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

Amersfoort, J.

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.

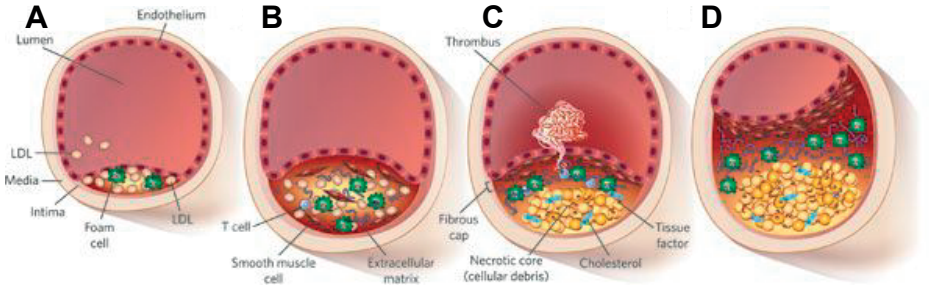

Figure 1 Development of atherosclerotic lesion. (A) Atherosclerosis is initiated after endothelial dysfunction causes monocyte recruitment and lipoproteins such as low-density lipoprotein (LDL) to infiltrate the subendothelial space (intima). Monocytes differentiate locally to macrophages which engulf 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

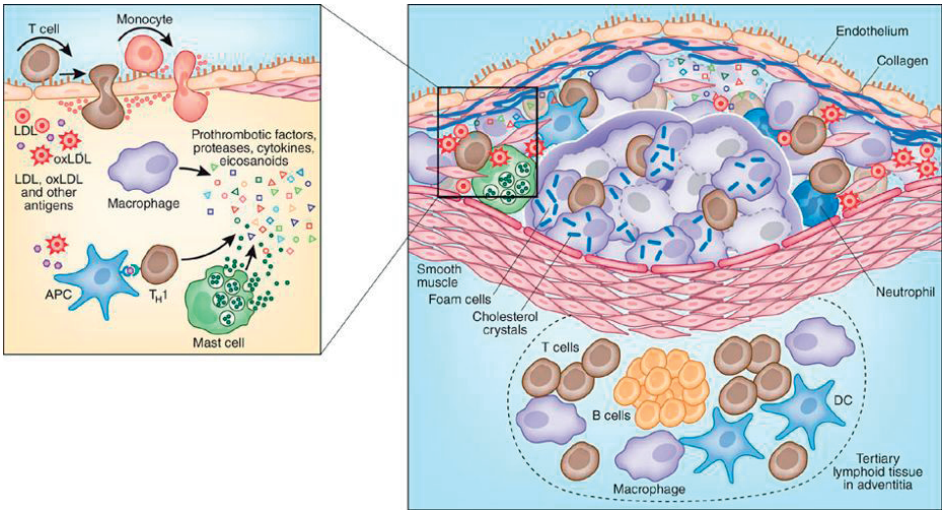

Figure 2 Immune cell types in atherosclerosis. Inflammation in the lesion is largely but not exclusively mediated by macrophages and T cells. Monocyte-derived macrophages take up (modified) lipoproteins such as oxLDL and secrete atherogenic factors such as proteases and inflammatory cytokines, thereby 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

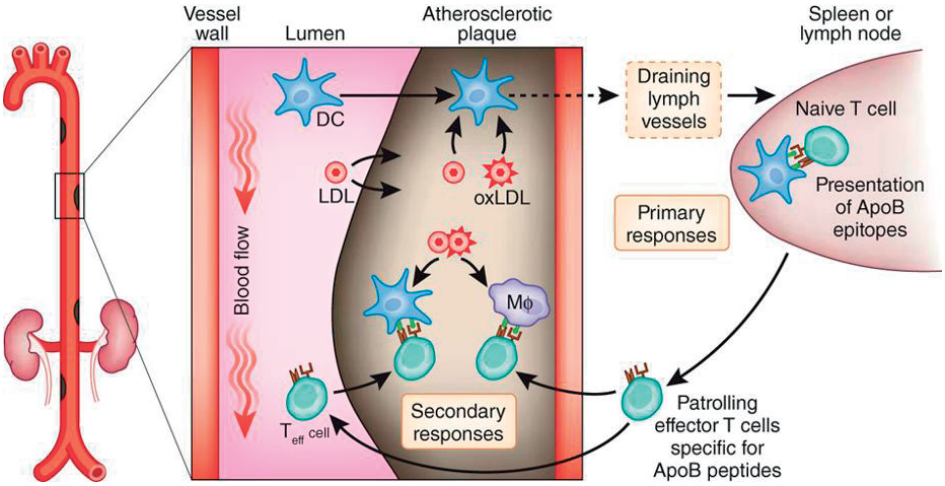

Figure 3 T cell response in atherosclerosis. A T cell response in atherosclerosis is initiated when DCs migrate to the atherosclerotic plaque and engulf native LDL or modified LDL (oxLDL) and subsequently migrate to a lymph node via the draining lymph vessels. Alternatively, blood-borne antigens can 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

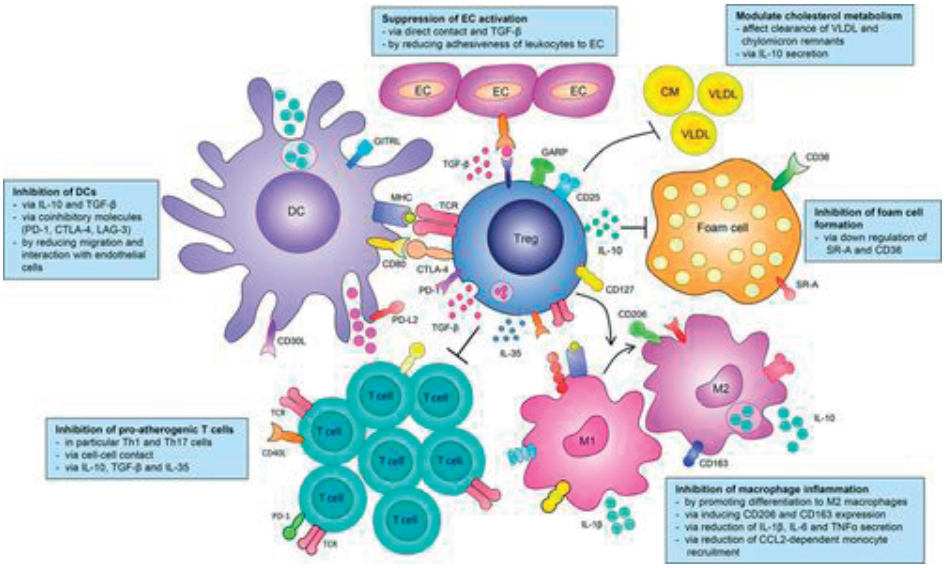

Figure 4 Treg cell suppression of atherogenic immune cell mechanisms. Through cytokine secretion and direct cell-cell interactions, Treg cells inhibit various atherogenic mechanisms and cell types. Treg cells can inhibit EC activation and foam cell formation which are both main mechanisms involved in 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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