Modulation of the immune system for treatment of atherosclerosis

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General Introduction

Cardiovascular Disease

Over the last century, through enhanced hygiene and introduction of vaccination programs, the number of deaths caused by infectious diseases has been reduced tremendously *(1–4)*. With resulting increased age, and changes in lifestyle, mortality due to cardiovascular diseases and cancer have become the most important causes of death in the developed world *(1–4)*. Cardiovascular diseases are currently the number one cause of death worldwide and comprise all diseases related to the heart and vasculature. Myocardial infarction and ischemic stroke account for the vast majority of cardiovascular disease related deaths *(1)*. For both conditions atherosclerosis is the underlying pathology *(5)*.

Major risk factors for cardiovascular disease through induction of atherosclerosis, include dyslipidemia and hypercholesterolemia in particular, smoking, sedentary lifestyle, hypertension, obesity, and stress *(6)*. To reduce mortality rates due to CVD, governments have imposed awareness programs, including advice on healthy food intake and lifestyle to reduce incidence of cardiovascular events. These awareness programs and introduction of cholesterol lowering drugs have reduced the number of cardiovascular deaths in Europe and the United States over the last decades *(1)*. In recent years however, the decrease in CVD related deaths has stalled and even increased again in the United States due to enhanced incidence of obesity and type 2 diabetes *(7)*, important risk factors for atherosclerosis. Cardiovascular diseases remain the primary cause of death worldwide and pose a great economic burden, costing $555 billion in 2015 in the United States and expected to rise in coming years *(8)*, and €210 billion in Europe *(9)*.

Atherosclerosis is characterized by the accumulation of lipids, including cholesterol, in the medium to large sized arteries, forming lipid rich gruel (“athere” in Greek)-like lesions. As cholesterol accumulates in the vessel wall, immune cells are attracted to these sites and take part in a pathogenic immune response *(10)*. Over decades of atherosclerotic lesion development, plaques can grow unnoticed in size and complexity, and can accumulate cholesterol crystals, and collagen and calcium deposits, causing hardening (“sclerosis” in Greek) of the vessel wall. Atherosclerosis becomes clinically relevant when the plaque has grown to such a volume that it directly limits blood flow towards down-stream tissues, or when atherosclerotic plaque components come in contact with the blood leading to formation of a (dissociated) thrombus which occludes an artery *(10)*. In both cases, arterial occlusion can lead to oxygen deprivation of down-stream tissues. Severe acute obstruction of arteries by a thrombus can lead to life threatening conditions, including myocardial infarction and ischemic stroke *(10)*.

Treatment of atherosclerosis has classically focused on adapting a healthy lifestyle and reducing cholesterol levels, ignoring the ensuing pathogenic immune response resulting from the cholesterol accumulation in the vessel wall. In line with an active atherogenic role for the

immune system in atherosclerosis continuous immune activation as present in, systemic lupus erythematosus (SLE), and HIV, Chlamydia pneumoniae, and even dental infection, have been suggested to be associated with atherosclerosis *(11–13)*. Only very recently, the potential for treatment of atherosclerosis by immunomodulation was shown in humans in the CANTOS trial, in which administration of neutralizing antibodies against IL-1β reduced major cardiovascular events by up to 15% *(14, 15)*. As many CVD patients, even after successful lipid lowering, have an increased risk for a cardiovascular event due to inflammation, developing strategies to control the pathogenic immune response in atherosclerosis is vital for a next step in the successful treatment of atherosclerosis *(16)*.

Cholesterol

As previously indicated, arterial accumulation of cholesterol is a hallmark of atherosclerosis, playing a causal role in the pathogenesis of atherosclerosis. Cholesterol is however a vital building block of cellular membranes, determining membrane fluidity *(17)* and essential for formation of lipid rafts *(18)* which are important in cell signaling. Moreover cholesterol is an essential precursor for bile acids and steroid hormones *(19)*. Intracellular free cholesterol is however toxic, and therefore unused intracellular is quickly esterified and overall cholesterol levels are tightly regulated. Most cells acquire cholesterol through uptake of cholesterol by uptake of cholesterol rich particles from circulation, and through de novo synthesis of cholesterol. Both pathways are promoted in cholesterol deprived cells by proteolytical activation of the sterol regulatory element-binding protein 2 (SREBP-2) transcription factor, upregulating the expression of proteins involved in cholesterol synthesis including the rate limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), and the upregulation of the LDLr which facilitates uptake of cholesterol rich particles from circulation *(20)*. Statins, which are currently used to lower cholesterol levels act through inhibition of HMGCR, thereby reducing de novo cholesterol synthesis, leading to a low intracellular cholesterol level and consequently increasing expression of the LDL receptor and uptake of cholesterol from the blood plasma *(21)*. When cellular cholesterol levels are high, cholesterol metabolites promote the activation of the liver X receptor (LXR) *(22)*. Since most cells do not possess the capability to catabolize cholesterol, reduction of the cellular cholesterol level is dependent on LXR mediated upregulation of cholesterol efflux transporters, including ATP-binding cassette transporters (ABC), promoting reverse transport of cholesterol *(22)*. LXR also promotes the 2 known pathways by which cholesterol can be eliminated from the body, namely through hepatic biliary cholesterol excretion and trans-intestinal cholesterol excretion *(23)*.

Since cholesterol and lipids are insoluble in blood plasma, transport of cholesterol and triglycerides in the body is mediated by particles called lipoproteins. Lipoproteins consist of a single outer layer of phospholipids, and cholesterol, with hydrophilic polar groups facing outwards, and lipophilic groups facing the core of the particle which contains triglycerides

and cholesterol esters. The outer layer also contains proteins important for the function of the lipoprotein, called apolipoproteins *(21)*. These apolipoproteins are essential for the formation of lipoproteins and aid in determining size, and structure of the lipoproteins, and harbor binding sites for receptors through which it allows the lipoprotein to deliver triglycerides and cholesterol to cells or can be taken up as a whole *(21)*. Through the apolipoprotein and lipid content, lipoproteins are often separated into distinct groups, including chylomicrons and chylomicron remnants, Very Low Density lipoprotein (VLDL), Intermediate low density lipoprotein, low density lipoprotien (LDL), and high density lipoprotein *(21)*.

In the small intestines, enterocytes take up dietary cholesterol and cholesterol from the bile. To transport the lipids taken up by enterocytes, enterocytes load lipids onto apolipoprotein B-48 (apoB48), forming nascent chylomicrons. Chylomicrons are the largest of lipoproteins and vary in diameter from 75 to 600 nm *(24)*. Chylomicron size depends on the flux of triacylglycerol through the enterocyte, resulting in larger chylomicrons during fat absorption *(25)*. The main protein constituent of chylomicrons is ApoB48, a truncated form of apolipoprotein B-100 (apoB100). In human apoB48 is solely produced in the intestines due to selective intestinal expression of apobec-1. Apobec-I is the catalytic subunit of a protein complex that executes the site specific C to U mRNA editing of ApoB100 mRNA at amino acid 2152, introducing a stop codon which results in production of the truncated 2152 amino acid long apoB48 protein, which thereby lacks the 2384 amino acid long C-terminal sequence of the full apoB100 protein *(26–28)*. Nascent chylomicrons are released in the lymph, and after entering the circulation chylomicrons obtain apolipoprotein C-II (apoC2) and apolipoprotein E (apoE) from HDL or VLDL. Interaction of apoC2 with lipoprotein lipase (LPL), primarily bound to the endothelium of capillaries from muscle and adipose tissue, promotes the hydrolysis of triglycerides by LPL, releasing free fatty acids (FFAs) from the chylomicron for local or systemic use *(29)*. Chylomicrons and chylomicron remnants are primarily cleared from circulation by the liver through apoE stimulated endocytosis *(30–32)*. ApoE can interact with several molecules promoting lipoprotein clearance, including heparan sulfate proteoglycans, the LDL receptor (LDLr), the LDL receptor-related protein (LRP), and the VLDL receptor in the liver *(30–33)*.

To ensure a steady supply of triglycerides and cholesterol to extrahepatic tissues, the liver packages triglycerides, and cholesterol, in nascent VLDL particles by loading lipids on apoB100. After the release of nascent VLDL into the bloodstream, apoC and apoE are quickly incorporated in the particle, thereby forming mature VLDL from which triglycerides can be hydrolyzed like is the case in chylomicrons *(34)*. Through hydrolysis of triglycerides VLDL is transformed into a smaller and more cholesterol rich IDL particle which can be cleared from circulation through its interaction of ApoE and Apob100 with previously mentioned hepatic receptors promoting lipoprotein clearance *(30–33)*. Alternatively, more TG is hydrolyzed from IDL by hepatic lipase, transforming IDL in cholesterol rich LDL particles *(35)* which also loose ApoC and ApoE in the process. The function of LDL is the delivery of cholesterol to

extrahepatic cells, which occurs through receptor mediated endocytosis of the LDL particle through interaction of apoB100 with the LDLr *(36)* and heparan sulfate proteoglycans *(33)*. Although all ApoB containing particles are considered atherogenic, especially the cholesterol rich LDL particle are deleterious in atherosclerosis development *(37, 38)*.

Excess cholesterol can be transported back to the liver by interaction of HDL with cellular cholesterol efflux transporters in a process called reverse cholesterol transport *(22, 39)*. In the liver cholesterol can be repackaged in VLDL or excreted with the bile. HDL is produced in the liver and intestine through interaction of apolipoprotein A-I (ApoA1) with ATB-binding cassette transporter A1 (ABCA1), resulting in the essential lipidation of ApoA1 and thereby forming nascent β-HDL *(40)*. Through uptake of cholesterol and phospholipids the HDL particle matures and is cleared from circulation by the liver through interaction with hepatic scavenger receptor class B type 1 (SR-B1) *(41)*. Since HDL transports excess cholesterol from peripheral tissues to the liver, HDL is considered anti-atherogenic.

Experimental models of atherosclerosis

A lot of our understanding of the pathogenesis of atherosclerosis is derived from studies with in vivo experimental models. Murine models of atherosclerosis are most commonly used in experimental atherosclerosis studies, among other things due to development of atherosclerotic lesions in a relative short time frame, easy handling, cheap housing, and availability of tools that allow manipulation of murine DNA. As C57/BL6 mice do not develop atherosclerotic lesions upon feeding them a western type diet (WTD), currently used models involve ApoE *(42, 43)* or LDLr deficiency *(44)*, or a functionally impaired isoform of ApoE (ApoE*3) *(45)* to promote hypercholesterolemia and atherosclerosis.

As previously discussed, ApoE is present in chylomicrons and VLDL but not in LDL, and promotes lipoprotein clearance through interaction with multiple receptors. Deficiency or sub-optimal ApoE function therefore leads to a rise in predominantly VLDL levels but also decreases HDL levels *(42, 43)*. Cholesterol levels in $ApoE^{-/-}$ mice on chow diet are approximately 5 times higher (~400 mg/dl) than in control mice and slowly lead to atherosclerotic lesions. Plaque development can be accelerated by feeding $ApoE^{-/-}$ mice a western type diet (WTD) containing cholesterol. Since ApoE has been reported to influence antigen presentation and other inflammatory processes, $LDLr^{-/-}$ mice are better suited to study T cells and the immune system in the context of atherosclerosis *(46)* .

Since the LDLr is the main mediator in ApoB100 mediated uptake of LDL, which is devoid of ApoE, $LDLr^{-/-}$ mice accumulate mainly LDL when being fed a western type diet *(44)*. LDLr deficiency induces only a very modest increase in cholesterol on chow diet (~225 mg/dl), and as a consequence $LDLr^{-/-}$ mice have marginal plaque development on chow diet and depend on a WTD containing cholesterol to develop advanced atherosclerotic lesions. As LDLr surface expression is lowered by PCSK9, adenoviral induced hepatic overexpression of PCSK9 can

reduce LDLr expression in mice otherwise expressing the LDLr *(47)*. This is particularly useful to circumvent extensive cross-breeding efforts for the assessment of the effect of certain genotypes in atherosclerosis.

To further study the function of lipoproteins and further humanize mouse models of atherosclerosis several adaptations have been made to the preexisting LDLr and ApoE based models. These experimental models include mice expressing solely ApoB48 or just ApoB100 *(36, 48)*, and mice expressing human ApoB100. Since the human liver does not express Apobec-1, whereas mice do, the human liver solely produces ApoB100 containing VLDL, while mice also produce VLDL containing ApoB48. Because of this, and since thymic selection reduces the frequency of T cell clones recognizing endogenously expressed antigens, we used $LDLr^{-/-}$ human ApoB100 transgenic mice (HuBL mice) *(36, 48)* in Chapter 4 in which we assessed the effect of modulation of the immune response to human ApoB100 derived peptide p210 on atherosclerosis *(49)*. To allow the study of CD8 epitopes relevant for vaccination in humans in the context of atherosclerosis (chapter 5), we crossbred HuBL mice with mice transgenic for the most common human MHC-I molecule (HLA-A2) *(50)*.

Atherosclerosis development

Endothelial dysfunction

Despite the even distribution of lipoproteins throughout the arterial system, atherosclerotic lesion development occurs in well-defined areas in the arterial tree. At branches, bends, and bifurcations of medium to large sized arteries, disturbed steady laminar flow predisposes the vessel wall to the development of atherosclerosis *(51)*. Even before initiation of atherosclerotic lesion development, thickening of the intima is visible at atherosclerosis prone region (Fig. 1A) *(52)* The innermost monolayer of the vessel wall, the endothelial cell layer, exerts some essential roles in vascular biology as it is involved in regulation of vascular tone, vessel remodeling, angiogenesis, nutrient permeability, coagulation, fibrinolysis, and inflammation *(53)*, and plays a key role in the predisposition of sites with disturbed laminar flow to develop atherosclerosis. For proper endothelial function, endothelial cells need to align with the laminar flow direction. To correctly align, endothelial cells possess mechanoreceptors through which they sense direction and force of the shear stress *(54)*. Correct alignment and constant laminar flow induces transcription factors Kruppel like factor (KLF)2, KLF4, and nuclear factor (erythroid-derived 2)–like 2 (NRF2), which in turn promote the upregulation of immunosuppressive, antioxidative, vasodilative, and antithrombotic gene expression in endothelial cells *(54–56)*. Endothelial cells facing low, multidirectional, or oscillatory flow, as present in atherosclerosis prone areas, fail to elongate and align to the flow direction, leading to endothelial dysfunction and upregulation of pro-inflammatory genes *(54)*. Deletion of the mechanosensory protein proteoglycan syndecan 4, essential for correct endothelial cell alignment with the laminar flow direction, induces atherosclerotic lesion formation in hypercholesterolemic mice in sites normally protected from

atherosclerosis *(54)*, stressing the importance of endothelial alignment and endothelial health to prevent atherogenesis.

Endothelial dysfunction promotes atherosclerosis in several ways. Through disturbance of intercellular connections, permeability of the endothelial layer is enhanced which allows plasma constituents to enter the subendothelial space *(57, 58)*. Serum derived lipoproteins have been reported to be preferentially retained in the subendothelial space over other serum constituents. Preferential retention of lipoproteins in the subendothelial space has been dedicated to different mechanisms. On the one hand apolipoproteins are known to harbor sites that have proteoglycan binding properties that could tie lipoproteins to extracellular matrix proteoglycans in the subendothelial space, causing entrapment of the lipoproteins *(59)*. On the other hand, the concentration of serum derived molecules in plaques strongly correlates with their plasma concentration and molecular size. This could indicate that the preferential accumulation of lipoproteins in the subendothelial space compared to other serum proteins is due to decreased egress of larger molecules from the subendothelial space depending on size in a process called molecular sieving *(60, 61)*. Moreover endothelial dysfunction goes hand in hand with upregulation of chemotactic molecules on endothelial cells, including CCL2, and adhesion molecules such as ICAM-1, VCAM-1, and E-selectin/P-selectin, recruiting atherogenic immune cells to the vessel wall *(62–64)*.

Early atherosclerosis

The lipoproteins entrapped in the subendothelial space are prone to undergo oxidative modifications which further activate the endothelium, which responds by upregulation of CCL2, E-selectin and P-selectin and upregulation of ICAM-1 and VCAM-1, promoting rolling of leukocytes, and tight adherence and leukocyte transmigration respectively *(65–69)*. In the initial stages of atherosclerosis primarily monocytes are recruited from circulation to lipid rich areas in the vessel wall, forming so called fatty streaks (Fig. 1B). Fatty streaks are asymptomatic and might be reversible by reduction of circulating cholesterol levels. Monocyte recruitment to these fatty streaks is clearly pathogenic, as lesion formation is nearly abolished by inhibition of chemokines and chemokine receptors involved in recruitment of monocytes *(70)*. Environmental cues in the subendothelial space, including oxidized lipoproteins and pro-inflammatory cytokines derived from activated endothelium, SMCs, and other immune cells, induce differentiation of monocytes into macrophages *(71–74)*. Oxidized LDL is a strong ligand for macrophage scavenger receptors including CD36 and SR-A1, and is often complexed with antibodies, leading to uncontrolled uptake of oxLDL by FC receptors and scavenger receptors on the phagocytic macrophages *(75)*. When the cellular intake of cholesterol exceeds the cellular capacity of the macrophage to use or efflux cholesterol, cholesterol esters accumulate in the macrophage in lipid droplets, giving the macrophages a foamy appearance. These so called foam cells secrete a plethora of cytokines

which promote the recruitment of other (immune) cells *(75)*, including neutrophils, dendritic cells, T cells, and smooth muscle cells (SMCs).

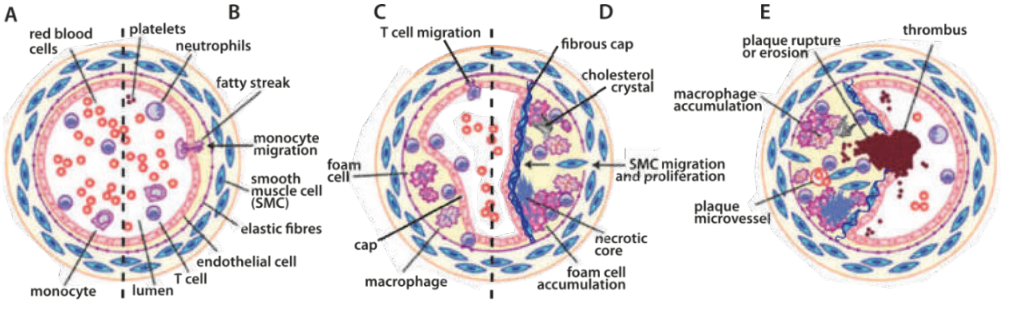

Fig. 1 Atherosclerotic lesion development. *Over decades of atherosclerosis development, atherosclerotic lesions can advance through various stages. (**A**) Initially atherosclerosis prone regions are marked by endothelial dysfunction and intimal thickening. (**B**) Endothelial dysfunction facilitates the entry of lipoproteins and monocytes into the subendothelial space. In the subendothelial space monocytes differentiate into macrophages and take up cholesterol rich lipoproteins. When more cholesterol is ingested than can be handled by the macrophage, macrophages convert to foam cells. The formation of foam cells and presence of small lipid droplets in the artery wall is characteristic for a fatty streak. (**C**) Foam cells attract more immune cells to the plaque, promoting further lipid accumulation in the plaque. Formation of a large lipid core marks the atheroma stage. The lipid core is shielded from the lumen by a cap structure, which prevents thrombotic events. (**D**) In the more progressed fibroatheroma, collagen, primarily derived from SMCs, and SMCs reinforce the cap structure. High cell-death in combination with insufficient efferocytosis leads to formation of large necrotic cores and calcifications in the advanced fibroatheroma. Over time, presence of free unesterified cholesterol in the vessel wall promotes the formation of cholesterol crystals, which are therefore more prominent in advanced atherosclerotic plaques. Another marker for advanced plaques is microvessels which are formed due to hypoxia in advanced plaques. (**E**) Thinning of the fibrous cap and plaque or erosion lead to the formation of vulnerable plaques. Plaque rupture and severe endothelial erosion cause plaque derived clotting factors to come in contact with coagulation factors in the blood, and leads to formation of a thrombus. Edited from: Inflammation and atherosclerosis in rheumatoid arthritis, R.J. Stevens, K.M.J. Douglas, A.N. Saratzis and G.D. Kitas. Expert Reviews in Molecular Medicine (2005), vol: 7 (7) pp: 1-24. Expert reviews in Molecular Medicine (2005)*

Advanced atherosclerosis

Often though, initial lesions are not resolved, and lipid deposition and chronic inflammation of the vessel wall persist over decades leading to growth and remodeling of the lesion, and attraction of more immune cells (Fig. 1C). When small lipid pools fuse to form a large lipid core the atherosclerotic lesion is classified as anatheroma. Initially, the lipid core is shielded from the vessel lumen by a thin layer of endothelial cells (Fig. 1C). Under influence of inflammatory cytokines released in the atheroma, SMCs dedifferentiate and migrate from the media into the lesion and secrete ECM components, including collagen, promoting

stability. Furthermore, SMCs can form cell layers below the endothelial cell layer, aiding in formation of a stable fibrous cap, the hallmark feature of fibro-atheroma's (Fig. 1D). Although SMCs are mainly considered to be atheroprotective due to their plaque stabilizing features, recent reports indicate that over half of lesional foam cells in advanced atherosclerotic lesions could actually be SMC derived *(76–79)*. The pro-inflammatory macrophage foam cell like SMCs can produce pro-inflammatory and plaque destabilizing factors, contributing to atherosclerosis *(79)*. Uptake of excessive amounts of cholesterol by cells in the atherosclerotic lesion ultimately leads to cell death of foam cells in the lesion *(80)*. Probably due to insufficient phagocytic uptake of apoptotic cells, called efferocytosis, the vast majority of dying cells in the advanced fibro-atheroma are necrotic *(81)*. Cell necrosis leads to formation of calcifications and necrotic cores in the advanced fibro-atheroma (Fig. 1D). Moreover, necrotic cells release inflammatory cytokines, matrix degrading proteases and pro-angiogenic factors, promoting atherosclerosis and inducing plaque instability (Fig. 1E) *(81)*. During atherogenesis, the accumulation of unesterified cholesterol in the lesion leads to the formation of cholesterol crystals *(82)*, piling up in advanced atherosclerotic lesions. Superficial localization of cholesterol crystals in the plaque was found to be correlated with plaque instability *(83)* likely by piercing through plaque stabilizing structures. Moreover, cholesterol crystals can promote inflammation by activating the NLRP3 inflammasome *(84)*, and thereby contribute to atherosclerosis.

The development of atherosclerotic plaques can remain asymptomatic throughout its development. Growth of the atherosclerotic lesion, protruding into the vessel lumen is initially effectively counteracted by vascular remodeling, allowing the vessel lumen to remain its original size *(85)*. The enlargement of blood vessels is however limited by the connective tissue surrounding it. When a growing atherosclerotic lesion restricts lumen size to an extent that blood flow towards downstream tissues is very much limited, this leads to deprivation of downstream tissues from oxygen and nutrients, leading to a condition called stable angina *(86)*. The majority of cardiovascular deaths are however not caused by stable angina. Plaque destabilizing factors including SMC and endothelial cell death, and breakdown of ECM including collagen, by matrix metalloproteinases secreted by neutrophils, macrophages, dedifferentiated SMCs, and mast cells can weaken the plaque to such an extent that the plaque ruptures *(87)*. Alternatively, the endothelial cell layer can undergo such extensive cell death that the endothelial monolayer is unable to cover the atherosclerotic lesion in a process called plaque erosion *(87)*. Through both mechanisms plaque constituents come in contact with blood platelets which initiates the coagulation cascade leading to thrombus formation. When the thrombus does not break away from the lesion, and does not restrict blood flow to such an extent that down-stream is deprived of oxygen, the thrombus can be resolved asymptomatically *(88)*. Alternatively, the thrombus might occlude the artery at the atherosclerotic site or may break away from the lesion and travel with the bloodstream until it gets stuck in the narrowing arterial tree. Depending on the location of the occlusion in the arterial tree, the occlusion can lead to acute deprivation of oxygen and nutrients in

downstream tissues and if persistent for extended time, leads to tissue necrosis and potentially irreversible tissue damage. The acute deprivation of oxygen can be life threatening in the brain, leading to ischemic stroke, and in the heart, leading to acute myocardial infarction *(89)*.

Since atherosclerosis can have severe consequences, treatment regimens have been established to treat atherosclerosis at all stages. To combat the acute occlusion of an artery, thrombus dissolving drugs such as recombinant tissue plasminogen activator are administered as fast as possible, or in some cases the thrombus is removed with a stent retriever to restore blood flow to the infarcted areas. Vulnerable lesions or lesions partly occluding an artery can be scooped out of the artery in a surgical procedure called endarterectomy *(90)*. Moreover lesions that occlude an artery can be bypassed or a stent can be placed to allow steady blood flow, however both bypasses and stents are at risk for restenosis *(90)*. The preventive measures to lower risk of (re)occurrence of major cardiovascular events predominantly evolve around lowering LDL cholesterol, and involve lifestyle changes such as dietary adaptations, regular physical exercise, and no smoking *(6)*. If these behavioral interventions do not lower LDL-cholesterol sufficiently, statins are the first choice of treatment to lower cholesterol levels though inhibition of HMGCR, the rate limiting enzyme of de novo cholesterol synthesis *(21)*. Statin treatment has reduced major adverse cardiovascular events (mace) by 25-40%, however in some patients statins fail to reduce cholesterol levels and other patients do not tolerate statins very well *(16)*. Another pharmaceutical option to lower LDL-cholesterol levels is the inhibition of intestinal cholesterol absorption with ezetimibe, which inhibits the Niemann Pick C1 like 1 protein (NPC1L1) cholesterol transporter *(91)*. Although lowering of cholesterol is effective in lowering cardiovascular risk, some patients do not respond to lipid lowering treatment and many patients carry a residual risk to cardiovascular events due to unresolved inflammation, even after successful lipid lowering *(16)*. Administration of neutralizing antibodies against IL-1β in the CANTOS trial reduced major cardiovascular events by up to 15% in the higher dose groups *(14, 15)*, suggesting that there is still a world to win by modifying the immune response in atherosclerosis.

Immune system

Retention of oxLDL in the subendothelial space leads to a pathogenic immune response, which is the other driving force of atherosclerosis besides dyslipidemia *(92)*. Over the course of evolution, the immune system has evolved into a sophisticated network of cells and proteins that protects the host organism from disease causing microorganisms. Key requisites of the immune system are the ability to distinguish pathogens from host derived structures and harmless molecules, such as food components and degradation products. The immune system can be divided into the innate immune system and adaptive immune system, which fundamentally differ in the way by which they recognize (pathogenic) antigens. To

identify pathogens the innate immune system relies on receptors that recognize pathogen associated molecular patterns (PAMPs), such as viral double stranded RNA or bacterial lipopolysaccharides, which are not generated by the host *(93, 94)*. Moreover, innate immune cells can sense danger and tissue damage by interaction of damage/danger associated molecular patterns (DAMPs) with receptors that i.e. detect endogenous molecules at aberrant locations, such as extracellular DNA *(95)*. Depending on the PAMPs and DAMPs, and other environmental cues such as cytokines and interactions with other cells at the site of inflammation, innate immune cells can modulate their response, fitting to the type of pathogen or injury *(93–95)*. Uptake of pathogens by (innate) immune cells, and subsequent presentation of parts of the pathogens to cells of the adaptive immune system is a pivotal step for the involvement of adaptive immune cells in the inflammatory response *(96)*. Adaptive immune cells recognize a specific part of a specific pathogen, referred to as an antigenic epitope or epitope, with an antigen receptor that is non-variable per cell but highly variable between cells. Adaptive immune cells perform important effector and regulatory functions in the immune response and are indispensable for resolving many infections. After dealing with an infection, pools of adaptive immune cells with memory function remain, which allow for a more robust and faster immune reaction to a specific pathogen after reinfection. The antigen specific immune reaction in atherosclerosis is poorly understood *(97)*. Since subendothelial accumulation of lipoproteins is a hallmark of atherosclerosis, and antibodies against ApoB100 and oxidized phospholipids have been detected, as well as CD4 T cell responses against ApoB100, the current paradigm is that LDL forms the main antigen in atherosclerosis for the adaptive immune system *(97, 98)*. However, also immune reactions against other plaque components have been detected, including heat shock proteins *(99)* and type V collagen *(100)*. The adaptive immune response in atherosclerosis is generally regarded to be atherogenic, as it is dominated by pro-inflammatory adaptive immune cells. Because adaptive immune cells require antigen presentation and subsequent proliferation to acquire sufficient cell levels to impact disease, the acute phase of inflammation is dominated by innate immune cells. Because in atherosclerosis accumulation of cholesterol in the vessel wall persists, a chronic inflammatory response develops which involves innate and adaptive immune cells *(97, 98, 101)*. Although mast cells *(102)*, eosinophils *(103)*, NKT cells *(104)*, NK cells *(105)*, MDSCs *(106)* and ILCs *(107)* influence atherosclerosis development, these immune cells are beyond the scope of this thesis. Below, the relevant immune cells for this thesis will be discussed, being monocytes and macrophages, neutrophils, dendritic cells, T cells, and B cells, as well as the immunoproteasome, an immerging immune regulator.

Monocytes and macrophages

The initial hallmark of early atherosclerosis is the formation of foam cells in the subendothelial space of the artery vessel wall, in response to lipoprotein accumulation. In early atherosclerotic lesions, foam cells are predominantly derived from macrophages *(108)*. Although resident macrophages are present in the adventitia of (healthy) arteries, atherogenesis is largely dependent on macrophages derived from a blood derived cell

population, called monocytes *(109)*. Monocytes are produced in the bone marrow and can be subdivided in a pro-inflammatory/classical population ($Ly6C^{+}$ $CCR2^{+}CX3CR1^{low}$ in mice, $CCR2^{high}CD14^{+}CD16^{-}$ in humans), and an anti-inflammatory/patrolling monocyte population ($Ly6C^{low}CCR2^{low}CX3CR1^{high}$ in mice, $CX3CR1^{high}CD14^{dim}CD16^{+}$ in humans) *(110)*. Patrolling monocytes crawl over resting endothelium, remove debris from circulation, and are probably among the first cells to respond to inflammatory signals on the endothelium through their close interaction with the endothelium *(111, 112)*. Extravasation of patrolling monocytes is reliant on CX3CL1, produced by activated endothelium *(113)* and neointimal smooth muscle cells *(114)*. Upon exposure to DAMPs derived from the endothelium patrolling monocytes quickly attract neutrophils which promote necrosis of damage or infected endothelial cells, after which patrolling monocytes clean up the cellular debris *(111)*. If inflammation persists classical monocytes are attracted by CCL2 secretion, which is secreted by neutrophils and later on in atherosclerosis predominantly secreted by activated SMCs and macrophages in the plaque *(115)*. CCL2 secretion also promotes the release of inflammatory monocytes from the bone marrow *(116)*. Predominantly classical monocytes migrate into the atherosclerotic lesion and are considered to have a more pro-inflammatory phenotype than patrolling monocytes, however atherosclerosis studies in mice deficient for CCR2 *(117)* or CX3CL1 *(118, 119)* indicate that both monocyte populations are pro-atherogenic.

In the subendothelial space monocytes encounter stimuli that induce the differentiation of monocytes into macrophages (and monocyte derived DCs). Because of the plastic nature of monocytes *(120)*, and presence of opposing polarizing factors in the atherosclerotic plaque environment, a heterogeneous macrophage population is present in the plaque. The macrophage population has classically been divided into a pro-inflammatory macrophage subset (M1) and an anti-inflammatory subset (M2), mainly based on in vitro polarization studies. In the atherosclerotic lesion oxidized and aggregated lipoproteins promote a pro-inflammatory macrophage phenotype by TLR4 activation *(71, 72)*. Moreover, lesional cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) *(73)*, and IFN-γ *(74)* promote M1 macrophage polarization. These inflammatory macrophages have been termed M1 macrophages for their ability to reinforce Th1 responses through secretion of pro-inflammatory cytokines including IL-1-beta, TNF-α, IL-12, IL-18 and IL-23, and express high levels of co-stimulatory molecules CD80 and CD86, and high MHC-II levels *(121)*. M1 macrophages sustain inflammation, reinforce the pathogenic Th1 response observed in atherosclerosis, and are therefore detrimental in the context of atherosclerosis *(110)*.

Immune complexes *(122, 123)*, apoptotic cells *(124)*, macrophage colony stimulating factor (M-CSF) *(73)*, and complement components *(125, 126)* in the atherosclerotic lesion promote the development of M2 macrophages, which secrete Th2 cytokines including IL-4 and IL-13, and the immunosuppressive IL-10 and TGF-beta *(121)*. Macrophages have a strong capacity for phagocytosis through expression of several scavenger receptors and TLRs, and take up large quantities of plaque material, including lipoproteins and dead cells *(71–75, 123, 124)*. M2 macrophages are associated with the wound healing response and are considered anti-

atherogenic by counteracting inflammation and by enhanced uptake of dead cells, in a process called efferocytosis *(127)*. Since the original dichotomous delineation of M1 and M2 macrophages, several distinct M2 macrophage subsets have been recognized and a macrophage subset induced by oxidized phospholipids (Mox) *(128)*. Mox macrophages have a distinct phenotype from M1 and M2 macrophages and comprise 30% of macrophages in advanced atherosclerotic lesions in $LDLr^{-/-}$ mice, however their exact role in atherosclerosis remains to be evaluated *(128)*. Macrophages can quickly adapt to environmental cues, and in the process completely switch phenotype *(120)* or adapt a phenotype with mixed characteristics.

The accumulation of macrophages in the subendothelial space sets in motion an immune response that is largely pathogenic. Atherosclerosis is dominated by macrophages with a M1 phenotype, , coinciding with the Th1 response in the pathogenesis of atherosclerosis. M1 macrophage plaque content has been linked to decreased stability of advanced human plaques *(129)*. Macrophages can affect plaque stability in several ways. Through secretion of cytokines, and especially TNF-α, cell death is induced which promotes necrotic core formation and can affect cells with structural importance for the plaque *(130, 131)*. Moreover, macrophages can produce metalloproteinases which degrade matrix proteins, but can also produce matrix metalloprotease inhibitors which could lead to plaque stabilization *(132)*. Because of the central role of macrophages in the pathogenesis of atherosclerosis, modulation of the macrophage quantity and phenotype remain interesting treatment strategies for atherosclerosis, however may be limited due to their importance to combat infections.

Neutrophils

Neutrophils are produced in the bone marrow, originating from the granulocyte monocyte progenitor (GMP), from which also monocytes originate. Part of the produced neutrophils is stored in the bone marrow and can be recruited in response to inflammatory signals. Still, neutrophils are the most abundant leukocytes in circulation in humans, and are among first cells to be attracted to sites of inflammation by chemokines such as CXCL1, CXCL2, interleukin (IL)-1α and CCL2 *(133–135)*. At initiation of endothelial dysfunction, these chemokines would likely be predominantly derived from patrolling monocytes which are activated by DAMPs on the endothelium *(111)*. Later in atherosclerosis development, primarily plaque neutrophils, (M1) macrophages and activated SMCs would attract (more) neutrophils to the atherosclerotic lesion. Neutrophils possess strong phagocytic activity and carry granules in their cytoplasm, which can be secreted upon activation, which possess strong microbicidal activity. The lifespan of neutrophils is traditionally thought to be short, probably to prevent excessive inflammation, however GM-CSF, granulocyte colony-stimulating factor (G-CSF) and TNF-α *(136)* prolong the lifetime of neutrophils. Similar to monocytes and macrophages, neutrophils adapt to environmental cues and have been described to stimulate macrophage M2 polarization during helminth infection by secretion of IL-13 *(137)*, facilitate wound healing

of the skin *(138)*, and can adapt a regulatory phenotype during chronic inflammation *(139)* but are mostly known to act pro-inflammatory and induce M1 macrophage polarization during infection *(137)*.

In atherosclerotic lesions neutrophil numbers are generally low, probably because their short lifetime, however depletion of neutrophils with a Ly6G specific antibody (1A8) reduced atherosclerosis by 50 %, indicating that neutrophils do play a significant pro-atherogenic role. Neutrophils can potently attract classical monocytes to inflammatory sites through release of chemokines including, CXCL1, 2, 3 and 8, and can release granules containing azurocidin, which upregulate ICAM-1, VCAM-1, and E-selectin on endothelium, and enhance vascular permeability *(136)*. In line with an important role for neutrophils in attraction of classical monocytes to sites of inflammation, depletion of neutrophils reduces classical monocyte infiltration (108). In human atherosclerotic plaques, neutrophils often co-localize with M1 macrophages in vulnerable shoulder regions of the plaque, and are often enriched in sites of plaque rupture *(140, 141)*. Although causality of neutrophil accumulation at sites of plaque rupture has not been established, it is likely that neutrophils can promote plaque rupture, as neutrophils carry granules containing MMPs which break down ECM and could thereby destabilize the plaque *(140, 141)*.

Moreover, neutrophils have been described to induce plaque erosion, among other things through release of ROS inducing myeloperoxidase, and through neutrophil extracellular traps (NETs), inducing endothelial cell death *(87, 142, 143)*. NETs, produced in a process called NETosis, are composed of neutrophil derived granule proteins and chromatin. The NETs, form an extracellular web-like structure which can capture pathogens, but can also bind circulating platelets, coagulation factors, and VWF, promoting coagulation and thrombus formation *(87)*. The short lifespan of neutrophils leading to low neutrophil numbers in the plaque, might have been a reason why neutrophils have been overlooked in the pathogenesis of atherosclerosis, however also association studies in humans suggest that neutrophils are pro-atherogenic, and clinically relevant *(144)*

Dendritic cells

As professional antigen presenting cells, dendritic cells play an important role in instructing adaptive immune cells. Several different DC populations are identified, being plasmacytoid DCs (pDCs), type 1 and type 2 conventional DCs (cDC1/2), monocyte derived DCs (moDCs), and Langerhans cells *(145, 146)*. DCs can roam through peripheral tissues, but can also be tissue resident. Unactivated, immature DCs are specialized at sampling the environment, taking up antigens. Upon activation by DAMPs and PAMPs, dendritic cells stop sampling and migrate to lymphoid organs where they present the molecules that they have been taken up in the periphery *(147, 148)*. Depending on the encountered DAMPs and PAMPs, DCs can upregulate different co-stimulatory and co-inhibitory molecules and can produce a range of cytokines, skewing the adaptive T cell response following antigen presentation *(149)*. Activation of T cells is a pivotal event in establishing an effective adaptive immune response.

DCs are able to activate CD4 T cells through effective presentation of endocytosed antigens on MHC-II molecules *(149)*. Activation of CD8 T cells is reliant on loading of endocytosed antigens on MHC-I, and seems to be mainly reliant on the cDC1 population in vivo *(150–152)*. Besides its involvement in effective CD8 T cell activation, cDC1s are generally considered to be tolerogenic, whereas moDCs are pro-inflammatory *(145, 146)*.

In the healthy murine aortic intima primarily cDC1 and moDC subsets are found, which expand during atherogenesis *(153, 154)*. In line with a tolerogenic role of cDC1s, depletion of cDC1s aggravates atherosclerosis and limited the induction of Tregs *(153)*. As moDCs are ususally pro-inflammatory, it can be expected that moDCs have an opposing effect, and stimulate atherosclerosis. Similar to macrophages, DCs can take up oxLDL and can turn into foam cells in hyperlipidemic environments *(155)*. Depending on the antigens presented by DCs and their tolerogenic or pro-inflammatory status, DCs are capable of promoting atheroprotective and pro-atherogenic adaptive immune reactions. In the intestinal tract DCs with a tolerogenic phenotype are located that are known to confer immune tolerance to ingested substances, preventing immune reactions against ingested non self-molecules e.g. food components. Using this tolerogenic immune population to modulate the immune response towards atherosclerosis relevant antigens, including oxLDL, collagen, and HSPs could be a powerful tool to favorably modulate the adaptive immune response in atherosclerosis.

B cells and antibodies

The adaptive immune system contains a large heterogeneous adaptive immune population that is capable of specific recognition of (extracellular) 3d structures, being B cells. Although B cells can present antigens on MHC-II to T cells and thereby function as an APC *(156)*, and can produce inflammatory and inhibitory cytokines, probably the main function of B cells is the production of antibodies to protect the host from infection. Antibodies recognize (extracellular) 3d structures through variable regions which are created through somatic DNA rearrangements, resulting in an astounding number of antibody specificities of over 10^{15} that can be created *(157)*. Besides a variable region, antibodies possess an Fc-region, which dependent on the particular antibody isotype, harbors binding sites for Fc-receptors promoting Fc-receptor mediated uptake of the antigen, and regions promoting complement activation which can lead to further opsonization and lysis of a target cell *(158)*. Furthermore, binding of antibodies to certain regions on an antigen, can physically block specific molecular interactions. Although B cell levels in the atherosclerotic plaque are very low *(159)*, large number of B cells can be found artery tertiary lymphoid organs in the adventitia of advanced atherosclerotic plaques *(160, 161)*, and correlations between various serum immunoglobulin levels and atherosclerosis have been reported *(162)*, justifying studies into the role of B cells in atherosclerosis.

Initial studies indicated that B cells are atheroprotective, as adoptive transfer of splenic B cells reversed accelerated atherosclerosis in mice that had received a splenectomy *(163)*.

Transferred B cells derived from ApoE$^{-/-}$ mice were more effective inhibiting atherosclerosis than WT B cells in this experiment, indicating that hypercholesterolemia improved the atheroprotective capacity of the B cell population. Similarly, bone marrow transfer of B cell deficient μMT donors into lethally irradiated mice LDLr$^{-/-}$ led to increased atherosclerosis compared to transfer of WT bone marrow *(164)*, indeed indicating a atheroprotective role for the B cell population. More recent studies have indicated that the heterogeneous B cell population contains atherogenic and atheroprotective subtypes *(165, 166)*.

The majority of B cells belong to the B2 cell lineage, which develops in the bone marrow from a common lymphoid progenitor. After somatic DNA rearrangement membrane bound immunoglobulin M (IgM) is expressed on the cell membrane, forming immature B cells *(167, 168)*. After negative selection of immature B cells recognizing self-antigens, B2 cells are released from the bone marrow, after which they further mature into follicular (FO) B cells or marginal zone (MZ) B cells *(169)*. B2 cells have a relatively short half-life of a couple of days, unless they are activated by antigen recognition or innate signals *(170)*. FO B cells represent the largest group of B cells. When FO B cells encounter an antigen binding their IgM, the antigen is internalized and epitopes from the antigen presented on MHC-II while the B cell migrates to the T cell zones of lymphoid organs mediated by CCR7 upregulation *(171)*. Depending on the signals received from T follicular helper cells recognizing the antigen presented on MHC-II, FO B cells are stimulated to proliferate, and express antibodies with another constant region in a process called class switching *(172, 173)*. Th2 cells are very potent at promoting humoral responses, and induce class switching to IgE, murine IgG1 and human IgG4 *(174–176)*. Th1 cells are less effective at B cell activation and promote class switching to the IgG2 isotype *(174)*. Upon B cell activation, over multiple rounds of proliferation B cells introduce point mutations in the DNA sequence encoding the antibody variable regions in a process called somatic hypermutation *(177–179)*. Through display of whole opsonized antigens on follicular dendritic cells, B cell clones best capable of recognizing the presented antigens are selected and are allowed to develop into high affinity antibody producing plasma cells and memory B cells *(177–179)*. MZ B cells are located in the spleen in the marginal zones at blood interface, allowing MZ B cells to quickly react to blood born antigens by producing IgM antibodies for which no T cell help is needed *(180)*. During atherosclerosis MZ B cells are known to accumulate. Similarly, B1 cells, which are derived from progenitors from the fetal liver and mainly reside in the peritoneal and pleural cavities, do not need T cell assistance upon antigen recognition and produce IgM in response to antigen recognition. Besides antigen evoked antibody secretion, B1 cells also constantly produce IgM with a broad specificity, called natural antibodies *(181)*.

Depletion and adoptive transfer studies have indicated that MZ B cells *(182)* and B1 *(166)* cells are atheroprotective. In animals deficient for B1 cells, adoptive transfer of B1 cells incapable of secreting IgM did not lead to the atheroprotection observed in mice treated with WT B1 cells *(166)*, indicating that IgM antibodies are pivotal in the atheroprotective effects of B1 cells and likely contribute to atheroprotective effects of MZ B cells. IgMs have been

reported to bind to oxidized epitopes on lipoproteins, possibly promoting clearance of damaged lipoproteins from the blood before they become entrapped in atherosclerotic plaques *(183)*. Beside IgM secretion, marginal zone B cells were found to provide atheroprotection through regulating TFH cells and thereby reducing induction of pro-atherogenic FO B cells *(165, 184, 185)*. Production of pro-inflammatory cytokines, IgG and IgE could mediate the general atherogenic properties of FO B cells *(162, 165, 184, 185)*. Surprisingly, germinal center derived antibodies were reported to promote a more stable plaque phenotype *(185)*. Although the enhanced stabilization was observed in larger lesions, this could also reflect a further advanced plaque phenotype induced by germinal center derived antibodies. Other recent studies have shown that induction of ApoB100 *(186)* and collagen *(187)* specific antibodies could reduce lesion size. This indicates that induction of humoral responses positively modulating pathogenic and beneficial atherosclerosis-specific molecular interactions could be an interesting vaccination strategy to treat atherosclerosis.

T cells

Naïve T cell development

T cells are important cells of the adaptive immune system, which exert important immuno-regulatory and effector functions, and can provide immunological memory. T cells recognize their antigen in a fundamental different way than innate immune cells and B cells, as T cells are equipped with a T cell receptor (TCR) which recognizes a specific linearized peptide displayed on a protein scaffold, namely Major Histocompatibility Complex (MHC), instead of recognizing a native 3d structure *(188)*. TCRs are constant on a single T cell, but vary between different T cells, allowing specificity from a T cell clone for a specific MHC-peptide complex. To avoid the release of T cells in the system which do not recognize MHC-peptide complexes, or T cells that recognize self-peptides on MHC, T cell precursors are educated in the thymus, hence the name T cell. T cells develop from committed lymphoid progenitors (CLPs) originating from the bone marrow which migrate via the bloodstream to the thymus *(189, 190)*. There, CLPs differentiate towards a committed T-cell precursor, losing the potential to develop into B-cell and natural-killer T cells *(191)*. Then, through somatic DNA rearrangements, TCRs are created that are variable between cells, but constant on the same cell. Survival of these double positive thymocytes ($CD4^+CD8^+$) is dependent on strength of TCR signaling *(190, 192)*. Very poor interaction of the TCR with self-peptide–MHC complexes results in cell death by neglect, occurring in approximately 90% of thymocytes *(192)*. Approximately 5% of T cells recognize self-peptide–MHC complexes too well, which could cause auto-immunity if released from the thymus, and therefore undergo apoptosis in a process called negative selection *(192, 193)*. Immature T cells that express TCRs that cause intermediate TCR signaling, so between neglect and negative selection, are positively selected and can further differentiate into naïve CD4 T cells or naïve CD8 T cells *(192)*.

Antigen presentation

CD4 is a co-receptor for the TCR which promotes interaction of CD4 T cells with MCH-II-peptide complexes *(194)*. MHC-II is only expressed by antigen presenting cells, and is a heterodimer of 2 homogenous proteins which assemble in the ER *(195)*. Between both protein chains of the MHC-II molecule an open peptide binding groove is present in which peptides bind which are typically between 13 and 24 amino acids long *(196)*. To prevent endogenous peptides from binding in the peptide groove, the MHC-II peptide associates with the invariant chain which occupies the peptide binding groove and also guides the MHC-II complex to the endosomes *(195, 196)*. In the endosome the invariant chain is proteolytically trimmed and can be exchanged with exogenous peptides that have been taken up by the APC *(196)*. MHC-II molecules can then be transported to the cell membrane making them available for interaction with the TCR of CD4 T cells. In this way the MHC-II pathway is tailored to present exogenous peptide antigens by antigen presenting cells to CD4 T cells *(196)*.

Specific recognition of a target cell by a CD8 T cell is established through positive interaction between the T cell receptor of the CD8 T cell, and a MHC-I–peptide complex. In contrast to MHC-II, MHC-I is ubiquitously expressed and interacts with CD8 instead of CD4 *(194)*. The peptide binding groove of MHC-I is closed, limiting the length of peptides that can bind in the peptide groove *(196)*. As CD8 T cell epitopes tend to be 8-10 amino acids long, most proteins need to be proteolytically cleaved in the target cell to fit onto MHC-I (source). Proteolytical cleavage of cellular proteins is largely dependent on large multi-subunit protein complexes, which are called proteasomes *(197–199)*. Loading of peptides on MCH-I takes place in the endoplasmatic reticulum (ER) of the target cell, which requires transport of cytosolic peptides over the ER membrane by specific peptide transporter proteins, namely Transporter associated with Antigen Processing 1/2 (TAP1 and TAP2) *(196)*. As predominantly cytosolic peptides are loaded on MHC-I in the ER, mainly peptide epitopes from proteins produced in the cell itself are presented on MHC-I. Some APCs are also capable of presenting peptide epitopes, which have been taken up on MHC-I in a process called cross-presentation. Efficient cross-presentation is believed to be restricted to mainly the cDC1 population *(150–152)* although macrophages are also known to be capable of activating CD8 T cells *(200)*. Cross-presentation requires that phagocytized antigens are not completely broken down in the endocytic compartment *(201, 202)*. Two pathways for cross-presentation have been reported although the exact mechanisms which allow these DCs to cross-present are not entirely clear. In the cytosolic cross-presentation pathway, proteins/peptides from the endocytic compartment are released in the cytoplasm in which they are handled just like endogenous cytosolic proteins *(201, 202)*. The other pathway is the vacuolar pathway, in which antigens are processed and loaded on MHC-I in the endosome or phagosome *(201, 202)*. Cross-presentation allows antigen presenting cells to instruct naïve CD8 T cells *(196, 199, 201, 202)*.

T cell priming

For a naïve T cell to mature to an effector or memory T cell it first must establish a positive interaction between its TCR and a MHC-peptide complex *(203)*. To find a MHC-peptide complex with which it positively can interact, a naïve T cell can circulate the bloodstream and cross high endothelial venules (HEVs), passing the spleen and lymph nodes respectively, where APCs can present antigens to the T cells *(204, 205)*. Upon establishing a positive interaction between TCR and MHC-peptide complex, naïve T cells further receive co-stimulatory or co-inhibitory signals from the APC, and receive APC derived and environment derived cytokine signals. T cells integrate the signals of TCR signaling strength *(203)*, co-stimulation/inhibition *(206)*, and cytokine environment *(207)*, ultimately leading to clonal expansion and differentiation of the naïve T cell towards a particular T cell subset, equipped to deal with the situation at hand. Moreover, depending on the stimuli, short-lived, but highly functional effector populations can be generated, but also memory precursor effector cells that can transition into memory T cells, and contribute to long-lived immunological memory and protection *(203, 206, 207)*.

CD4 T cell subsets in atherosclerosis

By integrating the signals derived from the APC and environment upon priming, and subsequent release of chemokines and cytokines by CD4 T cells upon secondary TCR stimulation, CD4 T cells play an important role orchestrating the immune response. CD4 T cells, derived from atherosclerotic plaques have been described to recognize LDL derived ApoB100 epitopes *(186, 208)*, heat shock protein 60 *(209)*, and type V collagen *(100)*, underscoring the auto-immune character of atherosclerosis. The antigen specific CD4 T cell reaction is likely to comprise more atherosclerotic plaque components, however detecting lesional CD4 T cell antigen specificity has proven to be laborious. The majority of CD4 T cells in the atherosclerotic lesion appears to be highly activated and is predominantly of the Th1 subset. Th1 development is promoted by IL-12 and IL-18 which are secreted by M1 macrophages and inflammatory DCs *(210)*. Th1 cells are pro-atherogenic *(98)*, as they secrete pro-inflammatory cytokines including IFN-γ and TNF-α, resulting in high lesional levels of IFN-γ and TNF-α *(211)*. IFN-γ promotes atherosclerosis through various mechanisms including the recruitment of immune cells through upregulation of chemokines such as CCL2 in immune and non-immune cells *(70, 212)*. IFN-γ furthermore affects transcription of a myriad of genes in macrophages *(213)* skewing macrophage polarization to the pathogenic M1 phenotype *(214)*, and promotes foam cell formation *(215)*. Demonstrating the pro-atherogenic actions of IFN-γ, injection of recombinant IFN-γ increased atherosclerotic lesion size 2-fold in ApoE$^{-/-}$ mice *(216)* while ApoE$^{-/-}$ IFN-γR$^{-/-}$ mice had 60% smaller lesions than ApoE$^{-/-}$ animals *(217)*. TNF-α also has a detrimental role in atherosclerosis, as it induces endothelial *(130)* and smooth muscle cell *(131)* dysfunction, promotes neutrophil survival *(136)*, and promotes necrotic core formation in the atherosclerotic plaque *(218)*.

Whereas a clear pro-atherogenic role for Th1 cells has been established, the role for Th2 and Th17 cells in atherosclerosis is much less solidly defined. Very few Th2 cells are present in mouse atherosclerotic lesions, probably indicating a limited role for Th2 cells in atherosclerosis. IL-4 drives Th2 cell differentiation and expression of Th2 transcription factor GATA-3, but also inhibits the Th1 response *(219)*, through which Th2 cells were hypothesized to inhibit atherosclerosis. Typical Th2 cytokines include IL-4, IL-5, IL-10, and IL-13. Through IL-4, Th2 cells promote B cell proliferation, Ig class switching and are therefore important for induction of humoral responses. Experimental studies have however described pro-atherogenic effects of IL-4 in IL-4 deficient mice *(220, 221)*. Furthermore, induction of a Th2 response and humoral response against LDL, inducing IL-4, IL-5, and IL-10 did not affect atherosclerosis *(222)*, despite previously reported atheroprotective effects of IL-5 *(223)* and IL-10 *(224)*. Similarly, in humans, high IL-5 levels were associated with decreased mean common carotid intima-media thickness in women *(225)*, and enhanced Th2 cell levels were associated with a reduced risk of acute myocardial infarction *(226)*. These current data do not provide a clear picture of the role of Th2 cells in atherosclerosis.

Enhanced levels of IL-17 producing Th17 cells have been reported in murine atherosclerotic plaques *(100, 227)*. In vitro, oxLDL exposure of dendritic cells was found to induce Th17 cells besides Th1 cells through TLR4 and CD36 mediated induction of IL-6 and IL-1β *(227)*, providing a mechanism for Th17 generation in atherosclerosis. Th17 cells have been found to be pathogenic in multiple auto-immune diseases including rheumatoid arthritis, multiple sclerosis, and were therefore hypothesized to be pathogenic in atherosclerosis. In line with a pro-atherogenic effect of Th17 cells, blockage of IL-17 and IL-17A with monoclonal antibodies reduced plaque development *(228, 229)* and reduced macrophage numbers in the plaque *(229)* (2009) compared to isotype treated controls. IL-17A knockout animals were however found to have increased atherosclerosis *(230)*, and intraperitoneal administration of recombinant IL-17A reduced atherosclerosis *(230)*. Moreover, IL-17 expression was found to correlate with lower macrophage content and more SMCs, and a more fibrous plaque phenotype of murine carotid plaques *(231)*. Similarly, in human carotid plaques, expression of RORγt and IL-17A was positively correlated with SMC marker ACTA2 and with collagen I, supporting a profibrotic effect of IL-17. Besides a potential beneficial plaque stabilizing effect of IL-17, IL-17 has been reported to lower endothelial VCAM-1 expression, reducing adherence of human mononuclear cells to pre-activated human umbilical vein endothelial cells in vitro. In line with the inconclusive results of experimental studies, association studies in human of circulating IL-17 levels and various atherosclerosis parameters have not resulted in a clear picture of the role of IL-17 and Th17 cells in atherosclerosis *(232–234)*.

In correspondence with the pro-atherogenic role of inflammation, regulatory T cells, which dampen inflammation, are known to reduce atherosclerosis. This was shown by adoptive transfer of $CD4^+CD25^+$ T cells, enriched for Tregs, leading to reduced atherosclerosis *(235)*, whereas depletion of $CD4^+CD25^+$ or Foxp3 expressing T cells increased atherosclerosis *(236, 237)*. $CD4^+FoxP3^+$ regulatory T cells (Tregs) are indispensable in the maintenance of

peripheral tolerance towards self-antigens, among other mechanisms through suppressive effects on antigen presenting cells and other effector T cells. Within the Treg population, two distinct origins are distinguished, namely the Tregs generated in the thymus and peripherally induced Tregs, refered to as natural Tregs (nTregs) and inducible Tregs (iTregs) respectively. Induction of natural Tregs is thought to be primarily dependent on strength of TCR signaling, being higher than for induction of naïve conventional CD4 T cells but below the negative selection threshold *(238)*. Peripheral induction of Tregs is promoted by TGF-β and IL-2, weak TCR stimulation *(239, 240)*, weak co-stimulatory signaling *(241, 242)* and strong co-inhibitory signaling *(243)*. Tregs can dampen the immune response through secretion of anti-infammatory cytokines IL-10 and TGF-β, but can also dampen the immune response with co-inhibitory molecules such as CTLA-4 in a cell contact dependent fashion.

Cytotoxic CD8 T cells in atherosclerosis

Upon priming, the vast majority of CD8 T cells differentiate into the cytotoxic CD8 T cell or cytotoxic lymphocyte (CTL) subset *(244)*. The primary function of CTLs is to protect the host from intracellular pathogens *(245)* and tumors *(246)*, accessing cellular proteins presented on MHC-I. MHC-I/peptide complex recognition by CTLs leads to the formation of an immunological synapse with the target cell in which granules are released which exposes Fas Ligand on the CTL surface in the synapse *(247)*, and releases perforin and granzyme B by in the synapse, which induce apoptosis of the target cell *(248)*. Moreover, CTL activation leads to inflammatory cytokine secretion, including TNF-α and IFN-γ, which as previously discussed are pro-atherogenic *(249, 250)*. Although large numbers of activated CTLs are present in human and murine atherosclerotic lesions, it is currently not known which antigens these CD8 T cells recognize and which antigens are predominantly (cross-)presented on MHC-I. Several experimental studies have indicated that depending on antigen, antigen specific CTLs can act atheroprotective *(251–253)* or atherogenic *(254)*, dependent on the role of the targeted cell population in the atherosclerotic lesion. Induction of CTL reactivity towards smooth muscle cells enhanced vessel inflammation and atherosclerosis *(254)*, whereas CD8 T cell mediated killing of macrophages *(251)* and activated endothelium expressing VEGFR2 *(252)* or CD99 *(253)* attenuated atherosclerosis. Recent studies however, suggest an overall pathogenic role for CTLs through secretion of pro-inflammatory cytokines, which corresponds with observations of increased activated, and cytokine producing CD8 T cells in peripheral blood of patients with coronary artery disease *(255–257)*. Although induction of CD8 T cells towards pro-atherogenic cell types has been found effective at reducing atherosclerosis in experimental models, the therapeutical use of such a mechanism is probably limited due to side effects that can be expected by targeting endogenous cells.

(Immuno)proteasomes

The majority of proteins presented on MHC-I are generated through degradation of cytosolic proteins in large barrel shaped multi-subunit protein complexes specialized in the proteolytical cleavage of proteins, called proteasomes *(197)*. The barrel like structure of the

proteasome consists of 4 stacked heptameric rings. The proteolytic activity of the proteasome is exerted by three active (β) subunits, with caspase-like, chemotrypsin-like, and chymotrypsin-like activities, which are located in the middle 2 heptameric rings. The active sites of the B subunits face the lumen of the barrel, so that only proteins that have entered the barrel are degraded *(197)*. Through regulatory subunits attached to the outer rings, next to the openings of the barrel, entry of substrate, including poly-ubiquitinated, misfolded and oxidized proteins, is promoted *(197)*. The ubiquitously expressed constitutive proteasome is however not very potent in the production of peptides fitting on MHC-I. The immunoproteasome, which carries 3 slightly different catalytic subunits, cleaves proteins at other sites, leading to the production of more epitopes suited for MHC-I presentation *(199, 258)*. Under basal conditions this immunoproteasome is mainly expressed in cells of hematopoietic origin *(197, 199)*. During infection and inflammation however, IFN-γ signaling can also induce expression of the immunoproteasome on cells of non-hematopoietic origin, presumably to promote antigen presentation of intracellular pathogens to CD8 T cells *(197, 199)*.

Because proteasomes are responsible for the vast majority of cellular protein degradation, proteasomes are involved in many cellular signaling pathways. Therefore (immuno)proteasomal subunit deficiency or inhibition have far more elaborate effects than just influencing MHC-I epitope generation *(198, 259–261)*. In multiple immune cells, including T cells, B cells, and DCs, inhibition of the immunoproteasomal subunits LMP7 and LMP2 with the inhibitor ONX-0914 reduces their activation *(198, 259–262)*. Immunoproteasomal inhibition reduced disease severity in various experimental models of autoimmunity *(263–271)*. Therefore we assessed the effect of ONX-0914 on atherosclerosis in this thesis. In line with the immune inhibitory effect of immunoproteasomal inhibition, ONX-0914 treatment reduced atherosclerosis.

The exact mechanism in which immunoproteasomal inhibition dampens inflammation is still enigmatic, however several mechanisms have been proposed. Since proteasomes can generate biologically active peptides *(272)*, it is possible that due to inhibition of (immuno)proteasomal subunits peptides are generated with different biological activity. Furthermore it has been reported that constitutive and immunoproteasomes are preferentially attached to different regulatory subunits *(273)*, which was later challenged *(274)*, but if true could lead to breakdown of different proteins by proteasomes and immunoproteasomes. Up to date, no evidence is present that differential breakdown of specific proteins or differential generation of particular biologically active peptides underlies the immunosuppressive effect of immunoproteasomal inhibition. Besides affecting degradation of specific proteins, or generating specific biologically active peptides, inhibition of (immuno)proteasomal protein degradation leads to accumulation of misfolded and ubiquitinated proteins, which activates the protein response (UPR) *(262)*. The UPR, among other things, upregulates the expression of (constitutive) active proteasomal subunits to restore proteostasis (protein homeostasis) in the cell *(275, 276)*. Several proteins taking part

in the UPR have been reported to mediate inhibitory effects in immune cells *(262, 275, 276)*. This could act as a mechanism to inhibit activation of immune cells unable to maintain proteostasis and thereby unable to accurately process incoming signals. UPR activation mediated immune inhibition could protect the host from tissue damage by unfit immune cells.

Thesis outline

Epidemiological and association studies in human, and experimental studies have drawn a clear picture of active of the immune system contributing to development of atherosclerosis. While lipid lowering drugs, including statins, have been somewhat effective at reducing risk for developing a major cardiovascular event, residual inflammatory risk is often present, even after successful lipid lowering. Besides immune cells being activated by hyperlipidemia, immune cells can also affect the systemic lipid homeostasis. These interactions between lipids and immune system are reviewed in **Chapter 2**. In this thesis we have aimed to beneficially modulate the immune response to treat atherosclerosis.

Administration of antigens through the oral route is known to induce a tolerogenic response, known as oral tolerance, through presentation of the administered antigens by tolerogenic DCs. Induction of oxLDL specific inducible Tregs trough oral administration of oxLDL has proven to reduce atherosclerosis through immunosuppressive mechanisms, despite Treg levels dropping to baseline levels after 2 weeks post oxLDL administration. In **Chapter 3**, we therefore aimed to maintain high levels of oral oxLDL-induced Tregs through administration of an IL-2 complex (IL-2 coupled to an antibody). IL-2 complex treatment has been described to induce specific expansion of the Treg population and confer atheroprotection. We hypothesized that combined oxLDL and IL-2 complex treatment would have additional beneficial effects. Although in the treatment groups receiving combined or separate treatments, clear indicators of reduced inflammation, and in IL-2 complex treated groups also enhanced regulatory T cell levels were observed, only separate oral oxLDL administration significantly reduced atherosclerosis.

In **Chapter 4** we assessed different formulations of a human ApoB100 derived peptide, referred to as p210, to confer atheroprotection in $LDLr^{-/-}$ mice expressing human ApoB100 (HuBl). P210 is a 20 amino acid long peptide that spans the LDLr binding site of ApoB100 and has reduced atherosclerosis in various studies using $ApoE^{-/-}$ mice. Probably due to use of different p210 formulations and administration schemes, atheroprotective effects of p210 have been dedicated to induction of antibodies, cytotoxic CD8 T cells, and Tregs. Through coupling of p210 to cholera toxin B (CTB), known to promote mucosal uptake and tolerance, and oral administration, we aimed to induce a tolerogenic Treg response against p210. We aimed to induce antibodies and CD8 T cell responses against p210 through coupling p210 to Pan HLA DR epitope (PADRE), a CD4 T cell epitope, which can aide in T cell help for antigen

production, and alum adjuvanted immunization. Although the p210 administration schemes induced p210 IgGs, no changes in atherosclerosis development were observed in HuBl mice.

Because we did not observe (CD8) T cell responses, which were previously reported after p210 vaccination, we assessed the effect of vaccination on atherosclerosis with in silico predicted ApoB100 derived CD8 T cell epitopes in HLA-A2 (human MHC-I allele) transgenic HuBl mice, described in **Chapter 5**. Despite positive binding of the peptides to HLA-A2 and induction of strong CD8 T cell responses upon immunization, we did not observe an effect of vaccination on atherosclerotic plaque development. Discovering which antigens are responsible for CD8 T cell activation in the lesion would mean a breakthrough for studying and understanding the role of CD8 T cells in atherosclerosis.

In **Chapter 6** we assessed the effect of inhibition of the immunoproteasome with ONX-0914, an immunoproteasomal LMP7 subunit and LMP2 subunit inhibitor, on atherosclerosis. Besides producing MCH-I epitopes, immunoproteasomes are important for maintaining proteostasis, mainly in cells of hematopoieitic origin. Immunoproteasomal inhibition is known to reduce immune activation and previously reduced disease severity in multiple experimental models of auto-immunity.Treatment with ONX-0914 reduced atherosclerosis and unexpectedly also white adipose tissue mass, which we further investigated.

In **Chapter 7** data from this thesis are discussed together with concluding remarks and future perspectives.

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