dyslipidemia in humans, and non-human primates would presumably be a better model to study the reported effects of dyslipidemia on T cells [49]. Compared to dyslipidemic *Ldlr*$^{-/-}$ mice which have total serum cholesterol levels > 1000mg/dL which is mostly present in the VLDL fraction, dyslipidemia in humans is generally much less severe [49]. Therefore, future research in patients with FH or obesity should be performed to identify whether mTORC1 signaling and PPARδ activity is altered human Treg cells as a consequence of dyslipidemia. A confounding factor in future research on this effect of dyslipidemia is that FH patients and (obese) CVD patients are often treated with statins which has metabolic implications for Treg cells [50].

Interestingly, a recent study by Christensen et al. reported that children with FH have elevated levels of circulating PPARδ ligands or their precursors, including omega-6 FA, omage-3 FA, docosahexaenoic acid (DHA), independent of statin use [51]. In the same study, statin use in FH children was associated with lower amounts of PUFAs which can act as PPARδ ligands, as compared to non-statin treated FH children. This suggests that dyslipidemia in FH patients can lead to PPARδ activation in Treg cells and alter their metabolic phenotype and that statins can counteract this. Certain PCSK9 inhibitors may be preferred over statins to control dyslipidemia in FH children as the PCSK9 inhibitor RG7652 reduced serum cholesterol without affecting the circulating levels of inflammation-related eicosanoids [52]. Based on our research in chapter 3, dietary intake of specific PUFAs may increase the serum abundance of PPARδ ligands which can have beneficial effects on atherosclerosis and CVD as they promote the migratory capacity of Treg cells. However, it is important to realize that certain PUFAs can act as ligands for other PPARs and can be metabolized to render them ineffective as PPARδ ligands. Nevertheless, clinical observations in the past have suggested that high levels of n-3 PUFA (such as

docosahexaenoic acid) in blood, for example by eating fish regularly, reduce the risk of coronary artery disease [53,54]. A large study following 44,895 men without CVD showed however that simply increasing the intake of n-3 polyunsaturated FAs by increasing the weekly intake of fish from 1-2 servings to 5-6 serving does not substantially protect against CVD [55].

Other studies have tried to unravel whether the increased intake of specific PUFA, instead of a dietary regime which prescribes a specific type of food, can reduce the risk of CVD. A recent study showed that 15-18 months of dietary supplementation with n-3 PUFA (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) did not decrease the progression of carotid-intima-media thickness [56]. Another study showed that daily intake of 1.86 grams of EPA and 1.5 grams of DHA significantly reduces the progression of fibrous coronary plaques in patients with a low-intensity statin treatment [57]. In line, serum abundance of EPA and DHA is negatively correlated with atherosclerotic plaque progression in DM patients which had undergone percutaneous coronary intervention of vessels with non-culprit lesions [58]. Other studies have shown that dietary supplementation of PUFA can have immunomodulatory effects in humans [59,60], as well as in mice [61]. In our study, many known PPARδ ligands were from the hydroxyeicosatetraenoic acid (HETE) subclass which represents a diverse subclass of lipid metabolites generated from EPA, arachidonic acid and dihomo-γ-linolenic acid. A study by Mallat et al. shows that atherosclerotic plaques which were removed from symptomatic patients through endarterectomy contain more HETEs than plaques from asymptomatic patients [62].

Altogether, further experimental research is required to elucidate the immunomodulatory effect of PPARδ in Treg cells, for example using Treg cell-specific PPARδ-deficient mice, in atherosclerosis. Next, a prospective study which uses extensive genomics and lipidomics profiling to fully characterize the lipidome of dyslipidemia patients and subsequently correlates this to the development of symptomatic CVD, cardiac events or cardiac death should lead to more effective dietary regimes to prevent CVD. It is unlikely that the protective effects of n-3 PUFA on CVD which have been reported are solely Treg cell-mediated and whether these effects are PPARδ-dependent remains elusive.

Nevertheless, the notion that dietary lipids can instruct Treg cell metabolism and migratory capacity is interesting and future research should elaborate whether this has any clinical relevance.

In chapter 4, we studied the antigen-independent effect of dyslipidemia on T cell-mediated autoimmunity by examining how dyslipidemia-induced priming of $CD4^+$ Tn cells alters their effector phenotype. Priming of $CD4^+$ Tn cells was performed by pre-incubating $CD4^+$ Tn cells with excess lipoproteins or dyslipidemic serum from WTD-fed *Ldlr*$^{-/-}$ mice in the presence or absence of compounds which modulate lipid metabolism. Subsequently, the cells were activated by suboptimal antibody stimulation or antigens presented on MHC molecules by bone marrow derived dendritic cells. These data

showed that the specific method to induce lipid accumulation and the activating stimulus which was used, determined whether priming affected the effector phenotype. The use of lipoproteins to induce lipid accumulation had the advantage that the observed effects of priming were lipid-specific. A disadvantage was that lipid accumulation occurred without the context of blood-borne inflammatory factors (such as cytokines), which did occur during dyslipidemic serum-induced lipid accumulation. Arguing against the use of serum-induced lipid accumulation is that serum induces confounding factors in the form of the aforementioned blood-borne inflammatory factors. Whether lipoprotein-induced priming of $CD4^+$ Tn cells altered the effector phenotype was most likely dependent on the TCR-stimulating signal (antibody/antigen), which suggests that the amount of antigen present determines whether priming of $CD4^+$ Tn cells with lipids affects their effector function. The lack of an effect on the effector phenotype after antigen-stimulation of primed $CD4^+$ Tn cells isolated from OT-II cells could also be explained by the fact that OT-II T cells have a functional LDL-receptor whereas *Ldlr*$^{-/-}$ T cells do not. This suggested that priming of *Ldlr*$^{-/-}$ during dyslipidemia has a larger effect as compared to wildtype T cells as the LDL receptor-mediated uptake of native lipoproteins is absent. These findings were relevant as mutations in the *Ldlr* gene is a frequent cause of FH [63].

Future research should focus on whether lipid accumulation occurs in human Tn cells residing in lymphoid tissues and how priming of human $CD4^+$ Tn cells contributes to inflammation when DCs and other APCs are also affected by priming. Regarding the latter, DCs loaded with oxLDL have enhanced antigen presentation and are capable of stronger induction of T cell proliferation [64,65], indicating that this is an additional factor in dyslipidemia-induced antigen-independent effects on T cell activation.

An interesting finding from chapter 4 is that modulation of lipid metabolism with both an LXR agonist as well as a lysosomal inhibitor had mild anti-inflammatory effects on the effector phenotype of $CD4^+$ T cells specifically under dyslipidemia-like priming conditions. From a translational point of view, this suggests that *ex vivo* modulation of peripheral $CD4^+$ Tn cells from patients with dyslipidemia using compounds which modulate lipid metabolism might have distinct effects from those observed in healthy, normolipidemic individuals. As mentioned above, patients at risk of developing CVD are often treated with lipid lowering therapies. Lipid lowering therapy in FH patients has already been shown to affect the degree of lipid accumulation in circulating monocytes and their inflammatory phenotype [66], which adds to the complexity of translating our findings to the human situation.

Dyslipidemia was not associated with increased autophagy in $CD4^+$ T cells, in contrast to acetylated-LDL which does induce autophagy in foam cells [67]. Our findings on autophagy reported in chapter 4 suggested that lipid accumulation does not require an autophagy-dependent response to prevent lipotoxicity in the total $CD4^+$ T cell population. However, it is possible that Treg cells, which have been described to affect systemic

lipoprotein metabolism [68] and are more sensitive to environmental lipid perturbations, do require enhanced autophagy-mediated degradation of lipid droplets to prevent lipotoxicity. Our results in chapter 4 support the hypothesis that modulation of autophagy might be feasible as a therapy to dampen T cell-mediated immunity and ameliorate atherosclerosis [69]. Naturally, this approach requires more research but seems feasible as chloroquine treatment of $CD4^+$ Tn cells during priming with dyslipidemia serum had anti-inflammatory effects on their effector phenotype. In addition, chloroquine has been described to induce tolerogenic DCs [70]. Chloroquine is safe when used in small dosage and is already applied in the treatment of other autoimmune diseases, such as rheumatoid arthritis, to inhibit the immune system and ameliorate disease.

Our fourth approach was based on studying the effect of dyslipidemia on autophagy in T cells and the effect of pharmacological and genetic blockade of autophagy on T cell-mediated inflammation in atherosclerosis. Here we studied the effects of pharmacological autophagy inhibition during priming phase of $CD4^+$ Tn cells, as described above. The effect of genetic autophagy blockade in T cells on the T cell-mediated contribution to atherosclerosis was described in **chapter 5**. We generated a model of genetic blockade of autophagy in T cells by generating mice with T cell specific deletion of autophagy related protein 7 (Atg7), as whole body deficiency of Atg7 is lethal in mice within 24 hours after birth [71]. We used a recombinant adeno-associated virus which induced overexpression of murine PCSK9 (rAAV2/8-D377Y-mPCSK9) to mimic the *Ldlr*$^{-/-}$ phenotype in mice [72] with wild type Atg7 or a T cell-specific deficiency of Atg7. To our surprise, T cell-specific Atg7 deficiency impaired the development of hepatic steatosis. Hepatic steatosis, also known as non-alcoholic fatty liver disease, is characterized by excessive hepatocellular lipid accumulation. Hepatic steatosis can be caused by metabolic diseases, such as dyslipidemia and hyperglycemia, and is thus associated with obesity and diabetes mellitus [73]. When steatosis persists it elicits an inflammatory response, thus leading to steatohepatitis which can subsequently lead to hepatic fibrosis [74]. Research has primarily focused on the contribution of T cells in the inflammatory response which progresses steatosis to steatohepatitis and hepatic fibrosis. Interestingly, it has been suggested that inflammation precedes the development of steatosis [75]. High fat diet-fed C57BL/6 mice without hepatic steatosis which are injected with casein, to induce inflammation, develop more severe hepatic steatosis than PBS-injected mice by disrupting fatty acid synthesis and oxidation [76]. A recent report described how nutrient excess in the liver induces DNA damage and promotes T helper 17 (Th17) cell-mediated inflammation of white adipose tissue (WAT), which subsequently increased insulin resistance and FFA release in the circulation. This increase in circulating FFAs increased the amount of esterified FFAs (triglycerides) in the liver, thereby promoting hepatic steatosis development [75]. Interestingly, systemic administration of chloroquine has been described to exacerbate hepatic steatosis development in a high-fat diet-induced hepatic steatosis

model [77], suggesting that chloroquine treatment to modulate the immune system in patients with dyslipidemia and hepatic steatosis may be troublesome. One of the limitations in the studies presented in chapter 5 is the lack of a T cell subset-specific knock-out of Atg7. Indeed, it would be interesting to generate mice which have Atg7 deficiency, specifically in Th1 cells or Th17 cells. A limitation in the generation of these mice is that activated $CD8^+$ T cells also express T-bet, which is the characteristic transcription factor for Th1 cells. A recent paper has shown that this limitation can be overcome through the use of two orthogonal recombination systems in which Cre recombinase is expressed only in cells where Dre recombinase is also expressed [78]. The paper by Pu et al. reports this approach in endothelial cells as an example to prove that this system works, but theoretically it could be applied to immune cells as well. Hence, a *CD4-Dre Tbx21-CreER Atg7$^{f/f}$* mouse would allow for temporal deletion of Atg7 more specifically in Th1 cells, although Tbx21 would also be deleted through recombination in double-positive T cells in the thymus. This approach would also be interesting to further unravel the role of Th2 and Th17 cells in different stages of experimental atherosclerosis as their contributions to inflammation in atherosclerosis remain unclear.

Finally, **chapter 6** described our studies in which we aimed to unravel some of the antigen-independent effect of dyslipidemia on T cell-mediated immunity. This chapter was specifically based on the effect of lipocalin-2 (Lcn2) deficiency on different metabolic parameters associated with atherosclerosis which could affect T cell metabolism. Lcn2 is an inflammatory mediator, which is produced by macrophages, neutrophils and epithelial cells in the gut, lungs and kidneys. It is associated with severity of atherosclerosis and CVD in humans and has been shown in mice to promote obesity, hepatic steatosis and the development of insulin resistance [79]. Obesity is associated with increased levels of the adipokine leptin, a hormone which is primarily secreted by adipocytes and regulates the sensation of satiety in the hypothalamus and can thereby regulate body weight. Moreover, leptin regulates glucose metabolism in activated T cells [80], mTOR signaling in Treg cells [81] and can regulate autophagy in conventional T cells after TCR stimulation [82]. Additionally, Lcn2 can affect macrophage phenotype as well as neutrophil recruitment and function [79]. Therefore, we hypothesized that Lcn2 is involved in atherosclerosis by impacting T cell-mediated inflammation through its effect on systemic lipid metabolism and the inflammatory environment.

Lcn2 deficiency in *Ldlr$^{-/-}$* mice did not affect the metabolic parameters which we examined, including body weight, inguinal white adipose tissue weight, serum cholesterol levels, serum triglyceride levels and fasting glucose levels. Additionally, we measured mRNA expression of *Lep*, the gene encoding leptin, in WAT from *Ldlr$^{-/-}$* and *Ldlr$^{-/-}$Lcn2$^{-/-}$* mice but this was also unaltered between both genotypes. Flow cytometry analysis of T cell activation status and T cell subset percentages also showed no differences between *Ldlr$^{-/-}$* and *Ldlr$^{-/-}$Lcn2$^{-/-}$* mice, suggesting that Lcn2 deficiency had no major T cell-mediated

effects in our studies. Interestingly, Treg cells express *24p3r*, the Lcn2 receptor, suggesting that Lcn2, which is usually generally considered to affect innate immune cells, might modulate Treg cells (data on the *Lep* expression in WAT, effects of Lcn2 deficiency and T cells and Lcn2 receptor expression in Treg cells were not included in chapter 6). Lcn2 deficiency did impact the size and morphological composition of atherosclerotic lesions as Lcn2 deficiency promoted lesion growth in initial stages of plaque development and decreased the necrotic core size in advanced lesions. Hence, we did not focus on T cells in this chapter but aimed to elaborate how Lcn2 deficiency affected the aforementioned lesion characteristics. We reported that Lcn2 deficiency most likely increased the size of moderately progressed lesions by increasing the recruitment of monocytes to atherosclerotic lesions as Lcn2 interacts with various inflammatory factors involved in atherosclerosis, including leukotriene B4 [83]. We indeed observed more inflammatory monocytes in the circulation. Further research is however required to identify what the exact effects are of the interactions between Lcn2 and certain inflammatory factors, such as leukotriene B4, on their functionality. A report by Fernandez-García et al. which responded to our publication suggested that the growth in moderate lesion size was smooth muscle cell (SMC)-mediated [84]. While we disagree that the lesion promoting effect of Lcn2 deficiency is exclusively SMC-mediated [85], Fernandez-García et al. had a valid point that further identification of the cells expressing Lcn2 in atherosclerotic lesion, and their dynamics in diet-induced atherosclerosis, would be relevant in order to elucidate the mechanism by which Lcn2 affects atherosclerosis in different stages. This is especially relevant as not only inflammatory cells but also SMCs and endothelial cells express Lcn2 in atherosclerotic lesions [86,87].

In advanced stages of atherosclerosis, Lcn2 deficiency was associated with decreased intraplaque matrix metalloproteinase (MMP) activity and decreased necrotic core size suggesting that Lcn2 affects atherosclerotic lesion stability by increasing MMP-induced cell death. These findings were in line with clinical observations that serum Lcn2 is a predictor of major adverse cardiac events [88,89] and serum Lcn2 levels are higher in patients with symptomatic carotid atherosclerosis as compared to patients with asymptomatic carotid atherosclerosis [90]. Moreover, serum Lcn2/MMP9 complexes are associated with major adverse cardiac events [91]. Active MMP9 degrades matrix proteins in the fibrous cap of atherosclerotic lesions [92,93] and Lcn2 stabilizes MMP9 by preventing its inhibition by tissue inhibitor of matrix metalloproteinase 1 [94]. This suggests that Lcn2 could be used as a biomarker to identify patients at risk of an acute coronary event which could be followed by additional treatment of these patients with MMP inhibitors to stabilize atherosclerotic lesions. Pharmacological inhibition of Lcn2 itself can compromise an individual's resistance to bacterial infection as Lcn2 is a crucial bacteriostatic agent in the acute phase of infection with *E. Coli* [95]. Therefore, we expect future research into Lcn2

in atherosclerosis and CVD will aim to improve its use as a biomarker for CVD rather than as a target for therapy.

In conclusion, the research in this thesis has provided novel antigen-independent pathophysiological mechanisms through which diet-induced dyslipidemia can affect T cell-mediated immunity in atherosclerosis. Moreover, it supports the notion that modulation of cellular metabolism and autophagy in T cells is feasible to dampen inflammation and inhibit atherosclerotic lesion development, potentially preventing CVD. Future research is required to further elaborate the effect of dyslipidemia on cellular metabolism and autophagy in specific T cell subsets in a temporal and site-specific fashion and to identify whether similar pathophysiological mechanisms are present in humans.

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Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Jaarlijks zorgen hart- en vaatziekten wereldwijd voor meer dan 15 miljoen doden volgens de World Health Organization. In Europa is bij 45% van de overledenen hart- en vaatziekten de doodsoorzaak, en in de Europese Unie is het de doodsoorzaak bij 37% van alle sterfgevallen. Volgens cijfers gepubliceerd door de Nederlandse Hartstichting is in Nederland tussen 1980 en 2016 het sterftecijfer voor hart- en vaatziekten bij mannen gedaald met 69% en bij vrouwen met 59%. Desalniettemin heeft Nederland zo'n 1,4 miljoen hart- en vaatpatiënten en sterven er dagelijks 106 mensen aan een hart- of vaatziekte. Op jaarbasis sterven er alleen al aan hartinfarcten en beroertes 15,000 mannen en vrouwen in Nederland. De belangrijkste onderliggende oorzaak van hart- en vaatziekten is slagaderverkalking, ook wel atherosclerose genoemd. Atherosclerose is een ziekte waarbij de vaatwand in slagaders verdikt is als gevolg van een ontsteking die het lumen van een slagader kan verkleinen en zo de bloedtoevoer naar achterliggend weefsel kan verstoren. Atherosclerose wordt veroorzaakt door een immuunrespons geassocieerd met hoge niveaus van lipiden, die zich ophopen in de vaatwand. Risicofactoren voor atherosclerose zijn dyslipidemie, obesitas, diabetes en een sedentaire levensstijl. Daarnaast is er een grote genetische risicofactor in de vorm van de ziekte familiaire hypercholesterolemie (FH), die zonder behandeling dyslipidemie en vervolgens premature ontwikkeling van hart- en vaatziekten veroorzaakt. De ophoping van lipiden en de daaropvolgende immuunrespons vindt voornamelijk plaats op specifieke plekken in het bloedvatensysteem, waar afwijkende stroming van bloed zorgt voor verstoorde functie van cellen die de vaatwand bekleden waardoor lipiden ophopen. De kransslagaders van het hart zijn bij uitstek een plaats in het bloedvatensysteem waar deze afwijkende stroming aanwezig is en waar een verhoogde kans bestaat om atherosclerose te ontwikkelen.

In de eerste fase van atherosclerose vormen zich zogenoemde fatty streaks in de vaatwand. Deze fatty streaks bestaan voornamelijk uit accumulerende lipiden en ontstekingscellen die vanuit het bloed de vaatwand inkruipen en daar de vetdeeltjes opnemen in een poging ze te neutraliseren. Deze fatty streaks zijn op zich onschadelijk, maar wanneer deze zich verder ontwikkelen en toenemen in grootte kan atherosclerose tot vernauwing (stenose) van slagaders zorgen. Wanneer de stenose van een dusdanige ernst is dat er sprake is van een significante vermindering in bloedtoevoer naar het achterliggende weefsel spreekt men van ischemie, ofwel een verstoorde balans tussen de vraag en toevoer van zuurstof en voedingsstoffen. Ischemie is symptomatisch als het zorgt voor verminderde hartfunctie, electrocardiogram afwijkingen en druk op de borst (angina pectoris). Wanneer symptomen ontstaan onder stress en verdwijnen bij rust spreekt men van stabiele angina. Een atherosclerotische laesie kan zich ook dusdanig ver ontwikkelen dat deze onstabiel wordt en scheurt, waardoor de inhoud hiervan aan

de bloedbaan wordt blootgesteld. Dit induceert de vorming van een trombus (stolsel) welke in de kransslagader de bloedtoevoer naar het achterliggend hartweefsel blokkeert en in de halsslagader (carotis) de bloedtoevoer naar een deel van het brein. Bij langdurige ischemie tijdens een hartaanval sterft het hartweefsel, wat voor (blijvend) verminderde hartfunctie zorgt en mogelijk de dood tot gevolg heeft.
Tijdens een acute klinische situatie zoals een hartaanval is directe interventie nodig in de vorm van ballon angioplastiek (dotteren), mogelijk gecombineerd met het plaatsen van een stent om het bloedvat open te houden na de ingreep. Atherosclerose in de kransslagaders kan in de niet acute fase behandeld worden met een bypass operatie (ook wel omleidingsoperatie genoemd) waarbij een ader uit bijvoorbeeld het been van de patiënt wordt verwijderd en vervolgens gebruikt wordt om de bloedtoevoer naar ischemisch weefsel te herstellen. Een andere operatie is de endarterectomie waarbij atherosclerotische laesies chirurgisch worden verwijderd na het open leggen van het verstopte bloedvat. Endarterectomiën worden in niet acute situaties voornamelijk uitgevoerd in de carotiden (ofwel halsslagaders) en *arteria femoralis (beenslagaders)*, die de benen voorzien van bloed. Bij een beroerte als gevolg van occlusie van een carotis worden endarteroctomiën ook in de acute fase uitgevoerd. Grote nadelen van bovengenoemde chirurgische interventies als behandeling voor atherosclerose is dat deze invasief zijn en er een relatief grote kans bestaat op restenose. Gezien de rol van lipiden bij de vorming van atherosclerose worden mensen met een geschiedenis van of verhoogd risico op hart- en vaatziekten, zoals hartpatiënten of FH-patiënten, vaak farmacologisch behandeld met statines. Statines remmen de synthese van cholesterol in de lever en verminderen derhalve de hoeveelheid schadelijke vetten in het bloed, de zogenoemde LDL-deeltjes en verhogen de LDL receptor expressie. Statines zijn succesvol gebleken in de preventie van hart- en vaatziekten gezien ze de kans hierop met 25-30% verminderen. Echter, statines hebben weinig effect op reeds ontwikkelde atherosclerotische laesies en slaan niet aan bij alle patiënten. Sinds kort wordt nog een medicijn om lipiden in het bloed te verlagen in de kliniek gebruikt in de vorm van antilichamen die PCSK9 remmen. PCSK9 zorgt normaal voor lysosomale afbraak van de LDL receptor dus remming van PCSK9 zorgt voor een verhoogde opname van cholesterolrijke LDL deeltjes uit het bloed door de lever. Naast het verlagen van circulerende lipiden is een ander therapeutisch aangrijpingspunt het immuunsysteem omdat modulatie hiervan de ontwikkeling en stabiliteit van atherosclerotische laesies zou kunnen beïnvloeden. Klinisch onderzoek heeft al aangetoond dat het remmen van de ontstekingsfactor interleukine-1β geassocieerd is met een verlaging van 15% in het aantal doden als gevolg van een hartaanval, beroerte en hart- en vaatziekten gecombineerd. Dit benadrukt dat onderzoek naar de rol van ontstekingscellen (immuuncellen) succesvol naar de kliniek vertaald kan worden als behandeling van atherosclerose en hart- en vaatziekten.

LDL-deeltjes die de bloedbaan verlaten en in het onderliggende weefsel in de vaatwand accumuleren kunnen cellen die de vaatwand bekleden, de zogenaamde endotheelcellen, activeren, wat leidt tot de rekrutering van immuuncellen om deze vetten op te ruimen. Een versterkende factor in deze initiele ontstekingsreactie is de lokale modificatie van LDL-deeltjes, die ervoor zorgt dat deze als lichaamsvreemd worden beschouwd door het immuunsysteem. Een van de eerste immuuncellen die vanuit het beenmerg en het bloed gerekruteerd worden naar de vaatwand zijn de monocyten. Onder invloed van lokale omgevingsfactoren rijpen monocyten verder in de vaatwand tot macrofagen. Macrofagen zijn cellen met grote 'fagocytotische capaciteit' hetgeen betekent dat ze gespecialiseerd zijn in het opnemen van extracellulair materiaal dat als lichaamsvreemd wordt herkend. Echter, de grote aanvoer van vetten en het onvermogen van de macrofagen om de vetten adequaat te verwerken en af te voeren zorgt ervoor dat de cellen zich verder ontwikkelen tot een specifiek type macrofaag, namelijk de schuimcel (foam cell). De hierboven genoemde fatty streaks bestaan voornamelijk uit schuimcellen en geaccumuleerde vetten. De macrofagen, endotheelcellen en schuimcellen scheiden ontstekingsfactoren uit die andere immuuncellen, zoals neutrofielen en T cellen, rekruteren om bij te dragen aan het neutraliseren van (gemodificeerde) LDL-deeltjes.

Het belangrijkste celtype dat in dit proefschrift aan bod komt is de T cel. T cellen zijn gespecialiseerde immuuncellen die voornamelijk geactiveerd worden door specifieke stukjes eiwit, zogenoemde peptiden. Mensen en muizen hebben een T cel repertoire waarbij elke T cel kloon een specifiek peptide herkent via zijn T cel receptor. Deze peptiden zijn vaak afkomstig van ziekteverwekkers en worden door andere immuuncellen, zoals macrofagen en dendritische cellen, verwerkt en aan T cellen gepresenteerd via zogenaamde MHC-moleculen. Het eiwit waarvan de peptide afkomstig is noemt men in de immunologie het antigeen. Antigenen die vanuit de omgeving zijn opgenomen worden gepresenteerd aan $CD4^+$ T cellen die aan de humorale immuniteit bijdragen, door bijvoorbeeld bij te dragen aan de productie van antilichamen door B cellen. Intracellulaire antigenen, zoals bij een virale infectie, worden gepresenteerd aan $CD8^+$ cytotoxische T cellen. Cytotoxische T cellen dragen bij aan de immuunresponse door middel van het uitscheiden van ontstekingseiwitten, of het doden van een cel die het peptide op zijn membraan presenteert waar de T cel tegen gericht is. Afhankelijk van de ziekteverwekker wordt een T cel respons geïnitieerd die voornamelijk bestaat uit $CD4^+$ of $CD8^+$ T cellen. Na activatie door een peptide gaan T cellen in de effector fase delen en verder ontwikkelen in gespecialiseerde subtypes die de ziekteverwekker efficiënt kunnen bestrijden. Uiteindelijk ontwikkelt een klein percentage zich verder in memory T cellen die, bij een volgende blootstelling aan hetzelfde pathogeen, deze efficiënter kunnen bestrijden.

In het geval van atherosclerose zijn de peptiden waar T cellen uit atherosclerotische laesies op reageren vaak afkomstig van het ApoB100 eiwit, het belangrijkste eiwit in

LDL-deeltjes. Tijdens de vorming van atherosclerose worden cellen die LDL deeltjes verwerken tot peptide fragmenten naar de ontstekingsplaats gerekruteerd. Hierna migreren ze naar lymfeknopen die de vaatwand draineert om de peptide te presenteren aan een naïeve T cel, een T cel die nog geen antigeen gezien heeft, die door het lichaam circuleert, welke hierop wordt geactiveerd, gaat delen en naar de vaatwand migreert om aan de ontstekingsreactie deel te nemen. Op deze manier zorgt de verhoogde aanwezigheid van vetten in de bloedbaan voor meer antigeen in de vaatwand en een versterkte T cel gemedieerde ontstekingsreactie. In atherosclerose is de meerderheid van de T cellen $CD4^+$ en in deze $CD4^+$ T cel populatie is de T helper 1 (Th1) cel in de grootste hoeveelheden aanwezig. Th1 cellen zijn pro-inflammatoir, of ontstekingsversterkend, voornamelijk door het uitscheiden van een cytokine genaamd interferon-gamma. Een ander cruciaal type $CD4^+$ T cellen in atherosclerose is de regulatoire T (Treg) cel die juist gespecialiseerd is in het onderdrukken van andere immuuncellen, voornamelijk door het uitscheiden van de cytokine interleukine-10. Omdat Treg cellen relatief weinig aanwezig zijn in atherosclerotische laesies is het bevorderen van de Treg cel populatie een veelbelovende invalshoek voor therapie gericht tegen atherosclerose en hart- en vaatziekten.

Het systemische metabolisme van vetten is een belangrijke determinant in de kracht van de T cel-gemedieerde immuunrespons in atherosclerotische laesies aangezien het de aanwezigheid van antigeen in de vaatwand kan beïnvloeden. Maar cellulair metabolisme, ofwel de stofwisseling die plaatsvindt binnenin cellen, is ook van grote invloed op T cellen. Cellulair metabolisme van glucose (suiker) en vetten in T cellen is cruciaal voor hun inflammatoire potentie. Metabolisme is grofweg te verdelen in katabolisme en anabolisme wat respectievelijk afbraak en opbouw van macromoleculen inhoudt. Bij katabolisme wordt bijvoorbeeld een vetzuur of glucose molecuul afgebroken tot metabole intermediairen die in de mitochondriën worden gebruikt om energie in de vorm van ATP op te wekken. Bij anabolisme worden metabole intermediairen gebruikt voor de vorming van biomoleculen zoals cholesterol. Tijdens de celdeling is metabolisme essentieel voor T cellen omdat er binnen korte tijd een hoop dochtercellen van een T cel kloon gevormd moeten worden. Dit betekent dat er veel celmembranen, organellen en nucleotiden gevormd moeten worden bestaande uit vetten, eiwitten en andere koolstofverbindingen. De activiteit van glycolyse, het biochemische proces van de afbraak van glucose, wordt verhoogd en dit zorgt voor de metabole intermediairen die nodig zijn als voorloper voor de synthese van de voorgenoemde biomoleculen. Ook de synthese van cholesterol, een belangrijk onderdeel van celmembranen, wordt geïnduceerd bij de activatie van T cellen.

Waar metabolisme een verzameling biochemische processen omvat is er nog een intracellulair proces dat cruciaal is voor T cellen tijdens een immuunrespons, namelijk macro-autofagie. Macro-autofagie (hierna autofagie genoemd) is een intracellulair

proces waarbij eiwitten en organellen door middel van isolatiemembranen naar lysosomen worden gebracht, waarin ze worden afgebroken door lysosomale enzymen en zo gerecycled kunnen worden. Autofagie wordt in brede zin geïnduceerd door stress, bijvoorbeeld in een omgeving met weinig nutriënten. In die context vormt autofagie een intrinsiek mechanisme waarmee cellen met nutriënt-stress kunnen omgaan om zo toch de nodige macromoleculen tot de beschikking te hebben. Echter, autofagie kan ook als beschermend mechanisme werken om juist met een overmaat aan nutriënten om te kunnen gaan. In schuimcellen wordt autofagie van lipide druppels, organellen gespecialiseerd in de intracellulaire opslag van vetten, geïnduceerd om zo de lipidenbelading en lipotoxische stress te verminderen. Daarnaast kunnen middels autofagie slecht functionerende mitochondriën afgebroken worden en gerecycled worden voor bijvoorbeeld de vorming van nieuwe en goed functionerende mitochondriën. Autofagie en metabolisme zijn dus nauw met elkaar verbonden. Bij geactiveerde T cellen die gaan delen is autofagie verhoogd maar worden mitochondriën juist uitgesloten voor lysosomale degradatie omdat mitochondriën nodig zijn voor de energiehuishouding tijdens T cel deling. Grof gezegd heeft het remmen van autofagie dan ook een inhiberend effect op inflammatoire capaciteit van T cellen. Samengevat verhoogt dyslipidemie de kans op atherosclerose en hart- en vaatziekten onder andere door de aanwezigheid van antigeen voor T cellen te verhogen en is daarnaast bekend dat intracellulaire metabole processen, zoals autofagie en glycolyse, cruciaal zijn voor de inflammatoire capaciteit van T cellen. Verrassend genoeg is er weinig onderzoek gedaan naar hoe dyslipidemie intracellulaire metabole processen in T cellen moduleert en hierbij effect heeft op de inflammatoire capaciteit van T cellen en de vorming van atherosclerose.

In dit proefschrift worden een aantal mechanismes beschreven waarin dyslipidemie metabolisme en autofagie in verschillende subsets van T cellen beïnvloedt en zo bij zou kunnen dragen aan de T cel-gemedieerde immuunrespons in de vaatwand. In **hoofdstuk 2** zijn de belangrijkste metabole processen beschreven en door welke eiwitten deze gemoduleerd worden. Daarnaast wordt in hoofdstuk 2 beschreven op welke manier een omgeving die metabool gezien afwijkend is T cellen kan beïnvloeden en welke reeds bestaande medicijnen gebruikt kunnen worden om T cel metabolisme te beïnvloeden en zo ontsteking te remmen. In **hoofdstuk 3 en 4** wordt beschreven hoe dyslipidemie het metabolisme van Treg cellen en naïeve T cellen beïnvloedt en hoe dit bijdraagt aan hun inflammatoire capaciteit wanneer ze geactiveerd worden. In een deel van hoofdstuk 4 wordt beschreven hoe dyslipidemie autofagie in T cellen beïnvloedt en in **hoofdstuk 5** wordt beschreven wat het effect is van deficiëntie van een cruciaal autofagie eiwit, genaamd Atg7, op de inflammatoire capaciteit van T cellen in de context van dyslipidemie en atherosclerose. In **hoofdstuk 6** wordt beschreven wat het effect is van deficiëntie van lipocalin-2 (Lcn2), een eiwit wat geassocieerd is met hart- en vaatziekten, op de vorming van atherosclerose.

In **hoofdstuk 2** hebben we samengevat dat pro-inflammatoire T helper cellen voornamelijk glycolyse nodig hebben voor hun ontwikkeling, celdeling en activiteit terwijl Treg cellen voornamelijk afhankelijk zijn van vetzuur oxidatie voor hun celdeling. Verder is er in hoofdstuk 2 gediscussieerd over de manieren waarop een metabool afwijkende omgeving het metabolisme, en hiermee de inflammatoire capaciteit, van T cellen kan beïnvloeden. Wij hebben 5 manieren voorgesteld waarop dit kan plaatsvinden, te weten: 1) omdat er meer substraat aanwezig is in de omgeving; 2) doordat er meer intracellulaire reserves aangelegd zijn; 3) doordat het cellulair metabolisme voornamelijk via een specifiek proces plaatsvindt wat de activiteit van bifunctionele enzymen kan beïnvloeden; 4) doordat het de differentiatie van naïeve T cellen in subsets van effector T cellen kan sturen, en 5) doordat cellen in de omgeving minder nutriënten krijgen door de veranderde metabole activiteit van T cellen. Belangrijke eiwitten in de regulering van metabolisme in T cellen zijn mammalian target of rapamycin (mTOR) en AMP-activated kinase (AMPK). mTOR is een cruciaal metabool eiwit wat bij activatie van T cellen verhoogde activiteit heeft en processen aanstuurt die glycolyse stimuleren en de biosynthese bevorderen. Remming van mTOR door middel van bijvoorbeeld rapamycine om T cel gemedieerde ontsteking te remmen in patiënten waarbij een metabole ziekte hieraan bijdraagt is een therapeutische invalshoek die in de mens nog onvoldoende belicht is. Een ander medicijn dat reeds in de kliniek wordt gebruikt is metformine. Metformine stimuleert de activiteit van AMPK, wat inhibitie van mTOR als gevolg kan hebben en op deze manier vergelijkbare therapeutische effecten zou kunnen hebben als rapamycine of andere mTOR remmers. Metformine, een antidiabeticum, zou derhalve gebruikt kunnen worden ter immunomodulatie in patiënten van wie de T cellen zich op een pathologische manier hebben aangepast aan een systemische metabole ziekte.

In **hoofdstuk 3** zijn de effecten van dyslipidemie op het metabolisme en de migratie capaciteit van Treg cellen beschreven. Regulatoire T cellen zijn minder functioneel in atherosclerose en zijn relatief weinig aanwezig in atherosclerotische laesies. In literatuur is gesuggereerd dat dit laatste kan komen omdat de Treg cellen minder naar de vaatwand migreren, daar aangekomen doodgaan of daar aangekomen differentiëren in een ander celtype onder invloed van lokale (ontstekings)factoren. In hoofdstuk 3 hebben we beschreven dat dyslipidemie zorgt voor vetstapeling in regulatoire T cellen en dat dit de activiteit van het mTOR complex 1 remt waardoor de Treg cellen minder glucose-afhankelijk metabolisme hebben. Daarnaast stimuleert dyslipidemie de activiteit van de transcriptiefactor peroxisome proliferator activated receptor delta (PPARδ), wat ervoor zorgt dat Treg cellen een meer vetzuur-afhankelijk metabolisme krijgen. Omdat het genereren van energie essentieel is voor het vervormen van het cytoskelet, en daardoor de migratoire capaciteit van cellen, hebben we ook beschreven wat activatie van PPARδ voor invloed heeft op migratie van Treg cellen. We hebben aangetoond dat activatie van PPARδ met een synthetische ligand de migratie van Treg cellen verhoogt wat afhankelijk

is van de verhoogde vetzuuroxidatie die PPARδ activatie als gevolg heeft. Daarnaast blijkt dat Treg cellen niet minder maar juist meer naar ontstekingshaarden, waaronder atherosclerotische laesies, migreren na langdurige blootstelling aan dyslipidemie. Als laatste hebben we aangetoond dat Treg cellen die in atherosclerotische laesies van muizen met dyslipidemie aanwezig zijn meer vetten bevatten dan Treg cellen van muizen met normolipidemie. Deze resultaten hebben aangetoond dat het metabolisme van Treg cellen wordt beïnvloed door het dieet van muizen en dat dit functionele gevolgen kan hebben voor hun migratie capaciteit.

In **hoofdstuk 4** hebben we onderzocht of en hoe het blootstellen van naïeve $CD4^+$ T (Tn) cellen aan een dyslipidemische omgeving invloed heeft op hun fenotype na activatie. Dit is interessant omdat we op deze manier het 'priming' effect van extracellulaire vetten op Tn cellen bekijken, die door de vergroting van intracellulaire vetophoping een andere inflammatoire capaciteit hebben na activatie dan een 'normale' Tn cel. In hoofdstuk 4 hebben we ook gekeken naar de modulatie van autofagie van lipide druppels (zogenoemde lipofagie) door dyslipidemie en wat voor effect de remming van lysosomen heeft op de inflammatoire capaciteit van Tn cellen. Tn cellen stapelen vetten als ze geïncubeerd worden met geïsoleerde lipoproteïnen of serum wat uit dyslipidemische muizen gehaald is. Dit stapelen van vetten gaat daarnaast gepaard met een reactie van de transcriptiefactor liver-X-receptor (LXR) die na activatie de expressie van genen vergroot die de afvoer van cholesterol en andere vetten tot gevolg heeft. Het primen van Tn cellen met geïsoleerde lipoproteïnen verhoogde de proliferatie maar had geen duidelijke effecten op de proliferatieve capaciteit van de cellen of op de differentiatie van Tn cellen in specifieke subtypen. Interessant genoeg had de activatie van LXR met T0901317 voordat Tn cellen gestimuleerd werden wel remmende effecten op de proliferatie en stimuleerde het de differentiatie in Treg cellen. Dyslipidemie moduleerde autofagie niet in T cellen, wat suggereerde dat autofagie (of lipofagie) geen coping mechanisme is voor T cellen tijdens lipide overbelading. Hoewel autofagie dus niet gemoduleerd werd door dyslipidemie had de remming van lysosomen in Tn cellen wel een effect op hun effector T cel phenotype. Het remmen van lysosomen tijdens priming, om de endolysosomale opname en autofagie van lipiden te inhiberen, zorgde voor verlaagde proliferatie en daarnaast verlaagde differentiatie van Th1 cellen, specifiek onder dyslipidemische omstandigheden. Ook als we muizen met dyslipidemie behandelden met de lysosoom remmer chloroquine zagen we een hoger percentage Treg cellen. Interessant genoeg zorgde chloroquine in de muis juist voor verhoogde proliferatie wat waarschijnlijk door T cel aspecifieke effecten van chloroquine kwam. De resultaten in hoofdstuk 4 suggereerden dat het sturen van lipide metabolisme in Tn cellen specifiek onder dyslipidemische omstandigheden effect heeft op hun fenotype in de effector fase.

In **hoofdstuk 5** is beschreven wat het gebrek van T cel autofagie na deletie van het eiwit Atg7 doet met de ontwikkeling van atherosclerose. Dit hebben we onderzocht met behulp van muizen met T cel specifieke deficiëntie van Atg7 (de Lck-Cre $Atg7^{f/f}$ muis) die we vergeleken hebben met muizen met Atg7 sufficiënte T cellen (de $Atg7^{f/f}$ muis). Aangezien deze muizen normaal geen atherosclerose ontwikkelen hebben we dit moeten induceren met behulp van een virus dat het eiwit PCSK9 tot overexpressie brengt, hetgeen, samen met een vetrijk dieet, zorgt voor een vervette lever en de vorming van atherosclerose. De ontwikkeling van een vervette lever was sterk verminderd in Lck-Cre $Atg7^{f/f}$ muizen. Na verder onderzoek naar de T cellen in de lever bleek dat er procentueel een stuk minder T cellen aanwezig waren bij Atg7-deficientie maar dat relatief meer T cellen ontstekingsfactoren uitscheidden. Het grootste verschil zat echter in de natural killer (NK) T cel populatie die na Atg7 deletie vrijwel afwezig was in de lever, hetgeen overeenkomt met literatuur. De verminderde inflammatoire capaciteit van de totale T cel populatie in de lever is wellicht mede debet aan de verminderde ontwikkeling van vervetting. Naast verminderde leververvetting was de totale cholesterol concentratie in het serum sterk verminderd in muizen met T cel-specifieke deletie van Atg7. De combinatie van verminderd circulerend cholesterol en verminderde inflammatoire capaciteit van de T cel populatie hebben bijgedragen aan de sterke vermindering in atherosclerose in Lck-Cre $Atg7^{f/f}$ muizen ten opzichte van $Atg7^{f/f}$ muizen. De bevindingen uit hoofdstuk 5 suggereren dat de T cel specifieke remming van autofagie de vorming van atherosclerose kan remmen. Daarnaast zou systemische behandeling met een autofagie remmer vanuit de invalshoek van de T cellen ontstekingsremmende effecten kunnen hebben op atherosclerose en daarom wellicht als medicijn tegen hart- en vaatziekten gebruikt kunnen worden. Remmers van autofagie, zoals chloroquine, worden reeds gebruikt ter behandeling van auto-immuunziekten zoals reuma. Het onderzoek in hoofdstuk 5 onderschrijft dat remming van autofagie middels therapeutische interventie het immuunsysteem kan onderdrukken en zo wellicht bij kan dragen aan de preventie van hart- en vaatziekten.

In **hoofdstuk 6** hebben we het effect van Lcn2 deficiëntie onderzocht op de ontwikkeling van atherosclerose en systemische metabole parameters die het metabolisme van T cellen kunnen beïnvloeden. Lcn2 is betrokken bij de ontwikkeling van obesitas, wat samenhangt met de circulerende levels van het eiwit leptine. Leptine beïnvloedt verschillende metabole processen in T cellen, te weten, glucosemetabolisme en autofagie. Daarnaast heeft het effect op de activiteit van mTOR. Lcn2 draagt ook bij aan de ontwikkeling van insuline resistentie en lever vervetting, wat suggereert dat deficiëntie van Lcn2 indirect effect kan hebben op T cel metabolisme. In hoofdstuk 6 hebben we echter aangetoond dat de afwezigheid van Lcn2 in ons atherosclerose model geen effect heeft op gewichtstoename, gevaste glucose waarden of circulerende cholesterol- of triglyceride niveaus. Literatuur heeft beschreven dat Lcn2 directe effecten heeft op

andere ontstekingscellen. Zo kan Lcn2 de differentiatie van verschillende macrofaagfenotypes moduleren en is het betrokken bij neutrofiel migratie en functie. De focus van hoofdstuk 6 lag dan ook niet op de effecten van Lcn2 deficiëntie op het metabolisme van T cellen maar op de ontwikkeling van atherosclerose. Muizen zonder Lcn2 ontwikkelden grotere atherosclerotische laesies na 6 weken vetrijk dieet wat suggereerde dat Lcn2 in vroege fase van atherosclerose ontwikkeling van laesies remt. Omdat we een verhoogde activatie van monocyten observeerden postuleren wij dat Lcn2 deficiëntie in de vroege fase van atherosclerose de laesie ontwikkeling stimuleert door monocyt migratie naar de vaatwand te bevorderen. In latere fases van atherosclerose ontwikkeling was Lcn2 deficiëntie geassocieerd met een kleinere necrotische kern van de plaque en met een verminderde activiteit van eiwitten die de extracellulaire matrix afbreken in de vaatwand. Deze twee observaties suggereerden dat Lcn2 in de latere fases van atherosclerose ontwikkeling een negatief effect heeft op stabiliteit van de laesie. Dit komt overeen met klinische observaties waar hoge niveaus van Lcn2 in het bloed en de laesie geassocieerd zijn met symptomatische atherosclerose. Dus, Lcn2 heeft in proefdiermodellen voor atherosclerose verschillende effecten in verschillende fases van de ontwikkeling van atherosclerotische laesies, wat wellicht bij kan dragen aan het gebruik van Lcn2 als biomarker voor vroege fases van atherosclerose ontwikkeling. Aangezien Lcn2 een essentieel bacteriostatisch eiwit is tijdens de acute fase van bacteriële infectie is de ontwikkeling van therapieën die Lcn2 remmen ter preventie van hart- en vaatziekten wellicht minder wenselijk.

Het moduleren van het immuunsysteem voor therapeutische doeleinden in hart- en vaatziekten heeft de afgelopen tientallen jaren steeds meer aandacht gekregen als aanvulling op bestaande behandelingen voor atherosclerose. In dit proefschrift is met een nieuwe invalshoek gekeken naar een bekende risicofactor voor atherosclerose, namelijk dyslipidemie. We hebben beschreven wat een aantal antigen-onafhankelijke effecten van dyslipidemie zijn op metabole processen in T cellen en de functionele effecten hiervan. Aangezien metabolisme in T cellen een cruciale factor is in hun inflammatoire capaciteit en het metabole fenotype mede bepaald wordt door de metabole omgeving, is het een logisch doelwit voor immunosuppressieve therapie. Een complicerende factor bij het moduleren van T cel metabolisme als therapie voor atherosclerose is dat het lastig is om intracellulaire processen specifiek in T cellen te moduleren. Meer onderzoek is nodig om methoden te ontwikkelen om dit te bewerkstelligen. Wanneer dit eenmaal mogelijk is, zal de volgende stap naar therapie wellicht minder ingewikkeld zijn omdat reeds in de kliniek gebruikte medicijnen, zoals rapamycine, gebruikt kunnen worden met een minimale kans op bijwerkingen. Een groot voordeel van het moduleren van T cel metabolisme als therapie voor atherosclerose, ten opzichte van bijvoorbeeld vaccinatie, is dat het niet afhankelijk is van een antigeen. Dit is een voordeel omdat voor een groot aantal welvaartsziektes met een T cel component hét pathogene anti-

geen nog niet bekend is. Dit komt deels omdat er in mensen een grote heterogeniteit bestaat in het type humaan leukocytantigeen molecuul, wat bepaalt welke antigenen gepresenteerd worden aan T cellen. Het fine-tunen van metabolisme in T cellen voor therapeutische doeleinden overkomt dit probleem maar verder basaal en translationeel onderzoek is nodig om vast te stellen of deze benadering geschikt is voor de behandeling van atherosclerose en hart- en vaatziekten.

Dit onderzoek heeft een aantal antigeen-onafhankelijke pathophysiologische mechanismen beschreven waarmee dyslipidemie de T cel-gemedieerde immuunrespons kan moduleren. Daarnaast onderbouwt het onderzoek in dit proefschrift dat het moduleren van cellulair metabolisme en autofagie in T cellen geschikt is om ontsteking te verminderen, atherosclerose te remmen en zo hart- en vaatziekten te voorkomen. Verder onderzoek is nodig om uit te zoeken wat de effecten van dyslipidemie zijn op andere typen T cellen op verschillende locaties in het lichaam en gedurende verschillende fases van de ontwikkeling van atherosclerose. Uiteindelijk zullen de bevindingen uit dit proefschrift vertaald moeten worden naar de humane situatie en zo hopelijk nieuwe therapeutische mogelijkheden voor de behandeling van atherosclerose opleveren.

Curriculum Vitae
Scientific publications
PhD portfolio

CURRICULUM VITAE

Jacob Amersfoort werd geboren op 8 maart 1990 in Leiden. In juni 2008 behaalde hij zijn VWO diploma aan het Andreas College te Katwijk. In datzelfde jaar begon hij aan de studie Biomedische Wetenschappen aan de Universiteit Leiden. Hij behaalde de Bachelor of Science graad in maart 2012. Van oktober 2012 tot maart 2013 deed hij zijn eerste masterstage bij het Edinburgh Cancer Research Centre onder begeleiding van dr. E. Murray en prof. dr. T. Hupp. Deze stage werd afgesloten met een verslag getiteld: The use of transcription activator-like effector nucleases as a novel tool for the manipulation of p53 key players. Van september 2010 tot april 2013 was hij in het kader van de Netherlands Epidemiological Obesity (NEO) studie werkzaam als student-assistent bij de afdeling Radiologie van het Leids Universitair Medisch Centrum. Van juni 2013 tot januari 2014 deed hij een tweede masterstage onder begeleiding van M.B. Bizino en prof. dr. H.J. Lamb bij de afdeling Radiologie van het Leids Universitair Medisch Centrum. De titel van het onderzoeksproject was "The Human Heart in 3D: MR Image Analysis and Acquisition Technique Development" en heeft geleid tot een publicatie in het blad *European Radiology*.

Van april 2014 tot november 2018 was hij als promovendus werkzaam bij de afdeling BioTherapeutics van het Leiden Academic Centre for Drug Research onder begeleiding van dr. I. Bot en prof. dr. J. Kuiper.

Per 1 februari 2019 is hij aangesteld als post-doctoraal onderzoeker bij het Center for Cancer Biology van de KU Leuven – Campus Gasthuisberg in Leuven onder begeleiding van prof. dr. P. Carmeliet.

SCIENTIFIC PUBLICATIONS

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PHD PORTFOLIO

Courses

2015 LACDR Data management course
2015 On being a scientist (Ethics in science)
2014 Introduction to teaching and supervision for LACDR PhD students
2014 Time management
2014 Effective communication
2014 PhD Introductory course on drug research

Presentations

2018 Scandinavian Society for Atherosclerosis Research, Humlebæk, Denemarken
2017 Rembrandt Symposium, Noordwijkerhout, Nederland
2017 LACDR Spring Symposium, Leiden, Nederland
2017 Keystone Conference Integrating Metabolism and Immunity, Dublin, Ierland
2017 Scandinavian Society for Atherosclerosis Research, Humlebæk, Denemarken
2016 Rembrandt Symposium, Noordwijkerhout, Nederland
2016 Seahorse User Meeting, Amsterdam, Nederland
2016 LACDR Spring Symposium, Leiden, Nederland
2015 Rembrandt Symposium, Noordwijkerhout, Nederland
2015 3rd International Conference on ImmunoMetabolism: Molecular and Cellular Immunology of Metabolism, Chania, Griekenland
2015 LACDR Spring Symposium, Leiden, Nederland
2014 Rembrandt Symposium, Noordwijkerhout, Nederland
2014 Seahorse User Meeting, München, Duitsland
2014 LACDR Spring Symposium, Leiden, Nederland