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).

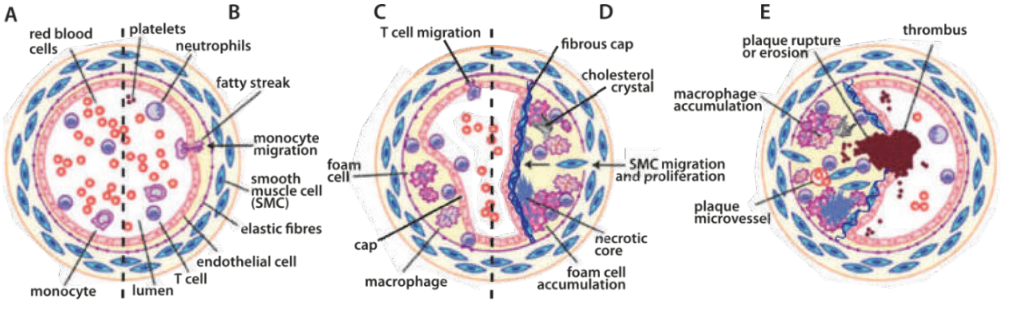

Fig. 1 Atherosclerotic lesion development. *Over decades of atherosclerosis development, atherosclerotic lesions can advance through various stages. (**A**) Initially atherosclerosis prone regions are marked by endothelial dysfunction and intimal thickening. (**B**) Endothelial dysfunction facilitates 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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Atherosclerosis: the interplay between lipids and immune cells

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Purpose of review

Cardiovascular disease is the leading cause of mortality worldwide. The underlying cause of the majority of cardiovascular disease is atherosclerosis. In the past, atherosclerosis was considered to be the result of passive lipid accumulation in the vessel wall. However, today's picture of the pathogenesis of atherosclerosis is much more complex, with a key role for immune cells and inflammation in conjunction with hyperlipidemia, especially elevated (modified) LDL levels. Knowledge on immune cells and immune responses in atherosclerosis has progressed tremendously over the past decades, and the same is true for the role of lipid metabolism and the different lipid components. However, it is largely unknown how lipids and the immune system interact. In this review, we will describe the effect of lipids on immune cell development and function, and the effects of immune cells on lipid metabolism.

Recent findings

Recently, novel data have emerged that show that immune cells are affected, and behave differently in a hyperlipidemic environment. Moreover, immune cells have reported to be able to affect lipid metabolism.

Summary

In this review, we will summarize the latest findings on the interactions between lipids and the immune system, and we will discuss the potential consequences of these novel insights for future therapies for atherosclerosis.

Keywords

atherosclerosis, immune system, lipids

Key points

- Dyslipidemia affects the adaptive immune response.
- T cells specific for modified lipoproteins aggravate atherosclerosis.
- The adaptive immune response modulates lipoprotein metabolism.
- Immune responses and lipid metabolism interact in a unique metabolic pathway underlying atherosclerosis.

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for 16.7 million deaths each year. The underlying cause of the majority of CVD is atherosclerosis, a disease that is characterized by the formation of lipid and (immune)-cell containing plaques in the intima of large and mid-sized arteries *(1)*. In the past decades, it was found that the immune system plays a crucial role in the development and progression of atherosclerotic plaques. By transforming immune cells into proinflammatory and anti-inflammatory chemokine and cytokine producing units, and by guiding the interactions between the different immune cells, the immune system decisively influences the propensity of a given plaque to rupture and cause clinical symptoms like myocardial infarction and stroke *(1–3)*. Although knowledge on immune cells and immune responses has progressed tremendously over the past decades, and has provided novel insights for many diseases, including atherosclerosis, it has become clear that atherosclerosis is not a 'standard' immunological disease. Recently, novel data have emerged that show that immune cells are affected, and behave differently in a hyperlipidemic environment. In this review, we will summarize the current knowledge on the effects of hyperlipidemia, and especially hypercholesterolemia and the effects of modified LDL, on immune cell development and function. The way the mature immune system reacts to challenges such as inflammation is largely defined by the self-renewal and multilineage capacity of a rare population of hematopoietic stem and progenitor cells (HSPCs), defined as $Lin^{-}Sca^{+}cKit^{+}$ cells, in the bone marrow *(4, 5)*. These HSPCs differentiate into leukocytes (including lymphocytes), dendritic cells, erythrocytes, and platelets, but also to endothelial progenitor cells. The self-renewal capacity and differentiation of HSPCs into mature blood cell lineages and the subset distribution of these separate blood lineages are tightly regulated by a combination of intrinsic and extrinsic signals such as growth factors, chemokines, and cell cycle proteins *(6)*. HSPCs are located in specialized microenvironments: the bone marrow niche. The bone marrow niche is composed of many cell types, including mesenchymal stem cells, CXCL12-abundant reticular (CAR) cells, osteoclasts, osteoblasts, adipocytes, and endothelial cells. The niche plays a critical role in the regulation of HSPC self-renewal, quiescence, and differentiation during hematopoiesis *(7)*. HSPCs are also activated during immunological challenges to replenish exhausted immune effector cells. These HSPC responses include expansion, mobilization, and differentiation and are regulated by systemic (cytokines, chemokines) and local, nichederived signals (chemokines, growth factors) *(6, 8, 9)*. In hypercholesterolemic ApoE $^{-/-}$ and $Ldlr^{-/-}$ mice, hyperlipidemia induces a substantial increase in the number of HSPCs, and a preference for myeloid skewing, which results in aggravated atherosclerosis *(10–12)*. Hypercholesterolemia causes HSPCs to lose their quiescence, characterized by increased proliferation and expression of cell cycle proteins such as cyclin B1, C1, and D1 *(10)*. Moreover, when hypercholesterolemia-primed HSPCs are transplanted into normocholesterolemic mice, they maintain their proliferative capacity and their preference to differentiate towards the myeloid lineage, resulting in monocytosis and granulocytosis *(10,*

11). Likewise, when normal HSPCs are transplanted into hypercholesterolemic mice, which contain a hypercholesterolemia-primed bone marrow niche, similar results were observed, proving that priming of either the HSPC itself or the bone marrow niche induces these effects *(10)*. Surprisingly, hypercholesterolemia also induces extramedullary hematopoiesis, and increased myelopoiesis was also found in the spleen *(13)*. Surprisingly, important mediators of HSPC biology are lipid mediators. For example, a predominant role for the cholesterol efflux pathways (ATP binding cassette transporters A1 and G1) and HDL was found for the maintenance of HSPC quiescence. Absence of ABCA1 and G1 induces HSPC mobilization, proliferation, and differentiation towards the myeloid lineage, and results in extramedullary hematopoiesis, revealing that disruption of cholesterol-efflux mechanisms play a major role in HSPC biology *(14)*. Moreover, this process is also mediated via proteoglycan bound apolipoprotein E that promotes cholesterol efflux via ABCA1 and ABCG1, thereby inhibiting HSPC proliferation *(11)*.

Lipids and innate immune responses

Monocytes

As described above, increased numbers of circulating monocytes are found in patients suffering from hyperlipidemia and atherosclerosis, as well as in experimental animal models of atherosclerosis, and are correlated with plaque size and plaque stage *(15–18)*. Monocytes can be divided into inflammatory monocytes (characterized by the expression of $CD14^{++}CD16^{-}$ or $CD14^{++}CD16^{+}$ in humans and $Ly6C^{high}$ in mice) and patrolling monocytes (which are $CD14^{+}CD16^{++}$ in humans and $Ly6C^{low}$ in mice) *(19)*. Hypercholesterolemia causes a profound increase in $Ly6C^{high}$ and $CD14^{++}$ monocytes, which is partly generated via extramedullary hematopoiesis *(10, 16–18, 20)*. Their fate to differentiate towards $Ly6C^{high}$ monocytes is driven by high cholesterol levels, as competitive bone marrow transplantation studies show that hypercholesterolemia-primed HSPC or a hypercholesterolemia primed bone marrow niche results in an increased fraction of $Ly6C^{high}$ monocytes *(10)*. Increasing evidence points out that the $Ly6C^{high}/CD14^{++}CD16^{-}$ monocytes do not only increase in number, they are also the monocyte subset that preferentially adheres to the endothelium, infiltrates the arterial wall, and is responsible for the generation of plaque macrophages. The role for the patrolling, $Ly6C^{low}/ CD14^{+}CD16^{+/+}$ monocytes is less clear. They are longer-lived, scan the endothelium for activation markers, and pathogens are able to phagocytose oxidative lipids, but do not seem to infiltrate atherosclerotic plaques *(17, 18)*.

Macrophages

Once monocytes have infiltrated the arterial wall, they differentiate into macrophages and become a key component of the atherosclerotic plaque. When exposed to a hyperlipidemic milieu, macrophages ingest and process (modified) lipids, predominantly modified LDL, which is a complex mixture of oxidation products and proteins, and store it in lipid droplets in their cytoplasm *(1)*. The uptake of LDL is mainly mediated by scavenger receptors such as

scavenger receptor A and CD36, and the efflux is mediated by ABC transporters, in particular, ABCA1 and G1 *(21)*. When lipid uptake exceeds efflux, or efflux is disturbed, lipids accumulate and macrophages become 'foam cells'. Initially, lipid uptake including oxLDL and phospholipids results in the activation of macrophages via pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPS), predominantly using TLR2 and TLR4, resulting in the release of a myriad of proinflammatory (i.e. interleukin-1 (IL-1), IL-6, IL-12, IL-15, IL-18, TNF-a, MCP-1) and anti-inflammatory (i.e. IL-10, TGF-b) cytokines and growth factors, thereby initiating/enhancing an inflammatory response which further regulates immune cell infiltration into the atherosclerotic lesion *(1, 22)*. Although many studies have found that proinflammatory cytokines prevail upon lipid loading *(1, 22)*, others claim that macrophage foam cell formation is associated with an anti-inflammatory response. Spann et al. *(23)* found that desmosterol plays a key role in the homeostatic response of peritoneal macrophages upon lipid loading, including activation of LXR target genes, inhibition of SREBP target genes, and suppression of inflammatory response genes. These results imply that macrophage activation in atherosclerosis results from extrinsic stimuli such as (lipid) debris, and inflammatory mediators derived from other cell types in the arterial wall. However, the stage of foam cell formation and the environment may also exert differential roles in macrophage activation. After massive uptake of lipids, cholesterol crystals can form in the macrophage foam cells. This crystalline material, but also the increased oxidative stress can lead to the formation of an inflammasome complex in macrophages. Inflammasome formation leads to activation of caspase-1 that rapidly cleaves pro-IL1b and pro-IL18 into their mature forms, which are both pathogenic inflammatory cytokines that drive atherosclerosis. Cholesterol crystals induce the nlrp3 inflammasome, which has been found to play a major role in atherosclerosis *(24)*. Within the atherosclerotic lesion, macrophages are exposed to sustained inflammation and oxidative stress, resulting in activation of endoplasmic reticulum stress pathways resulting in macrophage apoptosis and necrosis. The unfolded protein response (UPR), with factors like CCAAT-enhancerbinding protein homologous protein, $Ca2^{+}$/calmodulin-dependent protein kinase II, signal transducer and activator of transcription 1, and nitric oxides, play a major role in this process. Necrosis and apoptosis, and the subsequent defective efferocytosis of macrophage cell-rich and lipid-rich, sometimes crystalline, debris results in the formation of a necrotic lipid core and sustained atherosclerotic plaque inflammation *(25)*.

Lipids and adaptive immune responses

In addition to innate immune responses, hyperlipidemia can also trigger adaptive immune responses as illustrated by the presence of activated T cells in the atherosclerotic lesion as well as by the T-cell dependent induction of antibody production by B cells towards modified LDL *(1)*. Adaptive immune responses are initiated through antigen presentation, and in atherosclerosis, the antigen is often considered to be a (neo-)epitope of modified LDL.

Another proof that modified LDL drives adaptive immune responses is the existence of oxLDL specific T cells in experimental animal models and in patients *(1)*.

Dendritic cells

Although part of the innate immune system, dendritic cells have an important role in initiating the adaptive immune response towards atherosclerosis related antigens. In addition, dendritic cells also take up lipids (via scavenger receptors and efferocytosis) and form foam cells, contributing to atherosclerotic lesion development *(26–28)*. As they share phenotypic and functional properties with macrophages, which attain a dendritic cell-like phenotype upon foam cell formation *(29)*, it is complicated to dissect the role of dendritic cells and macrophages in the lesion. The uptake of oxLDL results in dendritic cells maturation, migration, and antigen presentation to T cells in the draining lymph nodes *(30, 31)*. On the other hand, oxidized phospholipids can impair maturation of dendritic cells *(32)*, possibly limiting excessive dendritic cell Interplay between lipids and immune activation *(32)*, and desmosterol may induce an anti-inflammatory response via LXR activation *(23, 33)*. Interestingly, circulating cholesterol levels and dendritic cells numbers do correlate as shown in DC-hBcl2 mice, which express the antiapoptotic Bcl-2 under the CD11c promoter *(34)*. Conversely, reduced levels of dendritic cells in ApoE$^{-/-}$ mice result in enhanced systemic cholesterol levels Conventional dendritic cells at the crossroads between immunity and cholesterol homeostasis in atherosclerosis *(34)*, whereas lesional lipid accumulation decreases *(27)*. The mechanisms behind this effect of dendritic cells on cholesterol metabolism have yet to be identified. Dendritic cells have been frequently used to modify the outcome of atherosclerosis. Adoptive transfer of dendritic cells pulsed with modified LDL into atherogenic mice may aggravate atherosclerosis via activation of T cells *(35)*, but may also inhibit atherosclerosis via the induction of antibodies specific for modified LDL. In addition adoptive transfer of tolerogenic dendritic cells (ApoB100-pulsed) can protect against atherosclerosis *(36)*. Similarly, transfer of oxLDL-induced apoptotic dendritic cells may form a novel therapy for both initial and advanced atherosclerosis since it induces tolerogenic dendritic cells, enhances regulatory T cells (Treg) numbers, and reduces inflammatory monocyte responses. T cells Initial reports on the presence of T cells in human atherosclerotic lesions initiated research into the role of T cells in atherosclerosis. T cells can differentiate into various subsets of T cells and a main driving force into their activation is the presentation of antigen by dendritic cells within the lymph nodes and their reactivation within the atherosclerotic lesion by the interaction of effector T cells with macrophages presenting antigen, which often is a (neo)-epitope of (modified) LDL.

Th1 cells

The predominant type of CD4^{+} T cells *(37–39)* within the atherosclerotic lesion is the Th1 cell. Th1 cells produce a plethora of proinflammatory cytokines (e.g. TNF-a, IFN-g, IL-2, and IL-12) and express the transcription factor T-bet. IFN-g promotes vascular inflammation by activating antigen-presenting cells (APCs), enhancing their lipid uptake, reducing collagen

production by smooth muscle cells, and enhancing leukocyte recruitment *(40–43)*. Th1 cells have been suggested to drive antigen specific immune responses in the atherosclerotic lesion and both oxidized LDL and heat shock proteins have been suggested as antigens *(44, 45)*. Initial reports show that oxLDL is recognized by T cells in human lesions, while in addition the adoptive transfer of T cells from atherogenic mice aggravates atherosclerosis. In line with the observation that these $CD4^+$ T cells recognize oxLDL in a human leukocyte antigen-antigen D related restricted manner, Paulsson et al. *(46, 47)* described the oligoclonal expansion of T cells in atherosclerotic lesions indicating the response towards modified LDL. More recent data identify T cells expressing TRBV31 that react to native LDL and ApoB100, possibly more than to modified LDL *(48)*. Interestingly next to antigen specific activation, hyperlipidemia may lead to lipid accumulation in T cells and activation of LXR leading to a decrease in the production of proinflammatory cytokines *(49)*.

Th2 cells

Th2 cells are known for their B cell help and the presence of immunoglobulin (Ig)G that recognizes native and modified LDL implies that T cells actively support isotype switching from IgM to IgG in B cells *(50)*. Isotype switching is also dependent on the co-stimulatory IL-4 and OX40-OX40L pathway and blockade of this pathway reduces atherosclerosis *(51)*. The importance of this pathway is demonstrated by the identification of immune response network associated with blood lipid levels *(52)*. This network shows a gene module, the lipid leukocyte module, which is replicated in T cells. Genetic variation driving lipid leukocyte module expression associate with serum IgE levels, which relate to mast cell activity. Lesional mast cell numbers and their activity are strongly related to the complexity of lesions and the outcome of CVD *(53)*. In addition to their effect on isotype switching, Th2 cells produce IL-5 and IL-13. IL-5 has been shown to be anti-atherogenic by promoting the development of B-1 cells that produce protective IgM antibodies, resulting in reduced atherosclerosis *(54)*. Recently anti-IL-5 autoantibodies have been shown to be associated with human atherosclerosis *(55)*. IL-13 has also been shown to reduce atherosclerotic lesion development by skewing macrophages towards an M2 phenotype *(56)*. Tregs Tregs are regulators of immune responses and their main function is inhibition of self-reactive T cells in the periphery, but Tregs have also a distinct effect on hyperlipidemia. Low levels of Tregs are associated with increased risk for myocardial infarction *(57)* and coronary syndromes *(58)* and during murine atherosclerosis Treg numbers significantly decrease with lesion progression *(59, 60)*. Interestingly, oxLDL negatively affects the suppressive capacity of Tregs *(59, 60)*. On the other hand, Tregs specific for oxLDL or peptides derived from apoB100 can be induced via oral or nasal tolerance induction and these induced Tregs inhibit lesion formation and progression. Overexpression of IL-10, a hallmark cytokine of Tregs reduces VLDL and LDL levels in serum of $LDLr^{-/-}$ mice *(61)*. Recent studies have revealed a direct role for Tregs in cholesterol metabolism because depletion of Tregs using DEREG mice significantly increases atherosclerosis associated with a 1.7-fold increase in plasma cholesterol levels. More specifically, VLDL levels were increased because the clearance of

VLDL and chylomicron remnants was inhibited in the absence of Tregs. They found reduced expression of sortilin-1 in the liver and increased plasma enzyme activity of lipoprotein lipase, hepatic lipase, and phospholipid transfer protein in Treg-depleted mice. In addition, Treg expansion in a regression model of atherosclerosis significantly reduced cholesterol levels when compared with control mice *(62)*.

B cells

Mature B cells can be categorized into B1 cells and B2 cells. The former are innate T-cell independent B cells capable of producing natural IgM antibodies. Conventional B2 cells are T-cell dependent and are important in adaptive immunity by production of specific IgG antibodies to their cognate antigen. OxLDL is highly immunogenic and anti-oxLDL antibodies can be detected in atherosclerotic plaques as well as in the circulation of mice and men. OxLDL-specific IgM titers, produced by natural antibodies, are associated with protection against atherosclerosis *(54)*. In experimental animal models, this protective role of natural anti-oxLDL antibodies produced by B1 cells was found to be mediated by IL-5 *(63)*. In contrast to B1 cells, B2 cells, T-cell-dependent antibody-producing cells, promote atherosclerosis, which is in line with the aforementioned role of the co-stimulatory OX40-OX40L pathway and the role of Th2 cells *(51)*. When B2 cells are depleted using anti-CD20, atherosclerosis decreases, and when B2 cells are transferred to atherosclerotic mice, atherosclerosis increases *(64)*. This also is consistent with the observation that anti-oxLDL IgG antibodies, derived from B2 cells correlate with the presence of CVD (54, 65).

Lipid-induced epigenetic changes in immune cells

In the past decade, it has become increasingly clear that many epigenetic pathways govern differentiation and activation patterns of immune cells. It was found that chromatin modifying enzymes, such as histone deacetylases (HDACs) or histone methyltransferases (HMTs) can modify lipid metabolism and inflammatory responses of macrophages upon oxLDL exposure *(66)*. For example, myeloid specific deletion of HDAC3 results in an anti-inflammatory macrophage phenotype that produces high amounts of TGF-b and thereby induces collagen production by SMCs and fibrous cap formation in an in-vivo atherosclerosis model *(67)*. Interestingly, when HSPCs, monocytes, and macrophages are exposed to oxLDL or hypercholesterolemia in vivo *(10, 68)*, differentiated macrophages exhibit a higher inflammatory status than the unexposed control groups. This 'trained immunity' response could be reversed by pretreatment with the methyltransferase inhibitor methylthioadenosine, suggesting an important role for epigenetic histone modifications in this process *(68)*. Also other epigenetic modulators such as micro-RNAs *(69)* and long non-coding RNAs *(70)* can be modulated upon lipid loading in monocytes/macrophages. Similar findings have been reported for other immune cells, such as T cells *(71)*, suggesting that lipid challenges induce epigenetic changes in immune cells that can mediate their differentiation, polarization or activation status, and thereby affect atherosclerosis (see also: Heijmans et al.,

The multifaceted interplay between circulating lipids and epigenetics. Curr Opin Lipidol, pp. 288–294).

Conclusion

In this review, we have outlined the current knowledge on how hyperlipidemia, and especially modified LDL, affects the immune system, and vice versa. As has become clear during the last decades, the immune system reacts to lipids and lipid modifiers, which drives the progression of atherosclerosis. However, although we know that both lipids and the immune system are major determinants of atherosclerosis, the majority of the mechanisms and pathways mediating the crosstalk between lipids and immune cells have still not been identified. However, understanding how the immune system is regulated in hyperlipidemic conditions and in the different stages of atherosclerosis, and how immune cells regulate lipid metabolism is of utmost importance to identify potential therapeutic targets to prevent or stabilize the disease process. During the last decades, treatment of atherosclerosis was predominantly focused on lipid lowering. Although our insights in cholesterol metabolism and the development of lipid lowering drugs, in particular 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins), has helped to lower the incidence of CVD, a substantial part of the population still suffers from CVD notwithstanding optimal lipid-modulating therapy *(72)*. Therefore, next to lipid lowering strategies, developing and testing of new anti-inflammatory protocols is needed in the future therapeutic approach of atherosclerosis. It may be anticipated that specific immunomodulatory therapies may not only correct derailed immune responses, but also correct dyslipidemia. Current therapies using statins effectively lower plasma LDL cholesterol but are also reported to have an anti-inflammatory effect, such as reducing intimal inflammation, lowering the lesional macrophage content *(73)*. From a scientific viewpoint it is interesting to dissect whether the anti-inflammatory effects of statins are the consequence of their lipid lowering effect or the consequence of, for example, inhibiting the mevalonate pathway. In this respect it will be of major interest to determine the effect of the strong lipid-lowering anti-PCSK9 treatment on the inflammatory status of patients. At present, various clinical trials are ongoing that directly focus on inhibiting the low-grade inflammation in CVD patients, which is illustrated by enhanced levels of IL-1, IL-6, and high sensitivity C reactive protein (hsCRP) *(1)*. The strategies include the use of a low dose of methotrexate or colchicine, comparable to the approach taken for the treatment of, for example, rheumatoid arthritis *(74)*. Currently the largest trial, the CANTOS trial (trial.gov: NCT01327846), is focusing on blocking IL-1b using canakinumab *(75)*. Experimental mouse data have shown that IL-1b blockade does diminish atherosclerosis and mechanistically it may be that reduction in IL-1b leads to lower IL-6 and hsCRP levels *(76)*. It is anticipated that once we understand the interactions between lipids and the immune system, the current anti-inflammatory approaches to address the low-grade inflammation will be combined with lipid lowering approaches to provide optimal treatment regimens for atherosclerosis.

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Conflicts of interest

There are no conflicts of interest.

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Protection from atherosclerosis induced by oxLDL tolerization is not reinforced by polyclonal Treg induction

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Abstract

Atherosclerosis is characterized by lipid accumulation in the arterial wall and an ensuing pathogenic immune response. Induction of immune tolerance towards oxidized LDL, through oral administration of oxLDL, was effective at reducing atherosclerosis in line with our previous results. As oral administration of oxLDL was found to induce Tregs and levels of Tregs returned to baseline levels within weeks after treatment, we hypothesized that the effect of oral oxLDL treatment on atherosclerosis could be improved by polyclonal stimulation of Tregs and to that end $LDLr^{-/-}$ mice were treated with IL-2 complexes after oral tolerance induction towards oxLDL. Only oral oxidized LDL administration reduced atherosclerosis, while trends towards reduced atherosclerosis were observed in the mice treated with IL-2 complex separately or IL-2 complex and oxidized LDL combined, compared to control treated mice. Our study suggest that specific tolerization towards plaque antigens is more beneficial than polyclonal Treg induction, and that a combination of both antigen specific tolerization and polyclonal Treg induction does necessarily lead to an additive beneficial effect on protection from atherosclerosis.

Introduction

Atherosclerosis is characterized by the retention of cholesterol rich low density lipoprotein (LDL) particles in the arterial wall. In the arterial wall LDL undergoes oxidative modifications which initiates local inflammation and attracts immune cells to the subendothelial space. Uptake of oxidized LDL (oxLDL) particles by antigen presenting cells (APCs), including macrophages and dendritic cells, results in processing of oxLDL derived proteins and presentation of oxLDL derived peptides on MHC-I and MHC-II molecules. T cell receptor (TCR) recognition of (ox)LDL derived peptide/MHC complexes induces activation and proliferation of (ox)LDL specific T cells , resulting in (ox)LDL reactive T cells in the atherosclerotic lesion *(1, 2)*.

Especially the induction of CD4 T helper 1 (Th1) cells, which are found at high levels in atherosclerotic lesions and mainly produce the inflammatory cytokines IFN-γ, TNF-α, IL-12, and IL-2, have been found pathogenic in the context of atherosclerosis *(3)*. Inhibition of the inflammatory Th1 response would therefore be an interesting option to treat atherosclerosis. $CD4^+FoxP3^+$ regulatory T cells (Tregs) are pivotal in the maintenance of peripheral tolerance towards self-antigens, among other mechanisms through suppressive effects on antigen presenting cells and other effector T cells, and were found effective at inhibiting autoimmune Th1 responses in experimental myasthenia *(4)* and autoimmune gastritis *(5)*. Supporting a therapeutic potential of Tregs for treatment of atherosclerosis, adoptive transfer of $CD4^+CD25^+$ T cells, enriched for Tregs, reduced atherosclerotic lesion development *(6)* whereas depletion of $CD4^+CD25^+$ or Foxp3 expressing T cells increased atherosclerosis *(7, 8)*.

Within the Treg population, two distinct origins are distinguished, namely the Tregs generated in the thymus and peripherally induced Tregs, referred to as natural Tregs (nTregs) and inducible Tregs (iTregs), respectively. Since antigen specific Tregs were found more potent at preventing autoimmune diabetes than polyclonal Tregs *(9)*, it can be therapeutically interesting to induce disease antigen specific Tregs to treat auto-immune diseases instead of increasing overall Treg levels. Antigen specific iTregs can be induced by delivery of a low quantity of antigen via oral administration. Presentation of antigen by the intestinal tolerogenic $CD11C^+CD103^+$ DC population, which expresses high levels of TGF-β, RALDH, and IDO promotes the induction of antigen specific immune suppressive regulatory B cells, Th3, Tr1 and Tregs *(10)*. Our group has previously shown that atherosclerosis could be inhibited through oral administration of oxLDL, a relevant antigen for atherosclerosis *(1, 2)*, inducing relatively low levels of oxLDL responsive inducible Tregs *(11)*.

Regulatory T cells can be polyclonally expanded in vivo by administration of IL-2, on which Tregs depend for optimal growth and survival. Complexing IL-2 with an IL-2 specific antibody (JES6-1A12) still allows IL-2 to bind to the high affinity IL-2 receptor which is mainly expressed on Tregs, but inhibits binding of IL-2 to moderate and low affinity IL-2 receptors, specifically expanding Tregs in vivo when administered repeatedly *(12, 13)*. Our group has previously

shown that atherosclerosis can also be inhibited through expansion of the entire Treg pool using IL-2 complexes *(14)*, leading to substantially higher Treg levels than observed after oral oxLDL tolerance induction. Since oral oxLDL treatment yields low levels but antigen specific Tregs and IL-2c treatment expands the overall Treg pool we hypothesized that combining both treatment regimens could lead to enhanced atheroprotection than separate treatment regimens.

Results

IL-2c treatment induces enhanced levels of Tregs

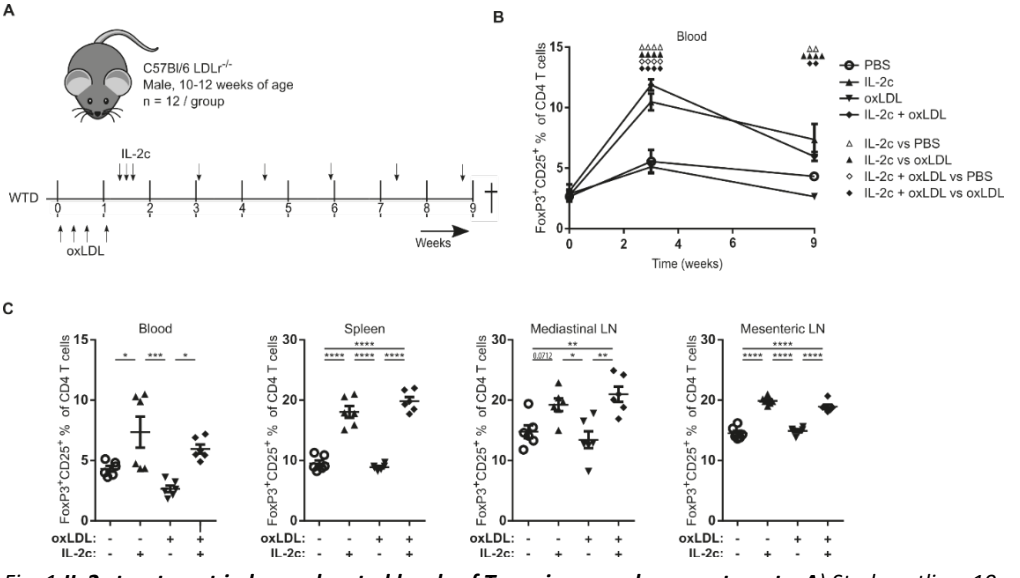

Fig. 1 ***IL-2c treatment induces elevated levels of Tregs in several compartments. A****) Study outline: 10-12 week old male LDLr$^{-/-}$ mice were fed WTD for 9 weeks. In the first 8 days of WTD, tolerance towards oxLDL was induced by 4 oral administrations of oxLDL (30µg) in oxLDL groups. Polyclonal expansion of Tregs was induced with 3 IL-2c injections in consecutive days starting 2 days after final oral gavage in IL-2c groups. After the last of the 3 consecutive IL-2c administrations, mice in the IL-2c group received a single IL-2c injection every 10 days. Vehicle injections served as control treatment.* ***B****) Quantification of FoxP3^{+}CD25^{+} regulatory CD4 T cells in circulation at the indicated timepoints as assessed by flow cytometry.* ***C****) Quantification of FoxP3^{+}CD25^{+} regulatory CD4 T cell levels in the blood, spleen, mediastinal and mesenteric lymph nodes after sacrifice measured by flow cytometry. Expressed as mean ± SEM,* ***B****) two way ANOVA* ***C****) one-way ANOVA with Sidak posttest, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

Since our lab has shown that non-specific Treg and oxLDL specific Treg induction, through intraperitoneal IL-2 complex administration and oral oxLDL administration of oxLDL reduced atherosclerosis we compared a combination of both treatments could complement each other by IL-2c mediated expansion of oral oxLDL induced oxLDL specific Tregs.

To induce oxLDL specific Tregs, tolerance to oxLDL was induced by oral administration of oxLDL (30 µg) 4 times in 8 days and atherosclerosis was induced by subsequent WTD feeding of male LDLr KO mice for 8 weeks. To induce polyclonal expansion of Tregs, mice received intraperitoneal IL-2 complex injections (1µg IL-2, 5µg anti IL-2 mAb, clone JES6-1A12) for 3 consecutive days, 2 days after the final oral oxLDL or oral control/PBS administration. To prevent elevated Treg levels from declining after the 3 consecutive days of IL-2c administration, mice received IL-2c injections every 10 days after the 3 initial IL-2c injections (Fig. 1A).

To assess whether treatment with IL-2c induced regulatory T cells we assessed Treg levels by flow cytometry in the blood, spleen, mesenteric lymph nodes (MLN) which drain the intestines, and mediastinal lymph nodes (HLN) which drain the heart. In the blood the Treg content of the CD4 T cell population increased over two-fold upon IL-2c treatment 3 weeks in the experiment, and slightly declined towards the end of the experiment (Fig. 1B). Furthermore IL-2c treatment successfully raised Treg levels in spleen, MLN and HLN at sacrifice, showing that we were indeed able to systemically enhance Treg levels by our IL-2c treatment regimen (Fig. 1C). Induction was observed in the control treted group and the oxLDL tolerance induced group. We did not observe enhanced Treg levels after oral oxLDL treatment alone in any of the assessed tissues/organs (Fig. 1C), in line with the previous oxLDL study in which elevated Tregs were measured maximally 14 days after final oxLDL administration in spleen and MLN *(11)*.

IL-2c and oxLDL treatment induce overall immune suppression

To assess whether the administration of oxLDL and IL-2c acted immunosuppressively, we assessed immune populations in circulation, spleen, HLN and MLN. IL-2c treatment alone reduced white blood cell counts (-37.7%, p = 0.0001) compared to the control treated group, similar to oxLDL treatment alone (-33.9%, p = 0.0005) (Fig. 2A). Combined oxLDL and IL-2c treatment (-58.0%, p = 0.0001) had an additional effect (vs oxLDL, p = 0.0209; vs IL-2c, p = 0.0593) on reduction of overall circulating leukocyte numbers (Fig. 2A). In all treatment groups a significant decrease in circulating neutrophils (Fig. 2B), classical monocytes (Fig. 2B) and B cells (Fig. 2D) was observed. Separate IL-2c administration induced patrolling monocyte and eosinophil levels (Fig. 2B). Overall CD4 T cells and CD4 Tem cells were significantly reduced in IL-2c+oxLDL treated mice and a trend towards reduction of CD4 T cells and CD4 Tem cells was observed in the oxLDL and IL-2c treatment groups, compared to control treated mice (Fig. 2C). In all treatment groups CD4 Tcm cell numbers were reduced compared to control treated mice, whereas naïve CD4 T cell levels were not affected (Fig. 2C). CD8 T cell

numbers were significantly reduced in the two IL-2c treated groups (Fig. 2E), with a trend towards reduction in the oxLDL treated group compared to control treated. Unlike in the CD4 T cell population, combined IL-2c and oxLDL also reduced naïve CD8 T cells and a trend towards reduction of naïve CD8 T cells was observed in IL-2c or oxLDL treated groups compared to PBS (Fig. 2C). Furthermore IL-2c treated groups had significantly reduced CD8 Tem and Tcm cell numbers in circulation, with a trend towards decrease for oxLDL treated (Fig. 2C).

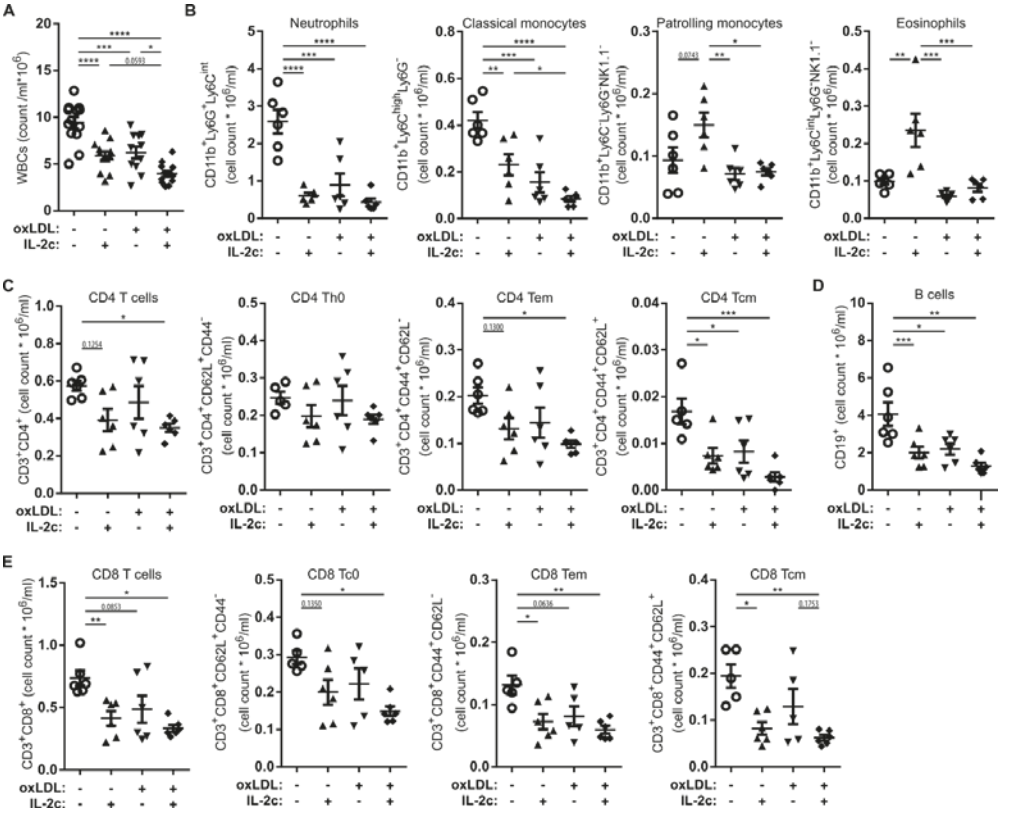

Fig. 2 ***IL-2c and oxLDL treatment induce overall immune suppression.*** *At sacrifice blood was collected and* ***A****) total white blood cell count was determined for all mice. On half of the mice flow cytometric analysis was performed and WBC counts were used to quantify the number of circulating* ***B****) major myeloid cell populations,* ***C****) CD4 T cells,* ***D****) B cells and* ***E****) CD8 T cells. Expressed as mean ± SEM, one-way ANOVA with Sidak posttest, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

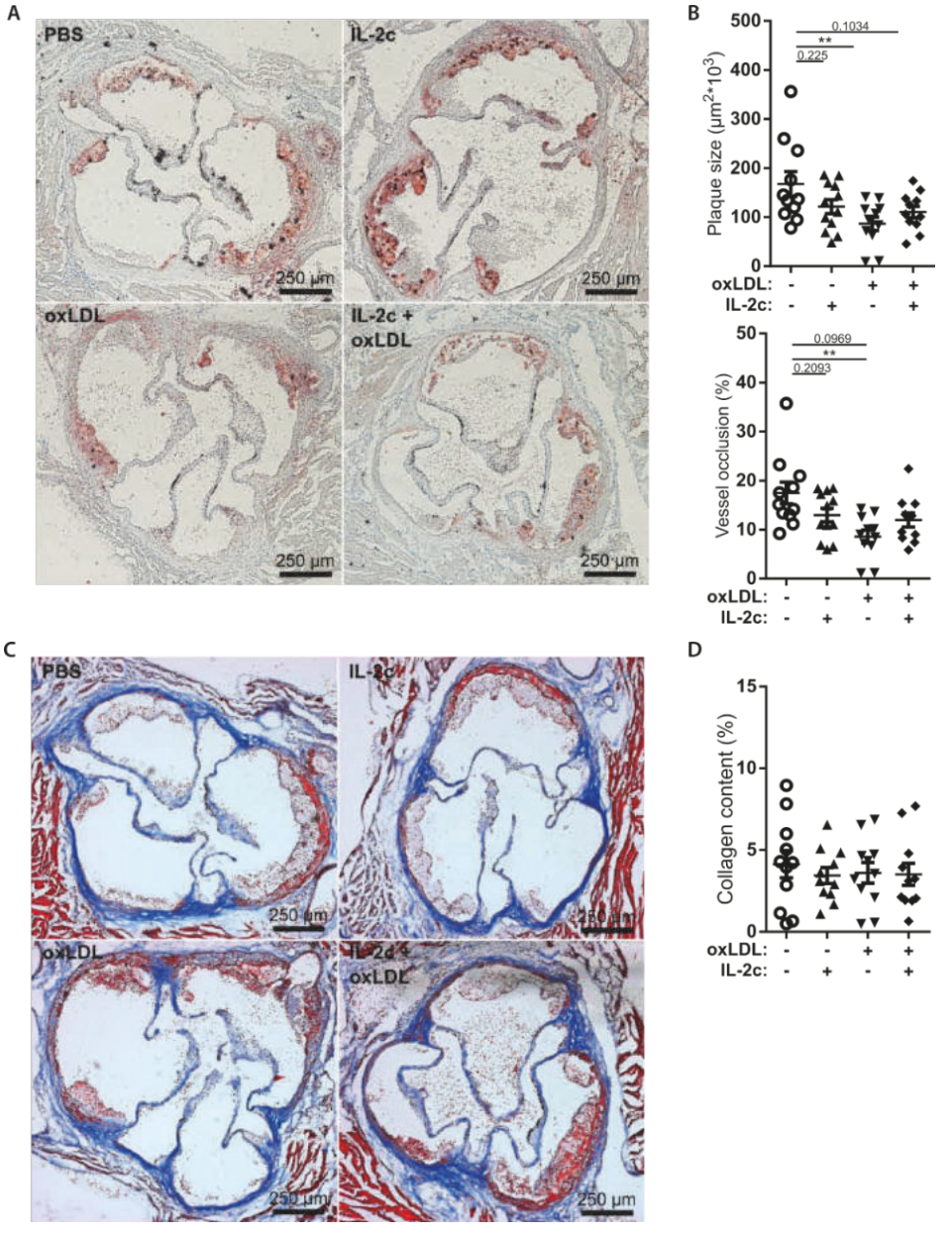

Fig. 3 ***Only separate oral oxLDL administration reduces aortic root plaque size.*** *To assess the effect of oral oxLDL and IL-2c administration aortic root sections were assessed.* ***A****) Representative microscopic images of neutral lipid staining with ORO used for* ***B****) quantification of atherosclerotic lesion size and vessel occlusion.* ***C****) Representative microscopic images of staining of collagen with Masson's Trichrome staining for the determination of plaque stability through* ***D****) quantification of collagen content. Expressed as mean ± SEM, one-way ANOVA with Tukey posttest, ** p < 0.01.*

The effect of oxLDL and IL-2c treatment on T cells was less evident in the lymphoid organs than in circulation. Neither treatment did alter overall splenocyte numbers or CD4 or CD8 T cell levels compared to control in the spleen (Fig. S1BC). oxLDL treatment alone significantly reduced the percentage of CD4 Tem cells and increased Th0 cells compared to all other experimental groups (Fig. S1B). Furthermore in all treatment groups a slight trend towards increased naïve CD8 T cells and reduced CD8 Tem and Tcm cells was observed, indicative of immune suppression by oxLDL and IL-2c treatment in the spleen (Fig. S1C). Furthermore we assessed T cell maturation in mesenteric lymph nodes and mediastinal lymph nodes but oxLDL and/or IL-2c treatment did not significantly impact the proportion of naïve, central memory, and effector memory T cells compared to control probably because the vast majority of T cells in the lymph nodes was already naïve in the control group (data not shown). Overall these data indicate that oxLDL and IL-2c treatment successfully induced immune suppression as seen from reduced circulating immune cell levels and reduced memory T cell populations in the IL-2c and oxLDL treated groups, compared to control treated mice.

Only separate oral tolerance induction to oxLDL significantly reduces aortic root plaque size.

To assess whether the immunosuppressive effects of the different treatment regimens translated in reduced atherosclerosis, aortic root tissue sections were stained with oil red o (ORO) and trichrome to assess plaque size and stability. The average lesion size in the control group was small (167,582 ± 84,079 μm^2) (Fig. 3AB) and low in collagen content (4.13 ± 2.78 %) (Fig. 3CD), characteristic for early atherosclerotic lesions. Surprisingly, despite increased Treg levels and clear signs of immunosuppression in IL-2c treated groups, only separate oral oxLDL treatment significantly reduced plaque size compared to the PBS group (48.2%, 86675 ± 45311 μm^2, p = 0.0095) (Fig. 3AB). A trend towards decreased plaque size was observed in IL-2c treated group (27.5%, 121342 ± 49079 μm^2, p = 0.2255), and the IL-2c + oxLDL combined group (33.9%, 110527 ± 39027 μm^2, p = 0.1034) compared to control treated (Fig. 3AB). Plaque size of the oxLDL treated group was not significantly different from that of the IL-2c treatment group (p=0.4714) or the IL-2c + oxLDL treatment group (p = 0.7602) (Fig. 3AB). Collagen content of the lesions was very similar in all groups (Fig. 3CD), indicating that at this early point of lesion development IL-2c and oxLDL treatment did not affect plaque stability (p = > 0.9 for all comparisons). Neither treatment did significantly affect body weight or cholesterol levels (Fig. S2).

IL-2c treatment induces pro-atherogenic cell populations.

Since treatment with IL-2c induced high levels of Tregs, and appeared to have induced immune suppression, we assessed whether IL-2c administration induced expansion of cell populations that could explain why IL-2c treatment was not more effective than oxLDL treatment and did not provide an additional beneficial effect when combined with oral oxLDL

treatment. Among other cell populations, IL-2c treatment increased the NK^+ cell number, comprised of the atherogenic NKT and NK cell populations *(15–17)*, and increased the number of atherogenic eosinophils *(18, 19)* in the blood (Fig. 4A). IL2c treatment also increased conventional DC *(20)* and patrolling monocyte *(21)* levels in the spleen (Fig. 4A), albeit their role in atherosclerosis is less clear cut. Furthermore we performed a peritoneal lavage to assess cell numbers at the site of IL-2c administration, and found increased leukocyte numbers in the peritoneal cavity (figure). Since these cells were not $CD4^+$, also in the peritoneal cavity IL-2c administration had off-target effects. The off-target induction of pro-atherogenic cell populations by IL-2c are likely to have contributed to the inability of IL-2c treatment to improve the atheroprotective effect of oral tolerance induction towards oxLDL.

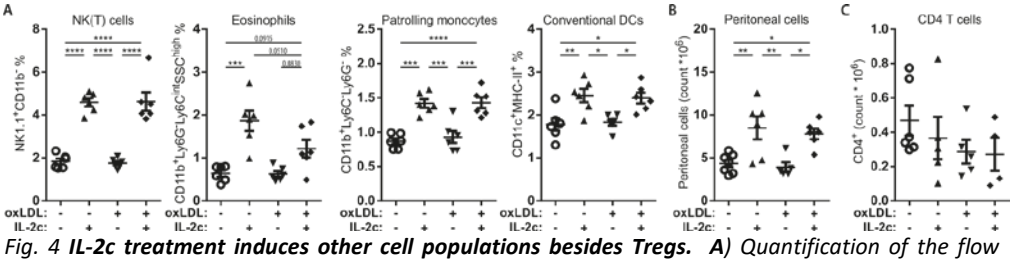

Fig. 4 ***IL-2c treatment induces other cell populations besides Tregs.*** ***A****) Quantification of the flow cytometric analysis of splenocyte populations increased by IL-2c treatment.* ***B****) Total count of cells obtained by peritoneal lavage.* ***C****) Quantification of the number of CD4 T cells in the peritoneal lavage fluid as determined by flow cytometric analysis. Expressed as mean ± SEM, one-way ANOVA with Tukey posttest, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

Discussion

Atherosclerosis is characterized by lipid accumulation and a chronic auto-immune like response in the vessel wall. Uptake of LDL, oxidized in the vessel wall, by macrophages is one of the hallmarks of atherosclerosis. Presentation of peptide epitopes from (ox)LDL derived ApoB100 by APCs is known to activate T cells leading to induction autoreactive (ox)LDL specific T cells. It is well established that the inflammatory response in atherosclerosis is skewed towards a Th1 response *(3, 22)*, resulting in high IFN-γ and TNF-α levels in the atherosclerotic lesion *(23)*.

$FoxP3^+$ regulatory T cells have been shown to be able to inhibit pathogenic Th1 skewed immune reactions in various experimental models of auto-immune disease *(4, 5)*, and therefore form an interesting treatment option. Antigen specificity is an important determinant for suppressive potential of Tregs *(9)*, however inducing and maintaining high levels of antigen specific Tregs has proven to be challenging *(24)*. In previous studies our lab has shown that oxLDL specific inducible Tregs and polyclonal Treg induction, induced through oral administration of oxLDL *(11)* and administration of IL-2c *(14)* respectively, effectively

inhibited atherosclerosis. Oral oxLDL administration led to increased Treg levels in MLN and spleen up to 2 weeks after final oxLDL administration but elevated Treg levels were not detected at sacrifice *(11)*. Therefore we hypothesized that maintaining or even increasing oxLDL specific Tregs with IL-2c could have an additional beneficial effect on atherosclerosis. Surprisingly only separate oxLDL administration significantly reduced atherosclerosis by 48.2%. A trend towards reduced plaque size of 27.5% and 33.9% was observed in the separate IL-2c, and IL-2c + oxLDL combination group respectively. Collagen content of the lesions was very similar in all groups, indicating that at this early point of lesion development, IL-2c and/or oxLDL treatment did not affect plaque stability. Similarly, treatment with IL-2c was only found to improve plaque stability in more advanced stages of atherosclerosis *(14)*.

Although in the IL-2c treated groups atherosclerosis was not significantly inhibited, we did observe enhanced $CD4^+FoxP3^+$ Treg levels in circulation, spleen, mesenteric lymph nodes, and mediastinal lymph nodes, indicating that IL-2c treatment had been effective. Furthermore lowered numbers of circulating immune cells were observed after IL-2c treatment, indicative that IL-2c treatment resulted in induction of functionally suppressive Tregs. In the group treated with only oxLDL we did not detect enhanced Treg levels in line with the aforementioned oxLDL tolerization study *(11)*. Oral oxLDL administration reduced white blood cell numbers compared to control treated similar to IL-2c treatment, indicative of ongoing immune suppression at sacrifice. This suggests that the suppressive capacity induced by oral oxLDL was not inferior to the suppressive capacity induced by IL-2c treatment, despite higher Treg levels in the IL-2c group than in the oxLDL group. The observation that antigen specific Tregs were found to have superior suppressive capacity compared to polyclonal Tregs *(9)* could explain this. The IL-2c mediated induction of atherogenic cell populations, including NK(T) cells *(15–17)* and eosinophils *(21)*, are likely to have inhibited the atheroprotective effect of IL-2c mediated Treg induction. In a previous study by Mahr *et al.*, treatment with JES6-1A12 – IL-2 complexes for 3 consecutive days doubled splenic cell numbers and enhanced NK cell, NKT cell, $FoxP3^-$ CD4 T cell, CD8 T cell, and B cell levels, 2 days after final injection, preventing engraftment of adoptively transferred bone marrow *(25)*. This suggests that over the course of the study the off-target effects of IL-2c treatment would have been even bigger than we observed at sacrifice and likely directly affected atherogenesis.

Furthermore it is possible that IL-2c administration interfered with the immune response induced by oral oxLDL administration which was likely dependent on oxLDL specific iTregs *(10, 11)*. Because of the plastic nature of Tregs *(26)*, IL-2c mediated induction of immune populations capable of secreting large amounts of cytokines, like eosinophils, could have affected the suppressive capacity and tissue distribution iTreg population. Furthermore we cannot exclude that IL-2c treatment expanded more specifically nTregs rather than iTregs in vivo, because these Treg populations cannot reliably be distinguished *(27)*, although in vitro both iTreg and nTreg populations are expanded by IL-2. Since adoptive transfer of nTregs led

to reduced iTregs *(28)*, it is possible that IL-2c mediated expansion of nTregs could have reduced iTreg numbers instead of expanding iTregs by IL-2c treatment. A difference in iTreg/nTreg balance would not only impact antigen specificity affecting immune suppression *(9)*, Treg subset specialization is dependent on environmental cues and is vital to suppress certain immune reactions *(29–31)*. Because of the different environmental origin of iTregs and nTregs, the function and tissue distribution of iTregs and nTregs are likely to be different. The observations that both iTregs and nTregs were needed to successfully suppress experimental colitis *(28)*, and that iTregs were more effective at inhibiting experimental asthma than nTregs *(32)*, indeed indicates functional differences between iTregs and nTregs. Therefore it would be interesting to assess whether iTregs and nTregs are similarly induced by IL-2c administration when reliable population specific markers are identified.

Treatment of autoimmune diseases using Tregs is among other things dependent on Treg antigen specificity and Treg quantity *(9, 24)*. Generating and maintaining high levels of antigen specific Tregs has proven to be challenging *(24)*. In this study we aimed to improve atheroprotection mediated by oral tolerance induction towards oxLDL through expanding and maintaining oral oxLDL induced Tregs by IL-2c administration in vivo. IL-2c administration did not improve efficacy of oral oxLDL administration despite enhancing Treg numbers.

Materials and Methods

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. LDLr$^{-/-}$ mice were originally purchased from Jackson Laboratories, and further bred in house. The mice were housed in groups of 2-4 animals in open cages with aspen bedding and were fed chow diet prior to the study. Mice were 10-12 weeks old at the start of the experiment and randomized based on age and weight (n=12).

Induction of atherosclerosis

LDLr$^{-/-}$ mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK) to induce atherosclerosis, and were provided with food and water at libitum. Directly after the first oral administration of oxLDL/PBS, mice were fed this WTD until sacrifice, 9 weeks later.

OxLDL tolerization

To induce oral tolerance mice were fasted for 4h with access to water, after which 2 mg of soybean trypsin inhibitor (Sigma) was administered orally to prevent antigen degradation. 10 minutes later mice orally received phosphate-buffered saline (PBS) or 30 µg of oxLDL. Induction of tolerance was repeated 3 times to a total of 4 injections in 8 days *(11)*. LDL was

isolated from blood plasma of a healthy volunteer by density gradient ultracentrifugation *(33)*, and oxidized with $CuSO_4$ (10 µM, 37°C, 20 hours) *(34)*.

Polyclonal Treg induction

To polyclonally induce Tregs mice intraperitoneally received IL-2c for 3 consecutive days, 2 days after final oral oxLDL administration. IL-2 complexes were generated by mixing recombinant IL-2 (1ug, Peprotech) with anti -IL-2 mAb (5 µg, JES6-1A12, R&D Systems) in sterile PBS and incubation at 37 °C for 30 minutes *(12–14)*.

Blood withdrawal and sacrifice

At intermediate timepoints blood was drawn by lateral tail cut and collected in EDTA coated capillary tubes (Microvette, Sarstedt). At the end-point of the study, mice were anesthetized by subcutaneous injection with a mix of ketamine (100 mg/mL), sedazine (25 mg/mL) and atropine (0.5 mg/mL), retro-orbitally exsanguinated, and perfused with PBS. TC levels were assessed with a commercially available kit (Roche) with precipath as an internal standard.

Histology

Hearts were cut in half and fixated in formalfix overnight. Hearts were incubated with OCT medium for at least an hour before cryosectioning (10 µm thick sections) and collected on Superfrost Plus™ Adhesion Microscope Slides (ThermoFisher). Neutral fats were stained with Oil Red O to assess lesion size in five subsequent sections of the heart at 70 µm intervals within the aortic root valve area. Collagen content of the lesions was quantified based on Masson's trichrome staining (Sigma-Aldrich). QWin software (Leica) was used to analyze the microscopic images (Leica DM2000).

Flow Cytometry

Cells from half of the animals (n=6 / treatment group) were stained extracellularly for 30 minutes in FACS buffer (PBS supplemented with 4% FCS) at 4° with the following antibodies (eBioscience/ThermoFisher Scientific): CD3-PerCP/Cy5.5 (145-2C11), CD4-PB (RM4-5), CD8-PE (53-6.7), FoxP3-PE (NRRF-30), CD25-FITC (PC61.5), CD44-FITC (IM7), CD62L-APC (MEL-14), CD19-PE (1D3), NK1.1-APC (PK136), CD11b-Pacific Blue (M1/70.15), Ly6C-PE (HK1.4), Ly6G-FITC (1A8), CD11c-FITC (N418), MHC-II- Pacific Blue (AF6-120.1). Cells were measured with a FACSCanto II (BD) flow cytometer. Analysis of flow cytometry data was performed with FlowJo software (Tree Star, inc.).

Statistical Analysis

Statistical analysis was performed with Graphpad Prism. Multiple group comparisons for a single timepoint were performed with a standard one-way ANOVA. For comparisons of multiple treatment groups with multiple timepoints, a standard two-way ANOVA was used.

The Tukey posttest was used to correct for multiple testing. A p-value < 0.05 was considered statistically significant.

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Supplementary figures

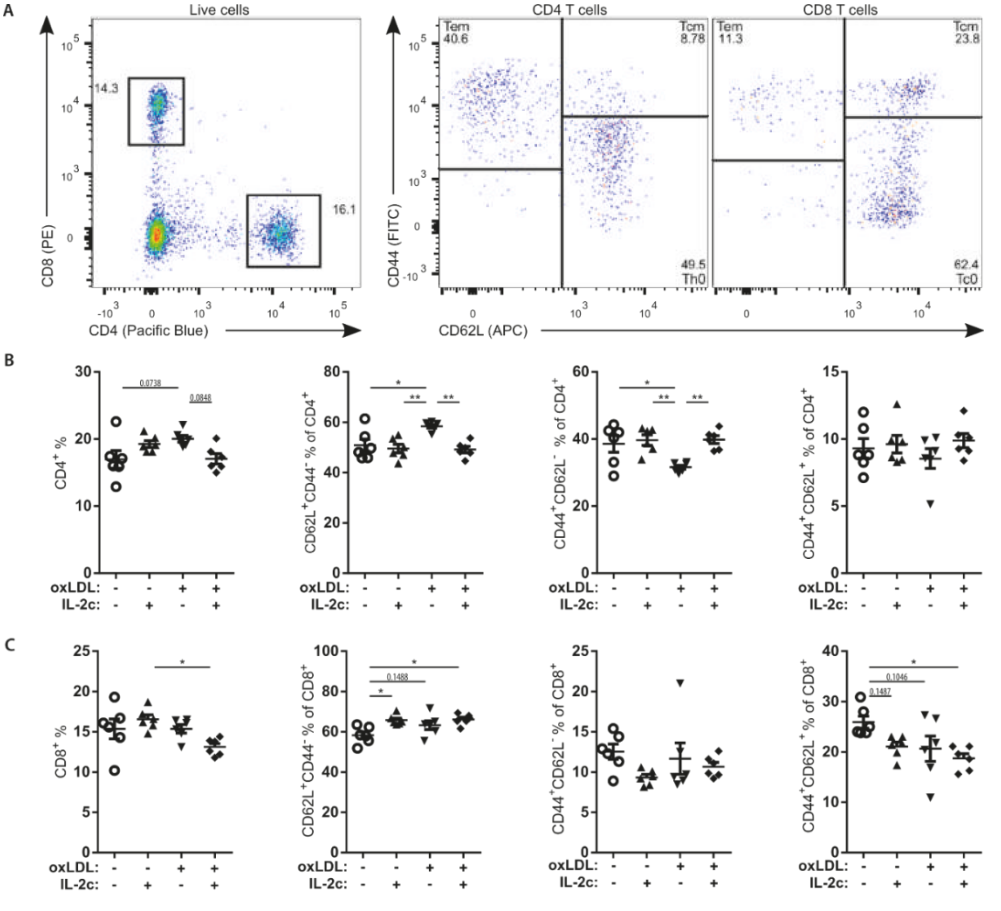

Fig. S1 ***Naïve and memory T cell subsets in the spleen. A****) Representative plots of the flow cytometric analysis of splenic naïve, Tem and Tcm of* ***B****) the CD4 T cell population and* ***C****) CD8 T cell population. Expressed as mean ± SEM, one-way ANOVA with Tukey posttest, * p < 0.05, ** p < 0.01.*

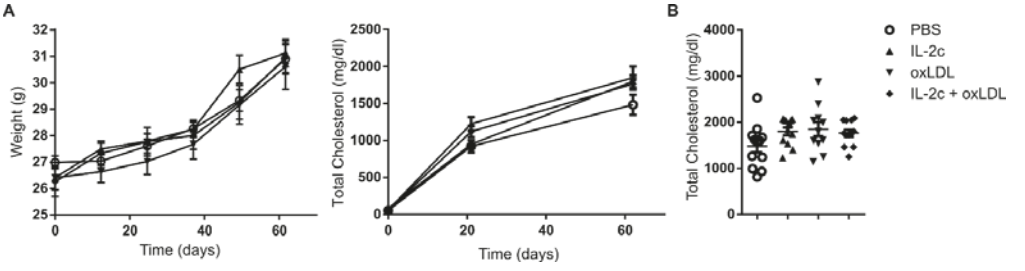

Fig. S2 ***Body weight and cholesterol are not affected by IL-2c or oxLDL treatment. A****) Over the course of the experiment body weight was monitored and cholesterol levels in blood plasma were determined.* ***B****) Cholesterol levels in blood plasma at sacrifice. Expressed as mean ± SEM,* ***A****) two-way ANOVA* ***B****) one-way ANOVA with Tukey posttest.*

4

Vaccination with ApoB100 derived peptide p210 does not provide atheroprotection in LDLr deficient, hApoB$^{100/100}$ transgenic mice.

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Abstract

Several vaccination strategies using ApoB100-derived peptide p210 have been found to be capable of reducing atherosclerosis. The atheroprotective mechanism of p210 vaccination is however disputed, and has been dedicated to induction of p210 antibodies, regulatory B cells and CD4 T cells, and cytolytic CD8 T cells. In this study we aimed to induce tolerance towards p210 through oral delivery of p210 coupled to the cholera toxin B-subunit (CTB), and aimed to stimulate a humoral and cellular response against p210 through subcutaneous alum-adjuvanted vaccination of p210 coupled to Pan DR-epitope (PADRE), in LDLr deficient, $hApoB^{100/100}$ transgenic (HuBL) mice.

CTB-p210 and PADRE-p210 administrations induced p210 IgG, and CTB-p210 also induced IL-10 producing regulatory B cells. We did not observe a significant impact of either p210 treatment on the CD4 T cell and CD8 T cell populations previously reported to mediate the atheroprotective effects of p210. The MHC binding properties of p210 make it unlikely that p210 effectively induces p210 specific T cells, however enhanced cell death in CTB-p210 but not in CTB or p210 stimulated splenocyte cultures is suggestive of adjuvant properties of p210, which could explain the divergent effects reported of vaccination with p210.

We did not observe reduced atherosclerosis upon p210 treatment, however as p210 is part of the LDLr binding site in ApoB100, it is possible that p210 antibodies affect atherosclerosis through modulation of the interaction between ApoB100 and the LDLr, which effect would have been missed with the LDLr deficient model used in this study. Alternatively, adjuvant properties of p210 could have induced atheroprotection in previous studies.

Introduction

Cardiovascular disease is still the primary cause of death in the world *(1)*. Atherosclerosis is the dominant underlying pathology of cardiovascular deaths. The pathogenesis of atherosclerosis is hallmarked by retention and accumulation of cholesterol rich LDL in the vessel wall of middle to large sized arteries. In the arterial wall LDL undergoes oxidative modifications, forming oxLDL, which triggers a chronic local immune response. Early atherosclerosis is dominated by the influx of monocytes in the atherosclerotic lesions which differentiate to macrophages and take up large quantities of oxLDL *(2)*. Presentation of LDL by dendritic cells and macrophages (antigen presenting cells, APCs) induces humoral and cellular adaptive immune responses to LDL, resulting in a pathogenic Th1 skewed immune response *(3, 4)*. Classically treatment of atherosclerosis has been focused on lowering lipid levels. This has been effective, however many patients carry a residual risk to cardiovascular events due to unresolved inflammation, even after successful lipid lowering *(5)*. Administration of neutralizing antibodies against IL-1β in the CANTOS trial reduced major cardiovascular events by up to 15% in the higher dose groups *(6, 7)*, supporting the rationale of reducing inflammation for treatment of atherosclerosis.

Since LDL has been identified as a major auto-antigen in atherosclerosis, multiple research groups have modulated the immune response towards LDL in preclinical models, successfully reducing atherosclerosis *(8)*. The clinical application of apoB100, the main protein in LDL, is hampered because of its large size and heterogeneous nature, and researchers have attempted to identify immunogenic epitopes in LDL for clinical purposes. The 20 amino acid long p210 peptide is one of the LDL ApoB100 derived antigens that in multiple different formulations and vaccination strategies, has proven to be capable of reducing atherosclerosis *(9–12)*. The protective mechanism(s) by which p210 vaccination induces atheroprotection are however still disputed *(8)*, which may hamper the clinical use of p210 vaccination. The p210 peptide was originally described as an antigen that is recognized by antibodies in serum of cardiovascular patients *(13)*, identifying p210 as an antibody epitope. In line with this, the atheroprotective effects of p210 have been attributed to induction of p210 specific antibodies *(12)*. However other papers have dedicated the atheroprotective properties of p210, utilizing several different formulations, administration routes and administration schedules, to induction of Bregs *(10)*, CD4 Tregs *(9, 10)*, and cytotoxic CD8 T cells *(11, 14)*.

In this study we aimed to further delve into the effects of p210 vaccination on the immune system and its atheroprotective effects. Since it is generally accepted that atheroprotection can be accomplished by induction of a regulatory response towards intact (ox)LDL *(15)* or parts of LDL *(16, 17)*, we first aimed to induce a tolerogenic response towards p210 by oral administration of p210 coupled to cholera toxin B (CTB). Repeated low dose antigen administration via the oral route is known to induce regulatory antigen specific adaptive immune cells, inducing so called oral tolerance *(18)*. CTB can induce tolerogenic dendritic

cells *(19)* and B cells *(10, 20)*, and enhances mucosal delivery of coupled antigens through interaction with GM1 gangliosides, greatly enhancing antigen specific regulatory CD4 T cell numbers in vivo *(21)*. To investigate the atheroprotective potential of p210 antibodies and p210 specific conventional effector T cells, we intraperitoneally vaccinated mice with p210 coupled to PADRE, adjuvanted with alum.

Since p210 is not completely homologous to the corresponding murine sequence in ApoB100 (90% homology, accession NP_033823.2 vs p210), we aimed to use a preclinical model with endogenous expression of human ApoB100, which could impact thymic selection of T cell clones specific for human apoB100 and p210, and in vivo presentation of the cognate antigen for specific T cell clones. Furthermore in all published studies regarding p210 immunization in atherosclerosis ApoE deficient mice were utilized. Since p210 is part of the LDL receptor binding site A in ApoB100 *(22, 23)*, we wondered whether the protective effect of p210 vaccination still was observed in the absence of LDLr. Therefore we used LDLr deficient and human $ApoB^{100/100}$ transgenic (HuBL) mice *(23, 24)* in the experiments described in this paper.

Results

Oral vaccination with CTB-p210 induces Bregs, and p210-specific IgG and IgA

To establish a regulatory response towards p210, female HuBL mice received an oral gavage with CTB-p210 (30 µg) every other day in the first week (4 times), after which p210-CTB was orally administered weekly to maintain a p210 specific immune response. In parallel to CTB-p210 administration, the control group orally received CTB (same molar amount). From the start of treatment animals were fed a western type diet to induce atherosclerosis, until sacrifice 8 weeks later. Since previously CTB-p210 was found to induce Bregs in vitro *(10)*, we assessed the splenic B cell population for IL-10^+ Bregs (B10 cells) with flow cytometric analysis of splenocyte cultures incubated with PMA/ionomycin and Monensin for 5h (Fig. 1B). The overall levels of splenic B cells (prior to culture) did not differ between CTB and CTB-p210 treated mice (Fig. 1A), but in line with the in vitro data of Rattik CTB-p210 treatment increased B10 cell levels compared to CTB treatment (Fig. 1C). Because p210 was initially identified as an antibody epitope *(13)*, we assessed the effect of oral CTB-p210 vaccination on the B cell population and p210 specific antibody levels. Oral CTB-p210 administration increased IgA and IgG levels, but not IgM levels, against native p210 (Fig. 1D) and malondialdehyde (MDA) modified p210 (Fig. 1E) compared to CTB administration.

Oral CTB-p210 administration does not induce Tregs

Because nasal CTB-p210 administration was previously found to induce CD4 Tregs in vivo *(9)* and CTB-p210 pulsed B cells induced CD4 Tregs in vitro *(10)*, we assessed Treg levels in several lymphoid organs. We did not observe enhanced levels of $FoxP3^+CD25^+$ CD4 T cells (Fig. 2A) and $FoxP3^+CD25^+$ CD8 T cells (Fig. 2C) in freshly isolated splenocytes, or enhanced levels of IL-10^+ CD4 T cells (Fig. 2B) and IL-10^+ CD8 T cells (Fig. 2D) in PMA/ionomycin stimulated

splenocyte cultures of P210-CTB treated mice. Since after oral administration of CTB-OVA the highest levels of Tregs were measured in the Peyer's patches and mesenteric lymph nodes draining the intestines *(25)*, we also assessed FoxP3^{+}CD25$^{+/-}$ CD4 T cell levels in Peyer's Patches (Fig. S1A) and mesenteric lymph nodes (Fig. S1B), and atherosclerosis relevant mediastinal lymph nodes (Fig. S1D), draining the aortic arch, and in circulation (Fig. S1C). In neither of the assessed organs we observed enhanced Treg levels, suggesting that oral CTB-p210 administration did not enhance Treg levels over oral CTB administration. Moreover, we did not observe a difference in IFN-γ^{+} CD4 T cells between CTB and CTB-p210 treated (Fig. 2B), indicating that the atherogenic Th1 response was not significantly altered by CTB-p210 compared to CTB administration.

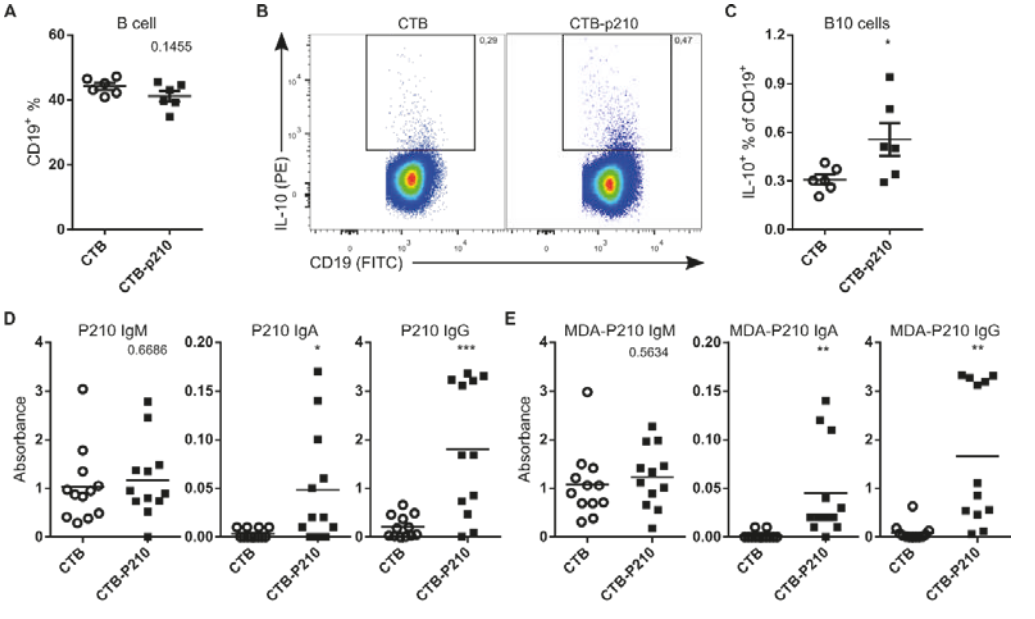

Fig. 1 ***Oral CTB-p210 administration induces B10 cells and anti-p210 IgA and IgG antibodies. A****) Quantification of B cell content in spleen as assessed by flow cytometry.* ***B****) Representative flow cytometry plots of the CD19^{+}CD4^{-}CD8^{-} population of splenocytes cultures incubated with PMA/IONO, and Brefeldin A and Monensin for 5h.* ***C****) Quantification of IL-10^{+} cells in the B cell population of PMA/IONO stimulated splenocytes cultures. Quantification of ELISA measurements of IgM, IgA, and IgG antibodies against* ***D****) native p210, and* ***E****) malondialdehyde (MDA)-modified p210, in blood serum (1:50 dilution) obtained at sacrifice. Represented as mean ± SEM, unpaired two-tailed T test, * p < 0.05, ** p < 0.01, *** p < 0.001.*

Oral CTB-p210 administration induces splenocyte reactivity towards oxLDL

To assess whether vaccination with CTB-p210 had affected the immune reactivity towards oxLDL, we subjected splenocytes to a thymidine incorporation assay. Surprisingly, both basal proliferation (Fig. 3A) and oxLDL induced proliferation (Fig. 3B) were increased in splenocyte cultures from CTB-p210 treated mice compared to splenocyte cultures from CTB treated mice. Furthermore overall thymidine incorporation was similar in αCD3/αCD28 cultures from both treatment groups (Fig. 2C), indicating that overall proliferative capacity of splenic T cells was similar in CTB-p210 and CTB treated mice. In an effort to quantify CTB-p210 treatment relevant antigen specific T cell responses, we exposed splenocytes from both treatment groups to freshly isolated LDL (5 µg/ml), copper oxidized oxLDL (5 µg/ml), p210 (2 µM), CTB (2 µM), or CTB-p210 (2 µM) for 5h after which levels of IL-2, IL-10 and IFN-γ producing CD4 and CD8 T cells were assessed by flow cytometry. We did not observe significant changes in cytokine production between the treatment groups (data not shown), however we did observe reduced cell viability in splenocytes cultured with CTB-p210 irrespective of in vivo treatment (Fig. 3DE).

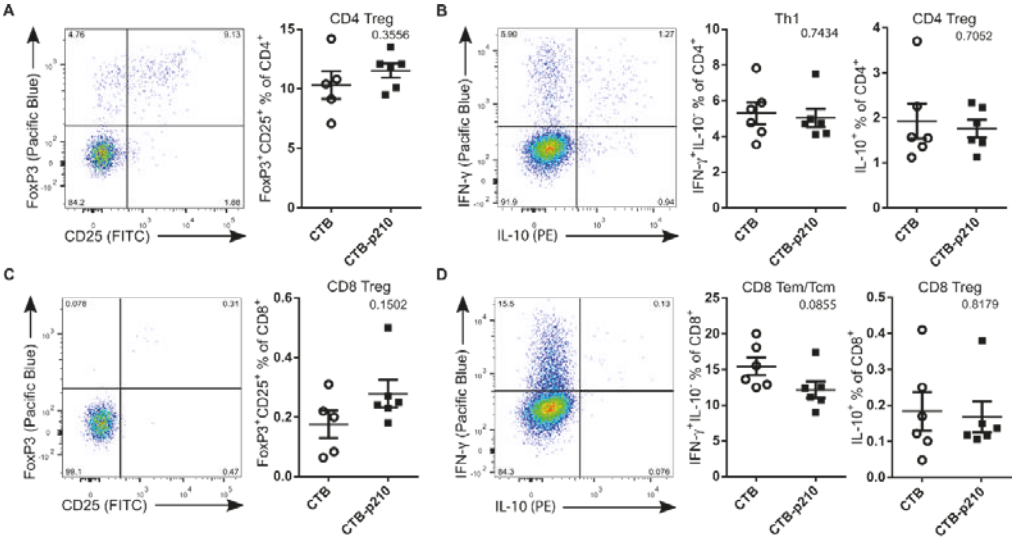

Fig. 2 ***Splenic regulatory T cell and Th1 cell levels are not affected by oral CTB-p210 administration.*** ***A****) Representative flow cytometry plots of the gating of regulatory CD25⁺FoxP3⁺ cells and quantification in the splenic CD4 T cell population and* ***C****) CD8 T cell population.* ***B****) 2*10⁶ splenocytes per well were incubated with PMA, ionomycin, Monensin and Brefeldin A for 5h after which IFN-γ⁺ and IL-10⁺ CD4 T cells and* ***D****) CD8 T cells were flow cytometrically assessed and quantified. Represented as mean ± SEM,*

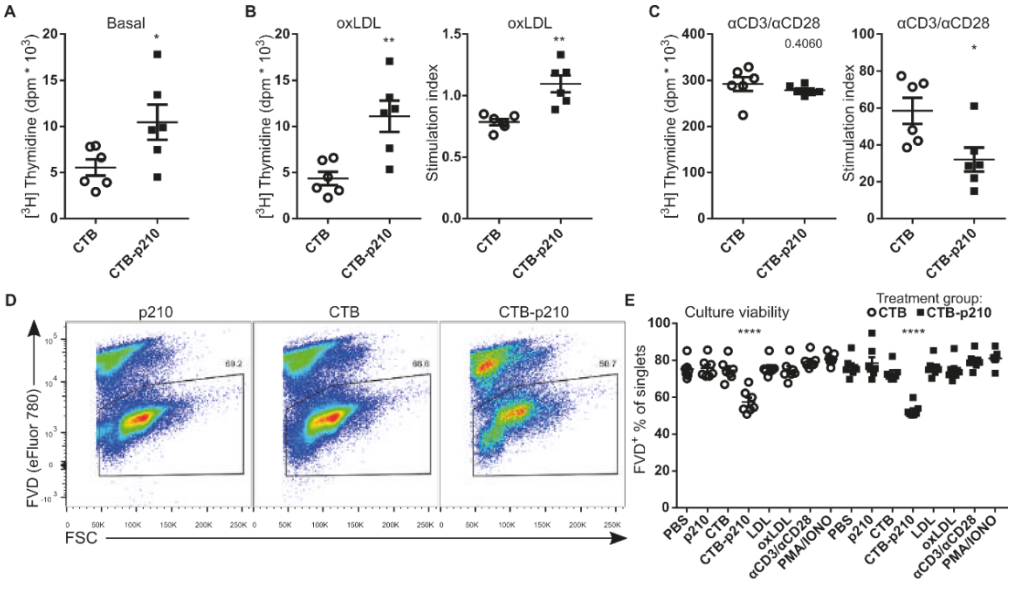

Fig. 3 ***CTB-p210 induces oxLDL reactivity in ex vivo splenocyte cultures and causes cell death in vitro.*** ***A****) Basal splenocyte proliferation* ***B****) oxLDL (5 µg/ml) induced proliferation and* ***C****) αCD3/αCD28 induced T cell proliferation as assessed by a thymidine incorporation assay in splenocyte cultures from CTB and CTB-p210 treated mice.* ***D****) Representative flow cytometry plots of the singlet population of splenocyte cultures (2*10^6 cells/well) from the same CTB treated mice, incubated for 5 hours with p210 (2 µM), CTB (2 µM), or CTB-p210 (2 µM), and gating for viable cells.* ***E****) Quantification of culture viability of splenocyte cultures from CTB and CTB-p210 treated mice, incubated for 5 hours with PBS, p210 (2 µM), CTB(2 µM), CTB-p210(2 µM), LDL (5 µg/ml), oxLDL (5 µg/ml), αCD3/αCD28, or PMA+Ionomycin, and Brefeldin A and Monensin, as assessed with flow cytometry. Represented as mean ± SEM,* ***A-C****) unpaired two-tailed T test,* ***E)*** *One-Way ANOVA with Holm-Šídák posttest, * p < 0.05, ** p < 0.01, **** p < 0.0001.*

Antibodies against p210 are not protective in LDLr deficient hApoB100tg mice.

To assess the effect of CTB-p210 vaccination on atherogenesis we histologically assessed the atherosclerotic lesion size in the aortic root (Fig. 4A) and brachiocephalic artery (Fig. 4C). The average aortic root lesion size was not different between the CTB treated group (636667 ± 170356 µm^2) and the CTB-p210 treated group (693083 ± 135938 µm^2) (Fig. 4B). Similarly brachiocephalic average lesion size and lesion volume did not differ between CTB (avg. lesion: 139790 ± 73282 µm^2; volume: 1.48*10^8 ± 1.29*10^8 µm^2) and CTB-p210 treated (avg. lesion: 94512 ± 69641 µm^2; volume: 9.46*10^7 ± 8.46*10^{7}µm^2) (Fig. 4D). Moreover, there was no correlation between the atherosclerotic lesion size in the aortic root and IgM, IgA, and IgG levels against native p210 (Fig. S2A) and MDA-modified p210 (Fig. S2B).

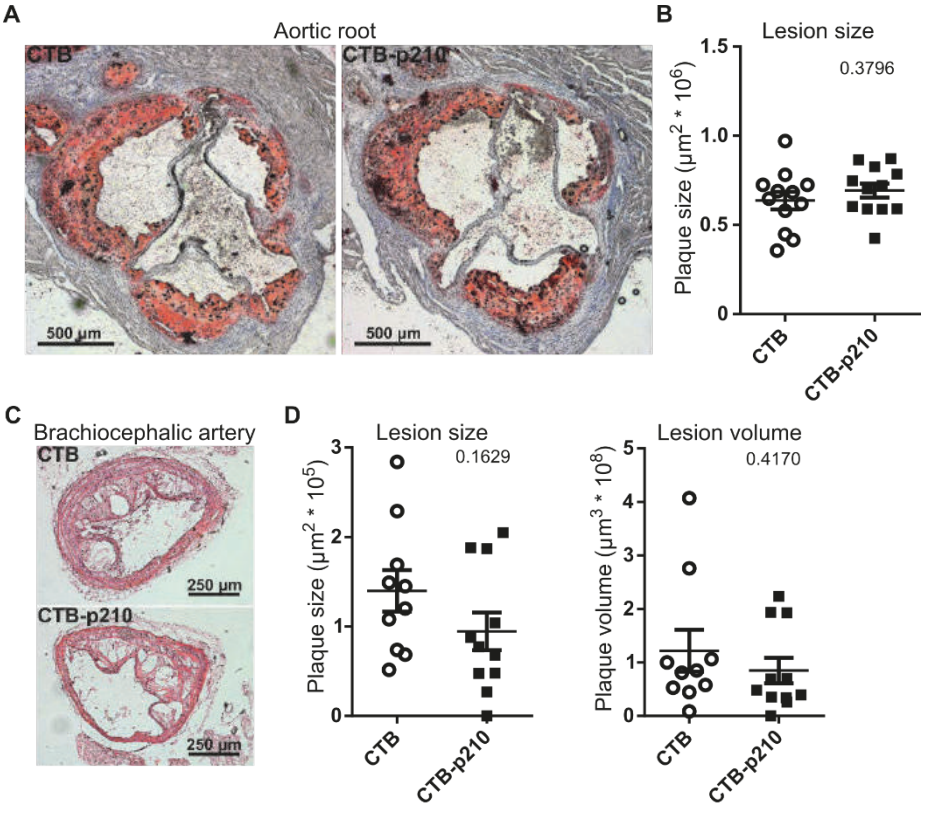

Fig. 4 ***Oral CTB-p210 vaccination does not reduce atherosclerotic plaque size in HuBL mice. A****) Transverse sections of the aortic root stained with oil red o* ***B****) and quantification of the average lesion size in the aortic root.* ***C****) Sections of the brachiocephalic artery stained with H&E and* ***D****) quantification of the average lesion size and lesion volume in the brachiocephalic artery. Represented as mean ± SEM, unpaired two-tailed T test.*

The only p210 specific response we could detect after oral CTB-p210 administration was the induction of p210 specific antibodies. To ensure that we did not underestimate the atheroprotective effect of p210 antibodies, possible due to induction of insufficient levels of p210 antibodies to significantly impact atherogenesis, we performed an additional experiment. In this experiment we subcutaneously immunized HuBL mice with p210 coupled to PADRE (Pan-DR-epitope), a strong MHC-II epitope which promotes T cell dependent antigen production *(26)*, and used alum as an adjuvant to stimulate a humoral response *(27)* and a conventional effector CD4 and CD8 T cell response against p210. Since a p210 specific atheroprotective CD8 T cell response was reported after alum adjuvanted vaccination with cBSA-p210, we were also cautious of a possible CD8 T cell response induced by our vaccination scheme.

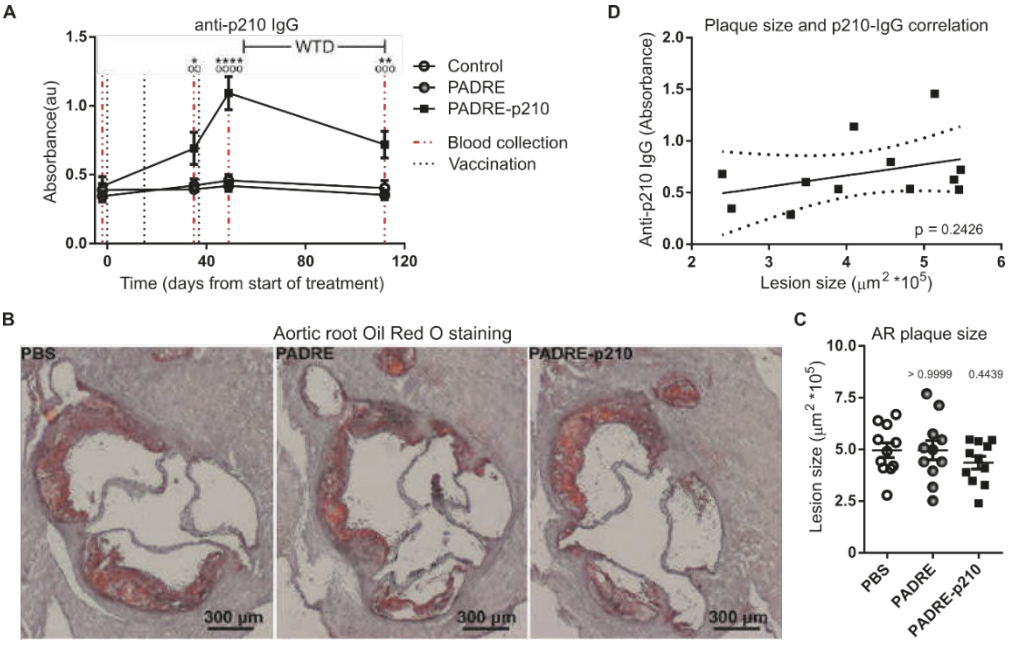

Fig. 5 ***PADRE-p210 vaccination induces high p210 specific IgG levels but does not act atheroprotective in HuBL mice.*** ***A****) Experimental setup and p210 IgG levels blood plasma (1:1000 dilution).* ***B****) Representative transverse sections of the aortic root stained with ORO,* ***C****) used for the quantification of the average atherosclerotic lesion size in the aortic root (AR).* ***D****) Correlation analysis of p210 antibody levels in blood serum obtained at sacrifice from PADRE-p210 treated animals, and the average atherosclerotic lesion size in the atherosclerotic root. Represented as mean ± SEM,* ***A****) Two-Way ANOVA with Holm-Šídák posttest, significance of control vs PADRE-p210 treatment depicted by (*) and of PADRE vs PADRE p210 by (°), * p < 0.05, **/°° p < 0.01, °°° p < 0.001 ****/°°°° p < 0.0001.* ***C****) One-Way ANOVA with Holm-Šídák posttest.* ***D****) Scatter dot plot depicted with trend line (solid line), 95% Confidence interval (dotted lines).*

Mice were subcutaneously vaccinated 3 times in 37 days with PADRE-p210 (25 µg, 6.7 nmol) in alum, PADRE (9.1 µg, 6.7 nmol) in alum, or PBS (Fig. 5A). Increased levels of p210 IgG antibodies were observed in the p210-PADRE treated group compared to PADRE and PBS treated groups after the second immunization and were further boosted with a third vaccination, after which mice received WTD until sacrifice (Fig. 5A). We did not detect differences between treatment groups in levels of CD8 T cells, $Ki67^+$ proliferating CD8 T cells, $CD44^+$ antigen experienced CD8 T cells, and $FoxP3^+$ CD8 Tregs in mediastinal lymph nodes (data not shown) and spleen (Fig. S3A). The effect of PADRE-p210 on atherosclerosis was assessed by histological analysis of aortic root tissue sections (Fig. 5B) p210-PADRE treatment did not affect aortic root lesion size (Fig. 5C) (PBS: 490517 ± 118811 μm^2 PADRE: 495962 ± 154416 μm^2 PADRE-p210: 436183 ± 102236 μm^2). Anti-p210 IgG levels of PADRE-p210 treated animals did not correlate with plaque size (Fig. 5D), similar to what we observed in CTB-p210 treated mice.

Discussion

Cardiovascular disease is the primary cause of death in the world with atherosclerosis as its main underlying pathology *(1)*. Atherosclerosis is driven by dyslipidemia and inflammation. The main focus of atherosclerosis treatment has classically been the normalization of circulating lipid levels. Normalizing lipid levels in circulation has reduced cardiovascular events by reducing the deposition and accumulation of cholesterol rich LDL in the artery wall *(28, 29)*. Furthermore normalization of lipid levels also reduces inflammation, however in many CVD patients in which treatment of dyslipidemia is successful, inflammation is not completely resolved and poses a residual risk for a CV event *(5)*. Recently, results from the CANTOS trial, in which a human IL-1β neutralizing antibody was used to dampen inflammation, provided proof that inhibition of inflammation is clinically effective in human, reducing major cardiovascular events by 15% *(7)*.

Preclinical studies have shown that inflammation in context of atherosclerosis can also be inhibited through modulation of the adaptive response to plaque constituents, including HSPs *(30, 31)* , complete (ox)LDL *(15)* and parts of (ox)LDL *(32)*, which lead to a reduction in atherosclerosis. Because LDL is heterogeneous containing lipids and a single, large apolipoprotein B-100, research groups have put effort into identifying immunogenic epitopes in the ApoB100 protein to use for modulating the immune response against LDL *(13, 16, 33)*. One of the epitopes that was identified by a peptide library screen for antibody binding derived from human pooled blood is p210 *(13)*. Since then, multiple immunization studies with p210 have been performed resulting in reduced atherosclerosis in ApoE deficient mice, although the mechanism of action is still disputed *(34, 35)*. To obtain new insights into the immunological mechanism of action of p210 we aimed to assess the effect of p210 vaccination on atherosclerosis and the immune system in an LDL receptor deficient mouse model with endogenous expression of human ApoB100, the HuBL mouse model.

Because p210 mediated atheroprotection has been suggested to originate from the induction of regulatory CD4 T cells *(9)*, regulatory B cells *(10)*, conventional effector CD8 T cells *(11, 14)*, and p210 specific antibodies *(12)* depending on formulation and mode of administration, we employed two vaccination strategies to promote induction of adaptive responses against p210. On the one hand, we opted to deliver p210 fused to the tolerogenic adjuvant CTB *(21)* and administered via the oral route to induce oral tolerance, which is known to induce regulatory B cells, and antigen-specific Tregs *(18)*. On the other hand, p210 was coupled to PADRE, adjuvanted with alum and injected subcutaneously to maximize p210 specific antibody production and induce conventional T cell responses.

Both vaccination approaches used in our study resulted in the induction of p210 specific antibodies in the treatment groups containing p210, indicating that both protocols broke tolerance towards a part of the endogenous protein apoB100. We did not observe an

atheroprotective effect of induction of antibodies against p210. In a recent study however, immunization with p210, and administration of antibodies against MDA-modified p210 resulted in reduced atherosclerosis due to p210-antibody mediated altered lipid handling and reduced inflammation *(12)*. Similarly, atheroprotection was observed in nasal CTB-p210 treated mice, inducing anti-p210 antibodies and CD4 Tregs *(9)*. The discrepancy in the observed atheroprotective effect of p210 immunization between the studies of Klingenberg and Zeng, and our study can be the result of the use of ApoE deficient mice in the previous studies, and the use of LDLr deficient animals in our study since p210 is part of LDLr binding site A in ApoB100 *(22, 23)*. In line with a role for the LDLr in the atheroprotective effects of p210 antibodies, LDLr mediated uptake of LDL by adipocytes was inhibited in vitro by p210 antibodies *(36)*. In macrophage cultures incubated with oxLDL, addition of p210 antibodies inhibited the formation of foam cells *(36)* and upregulated the expression of cholesterol efflux genes *(12)*, beneficial in the context of atherosclerosis. Induction of p210 and LDL cross-reactive antibodies with a p210 mimotope, was found to prevent high fat diet induced weight gain and liver steatosis in wild type C57BL/6 mice *(36)*, showing that anti-p210 antibodies can also improve lipid handling in vivo in WT animals. Through immunization with a p210 mimotope in the study of Kim, a peptide with a different amino acid sequence than p210 but capable of inducing antibodies cross reactive with p210 and LDL, the possibility that p210 specific T cells were induced and affected lipid handling was excluded. Moreover in humans, anti p210 IgM and IgG levels were correlated with improved carotid intima-media thickness parameters *(37)*, indicating atheroprotective properties of p210 antibodies in human. Interestingly the inverse correlations between anti-p210 IgG levels and baseline composite measures of carotid intima-media thickness disappeared when adjusted for known risk factors *(37)*, suggesting that improved lipid handling induced by anti-p210 antibodies *(12, 36)* might also occur in human.

Besides induction of protective antibodies, the protective effect of vaccination with p210 formulations has been dedicated to the induction of atheroprotective T cell populations *(9, 11, 14)*. However, after neither of the p210 immunizations we detected alterations in CD4 T cell or CD8 T cell populations, nor did we detect an atheroprotective effect which, if T cell dependent, should not have been affected by the use of an $LDLr^{-/-}$ or $ApoE^{-/-}$ model. Furthermore, immunomodulation using ApoB100 derived CD4 T cell epitopes was shown to be capable of reducing atherosclerosis in $ApoE^{-/-}$ mice *(16)* and $LDLr^{-/-}$ mice *(17)* before. It would be interesting to assess whether this favorable immunomodulation can still be achieved when commenced at later stages of atherosclerosis development, when immune tolerance towards plaque antigens like ApoB100 might have been eroded *(38)*. Lack of robust demonstration of p210 specific T cells is in line with in silico prediction tools of MHC binding which indicate that it is very unlikely that p210 itself or p210 derived peptides bind murine MHC-II (IAb) *(35)*, or MHC-I (H-2Kb and H-2Db) (Supplementary Table 1). This suggests that the previously observed T cell responses after p210 vaccination are likely dependent on an indirect mechanism. Similarly we observed enhanced cell death in splenocyte cultures

incubated with CTB-p210 irrespective of the treatment group, which was unlikely the result of a cytotoxic T cell response or regulatory T cell response towards CTB-p210, because flow cytometric analysis of the cultures did not reveal enhanced IFN-γ^+ or IL10$^+$ CD4 T cells or CD8 T cells, nor did p210 or CTB induce enhanced cell death. It is therefore conceivable that p210 potentiated the known pro-apoptotic properties of CTB *(39)*.

Adjuvant properties of p210 could explain the divergent immunological effects which have been described upon administration of different p210 formulations. As a matter of fact, the heparan sulfate proteoglycan and LDLr binding properties of the LDLr binding sites of ApoB100 were previously utilized to promote the uptake of the SIINFEKL peptide, promoting cross priming of CD8 T cells *(22)*. Furthermore, an enhanced FITC signal was detected in DCs cultured with p210-FITC but not in DCs incubated with FITC alone *(11)*, strongly suggestive that p210 can promote uptake of coupled proteins via heparan sulfate proteoglycans and the LDLr. Enhanced p210 mediated uptake and cross-presentation of cBSA, containing T cell epitopes, could therefore explain the observed induction of CD8 T cell activity in CTB-p210 vaccinated mice *(11)*. Due to the 70% homology between bovine albumin and murine albumin (accession M73993.1 vs accession BC049971.1), enhanced cBSA presentation is likely to cause induction of alloreactive and auto-reactive CD4 and CD8 T cell clones, and cross-reactive antibodies against murine albumin which is present in large quantities in atherosclerotic plaques *(40)*. Since immunization with cBSA reduces atherosclerosis *(41)*, the proposed adjuvant properties of p210 could therefore explain the atheroprotective effect of cBSA-p210 vaccination. Similarly, enhanced p210 mediated uptake of CTB, enhancing its tolerogenic activity, could also explain the enhanced induction of regulatory TGF-β^+ B cells in vitro with CTB-p210 compared to CTB-OVA *(10)* and the enhanced regulatory B10 cells that we observed in spleens of CTB-p210 treated mice.

In our immunization studies with CTB-p210 and PADRE-p210 we did not observe any improvement in plaque parameters after vaccination with p210. We did not observe previously reported atheroprotective CD4 and CD8 T cell responses, likely due to absence of T cell epitopes in p210. We did observe induction of anti-p210 antibodies. Failure of anti-p210 antibodies to modulate atherosclerosis in LDLr deficient HuBL mice used in this study, support the notion that anti-p210 antibodies act through modulation of the interaction of LDL with the LDLr. A conceivable explanation for the divergent immunological effects of vaccination with p210 which have been reported, are the strong indications that p210 possesses adjuvant properties and could thereby promote the activity of p210-coupled adjuvants and promote immune reactions against p210-coupled antigens.

Materials and methods

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Breeding pairs from human ApoB100 transgenic LDLr$^{-/-}$ (HuBl) mice *(23, 24)* were a kind gift from prof. Jan Nilsson (Lund University), and further bred in house. HuBl mice were housed in individual ventilated cages with aspen bedding, in groups of 2–4 mice. Prior to inclusion of mice in experiments, expression of human ApoB100 was confirmed in blood plasma with a human ApoB100 ELISA (Mabtech). Mice were fed a regular chow diet prior to initiation of the *in vivo* experiments after which they were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Purified p210 (human apoB-100 amino acids 3136 to 3155; KTTKQSFDLSVKAQYKKNKH) *(13)*, p210 gene fusion proteins with CTB and PADRE, and CTB and PADRE, derived from cytoplasmic inclusion bodies in E. coli like previously described *(42)*, were a kind gift from dr. Lebens, (University of Gothenburg), and administered as indicated in the results section. At the end-point of the studies, mice were anesthetized by subcutaneous injection with a mix of ketamine (100 mg/mL), sedazine (25 mg/mL) and atropine (0.5 mg/mL), retro-orbitally exsanguinated, and perfused with PBS.

In vitro assays

Basal splenocyte proliferation and proliferation in response to oxLDL (5ug/ml) or anti-CD3e (1 µg/mL) and anti-CD28 (0.5 µg/mL) (both from Thermo Fischer) were assessed by a ^{3}H-thymidine incorporation assay. Splenocytes (2*10^{5} cells/well) were cultured for 72h in RPMI (Lonza; supplemented with 10% FCS (GE Life Sciences), pen/strep Lonza) with mentioned stimuli or PBS (control) and incubated with 0.5 µCi/well ^{3}H-thymidine (Perkin Elmer) for the last 16 h. Cells were thoroughly washed with PBS and thereafter lysed with natriumhydroxide and taken up in Emulsifier-Safe™ (Perkin Elmer). ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as the mean disintegrations per minute (dpm). The stimulation index (s.i.) was defined by dividing the dpm of stimulated conditions by the dpm of the PBS condition per mouse. Moreover, responsiveness of splenic CD4 T cells, CD8 T cells, and B cells, towards p210 (2 µM), CTB(2 µM), CTB-p210(2 µM), LDL (5 µg/ml), oxLDL (5 µg/ml), αCD3 (1 µg/mL)/αCD28(0.5 µg/mL), or PMA+Ionomycin, was assessed by incubation of splenocytes (2*10^{6} cell /well) with the indicated stimuli or PBS and incubation with Monensin and Brefeldin (Biolegend) for 5 hours, after which splenocyte cultures were assessed with flow cytometry.

Flow Cytometry

Extracellular staining (ECS) of single cell suspensions was performed in PBS with 2% FCS and αCD16/32 antibody (93, Biolegend) and eBioscience™ Fixable Viability Dye eFluor™ 780

(ThermoFisher) to discriminate between living and dead cells for 30 minutes at 4°C. For intracellular transcription factor staining after ECS, cells were fixed and permeabilized with the FoxP3 transcription factor buffer set (Thermofisher/eBioscience) according to manufacturer's instructions, and incubated with flow cytometry antibodies for 45 minutes at 4°C. Spleen and lymph nodes were mashed over a 70 µm cell strainer (Greiner) to obtain single cell suspensions and red blood cells were lysed with ACK lysis buffer if necessary.

The following antibodies were purchased at BD biosciences: The following antibodies were purchases at BD Biosciences: CD4 PerCP and Pacific Blue (RM4-5), CD44 PE/Cy7 (IM7) Biolegend: CD8 BV510 and APC (53-6.7); eBioscience/Thermofisher scientific: CD19 Pacific Blue and FITC (eBio1D3), CD19 PE/Cy7 (eBio1D3), CD25 APC (PC61.5), CD25 FITC (PC61.5), CD8-APC (16-10A1), FoxP3-PE (NRRF-30), IFN-γ (XMG1.2), IL-2 APC (JES6-5H4), IL-10 PE (JES5-16E3), Ki67 FITC (SolA15). Compensation measurements were performed using UltraComp eBeads (ThermoFisher) and ArC Amine-Reactive Compensation Beads (ThermoFisher). Cells were measured with a FACSCanto II (BD Biosciences) and a Cytoflex S flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star, inc.).

P210 autoantibody determination

Native p210 and MDA-modified p210 (MDA-p210) antibodies were determined with an ELISA approach. MDA-p210 was obtained through incubation of p210 with 0.5M MDA for 3h at 37°C, and dialysis with PBS containing 1mM EDTA. High protein binding plates (Corning Costar) were coated with native p210 (20ug/ml) and MDA-p210 (20ug/ml) in PBS overnight at 4°C. Coated plates were washed with 0.01% Tween-20 (PBS-T) and blocked with PBS-T with 1% BSA for 1-2h at RT. Thereafter plates were incubated with plasma diluted 50 times with PBS-T (CTB-p210 study), or 1000 times with TBS-T + 1% BSA (PADRE-p210 study) for 2h at RT. Thereafter plates were incubated with secondary HRP conjugated Goat anti mouse IgM, IgA, and IgG antibodies (Thermo Fisher Scientific) for 1-2h at RT. A color reaction was developed with TMB substrate solution (Thermo Fisher Scientific), which was stopped with sulfuric acid (0.16M) and measured at 450nm using a spectrophotometer (Powerwave 340, Biotek).

Histology

Hearts were transversally cut in half and incubated in OCT medium for 30 minutes. After 30 minutes hearts were fast frozen on dry ice, and stored at -80°C until cryosections (10 µm) of the aortic root were made on collected on Superfrost Plus™ Adhesion Microscope Slides (ThermoFisher) and analyzed at 70 µm intervals. Neutral fats were stained with Oil Red O to determine the average lesion size of five subsequent sections of the aortic root containing 3 valvular leaflets with largest plaque area, as a measure of atherosclerotic lesion size.

Murine MHC-I binding prediction

The immune epitope database and analysis resource (iedb.org) was used to predict binding capacity of peptide sequences in the p210 peptide to H-2Db and H-2Kb. The 5 peptides with

the highest predicted binding affinity for H-2Db and H-2Kb according to the artificial neural network binding model *(43–46)* were depicted in the table. There is a strong correlation between binding MHC-I binding affinity and the capacity of peptides to induce a CTL response, with a proposed affinity threshold of 500 nM *(47)*.

Statistical Analysis

Statistical analysis was performed with Graphpad Prism. For comparisons of multiple treatment groups with a control group a one-way ANOVA was performed. For comparisons of data from multiple treatment groups and multiple time points, a two-way ANOVA was used. The Holm-Šídák posttest was used to correct for multiple comparisons. An unpaired two-tailed T test was used for pairwise comparisons. A p-value < 0.05 was considered statistically significant.

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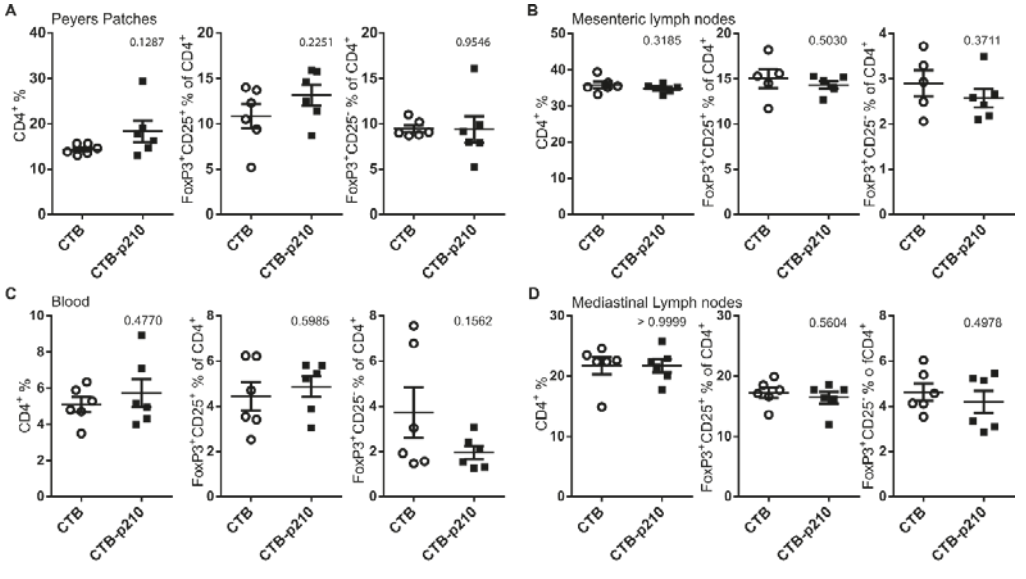
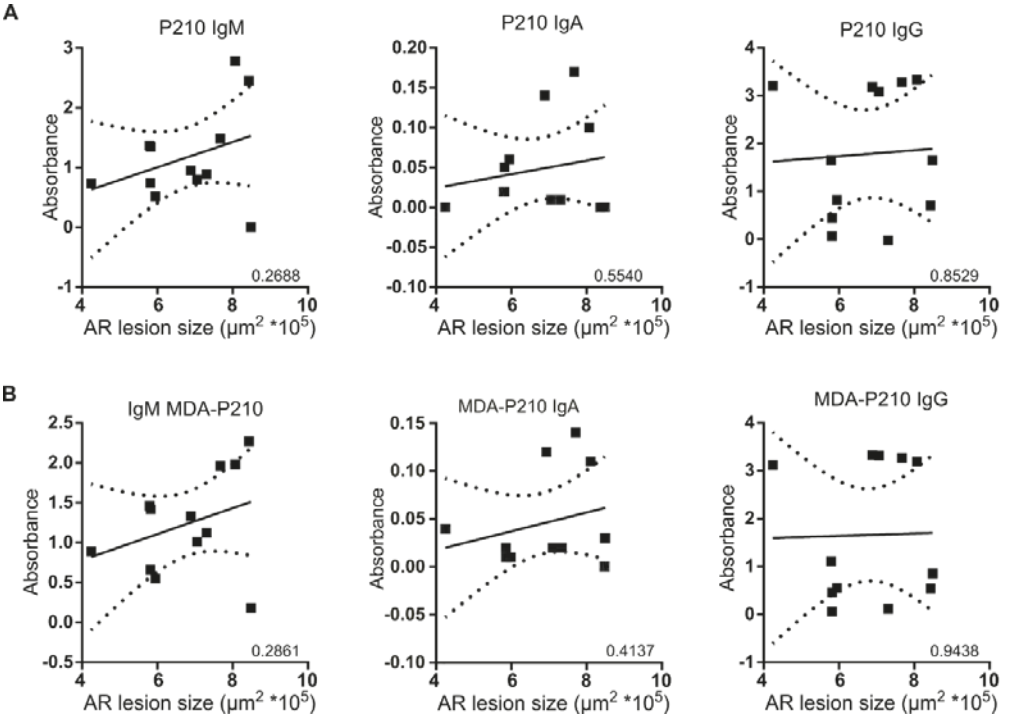
Supplementary figures



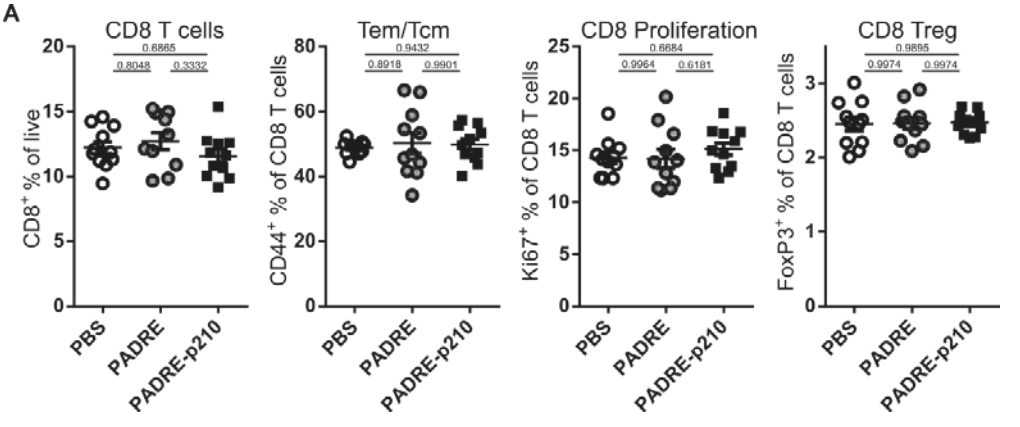
Supplementary Fig. S1 ***Oral CTB-p210 administration does not affect CD4 Treg levels.*** *Quantification of CD4 T cell levels, and FoxP3⁺CD25⁺ and FoxP3⁺CD25⁻ cell content of the CD4 T cell population based on flow cytometry measurements, of* ***A****) Peyers patches,* ***B****) mesenteric lymph nodes,* ***C****) the blood, and* ***D****) mediastinal lymph nodes. Represented as mean ± SEM, unpaired two-tailed T test.*



Supplementary Fig. S2 ***Anti-p210 antibody levels do not correlate with lesion size in CTB-p210 treated HuBL mice.*** *Correlation analysis of the average atherosclerotic lesion size in the aortic root and* ***A****) anti-p210 antibody levels and* ***B****) anti-MDA modified p210 antibody levels in blood serum from CTB-p210 treated animals obtainded after sacrifice. The trend line (solid line), 95% confidence interval (dotted lines), and p value (bottom right) are depicted in the scatter plots.*

Supplementary Table 1 **Best predicted H-2Kb and H-2Db binding peptides in p210**

Allele	Sequence	IC50 (ANN)	Allele	Sequence	IC50 (ANN)
H-2Kb	QSFDLSVKA	3986.06	H-2Db	TTKQSFDLSV	32470.27
H-2Kb	TKQSFDLSV	5484.41	H-2Db	TTKQSFDL	34542.56
H-2Kb	TTKQSFDLSV	9584.8	H-2Db	KTTKQSFDLSV	40485.33
H-2Kb	KTTKQSFDL	9731.83	H-2Db	KQSFDLSVKA	41053.05
H-2Kb	KTTKQSFDLSV	14409.83	H-2Db	KTTKQSFDLS	42345.55



Supplementary Fig. S3 ***Alum adjuvanted vaccination with PADRE-p210 does not change splenic CD8 T cell populations. A****) Splenic overall CD8 T cell levels, antigen experienced CD8 T cells (CD44⁺), proliferating CD8 T cells (Ki67⁺), and regulatory CD8 T cell populations (FoxP3⁺) were quantified by flow cytometric analysis. Represented as mean ± SEM, One-Way ANOVA with Holm-Šídák posttest, p values*

Induction of HLA-A2 restricted CD8 T cell responses against ApoB100 peptides does not affect atherosclerosis in a humanized mouse model.

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Abstract:

Cardiovascular diseases form the most common cause of death worldwide, with atherosclerosis as main etiology. Atherosclerosis is marked by cholesterol rich lipoprotein deposition in the artery wall, evoking a pathogenic immune response. Characteristic for the disease is the pathogenic accumulation of macrophages in the atherosclerotic lesion, which become foam cells after ingestion of large quantities of lipoproteins. We hypothesized that, by inducing a CD8 T cell response towards lipoprotein derived apolipoprotein-B100 (ApoB100), lesional macrophages, that are likely to cross-present lipoprotein constituents, can specifically be eliminated. Based on *in silico* models for protein processing and MHC-I binding, 6 putative CD8 T cell epitopes derived from ApoB100 were synthesized. HLA-A2 binding was confirmed for all peptides by T2 cell binding assays and recall responses after vaccination with the peptides proved that 5 of 6 peptides could induce CD8 T cell responses. Induction of ApoB100 specific CD8 T cells did not impact plaque size and cellular composition in HLA-A2 and human ApoB100 transgenic LDLr$^{-/-}$ mice. No recall response could be detected in cultures of cells isolated from the aortic arch, which were observed in cell cultures of splenocytes and mesenteric lymph nodes, suggesting that the atherosclerotic environment impairs CD8 T cell activation.

Introduction

Cardiovascular disease is the most common cause of death in the western world with atherosclerosis as the most common etiology *(1)*. Atherosclerosis is characterized by lipid deposition in the intima of medium to large-sized arteries, evoking immune infiltration in the vessel wall and inflammation. Among the immune cells attracted to the atherosclerotic lesions are CD8 T cells *(2)*. CD8 T cells in atherosclerotic lesions appear highly activated *(3)* suggesting a pathogenic role for CD8 T cells atherosclerosis. Recent studies indeed support the notion of an overall pro-atherogenic role of CD8 T cells in atherosclerosis through the secretion of pro-inflammatory cytokines *(4–6)*. Nonetheless, atheroprotective effects of CD8 T-cells in the lesion have been reported as well *(7)*.

These conflicting reports may result from the fact that CD8 T cells form a heterogeneous population consisting of different subsets and cells recognizing different antigens. The largest CD8 T cell subset is the cytotoxic CD8 T cell or cytotoxic lymphocyte (CTL) *(8)*. The primary function of CTLs is to protect the host from intracellular pathogens *(9)* and tumors *(10)*. Specific recognition of a target cell is established through positive interaction between the T cell receptor of the CTL, which is variable between individual CD8 T cells, and MHC-I complexed with a particular antigen derived peptide on the target cell. MHC-I/peptide complex recognition results in TNF-α and IFN-γ production by CTLs and leads to FAS ligand or perforin and granzyme B mediated cell death of the target cell *(11)*. The specificity of the TCR heavily impacts its pathogenicity, as depending on target antigen, vaccination induced CTLs can be atheroprotective *(12–15)* or atherogenic *(16)*. Induction of CTL reactivity towards vascular cells like smooth muscle cells, enhanced vessel inflammation and atherosclerosis *(16)*. As suppressing apoptosis of macrophages was found to enhance atherosclerosis *(17, 18)* and the absence of MHCI molecules aggravates atherosclerosis *(19)* we hypothesize that CD8 T cell mediated killing of macrophages is an essential process controlling progression of atherosclerosis. Intra-lesional TLR activation *(20)* and presence of apoptotic bodies *(21)*, both potent inducers of cross presentation *(22, 23)*, are likely to induce cross presentation of plaque antigens by lesion macrophages. Presumable cross-presentation of LDL derived apolipoprotein-B100 (ApoB100) epitopes on MHC-I by lesional phagocytes, suggests that inducing ApoB100 specific CD8 T cells could lead to killing of lesional macrophages and reduce atherosclerosis.

Since only a very small fraction of peptides binds to MHC-I *(24)*, we set out to identify CD8 T cell epitopes in ApoB100 to test this hypothesis. With *in silico* prediction models for HLA binding and antigen processing, human HLA-A2 restricted epitopes derived from human ApoB100 were predicted for translational relevancy. 6 ApoB100 derived peptides were selected and synthesized and binding of all peptides to HLA-A2 was confirmed with HLA-A2 assays in T2 cells *(25)*. Thereafter we performed vaccination studies using these peptides, inducing substantial levels of peptide specific memory CD8 T cells in HLA-A2 and human

ApoB100 transgenic LDLr$^{-/-}$ mice. Although ApoB100 specific CTLs were induced by ApoB100 peptide vaccination, these CD8 T cells did not change cellular plaque composition, plaque collagen content, and plaque size, indicating that induction of ApoB100 specific CD8 T cells does not affect atherosclerosis.

Results

Predicted HLA-A2 restricted epitopes stabilize HLA-A2 and induce peptide specific CD8 T cell responses after DC vaccination.

To target CD8 T cells towards plaque macrophages which are likely to cross-present plaque derived antigens, we predicted putative HLA-A2 restricted CD8 T cell epitopes in human ApoB100 using in silico models for immunoproteasomal processing and TAP binding *(26–28)* and HLA-A2 binding models *(28–35)*. We synthesized 6 peptides with the highest putative HLA-A2 binding and processing score (Table 1).

Table 1: **ApoB100 derived CD8 T cell epitope processing and HLA-A2 binding**

Peptide	Amino Acid Sequence	NetMHCpan (IC50)	Consensus percentile	Processing Score	Final Score
$ApoB_{2356-2364}$	VLMDKLVEL	2.95	0.2	2.32	1.85
$ApoB_{406-414}$	LLIDVVTYL	2.48	0.2	1.9	1.5
$ApoB_{406-417}$	LLIDVVTYLVAL	3.96	0.2	1.89	1.3
$ApoB_{2524-2532}$	YQMDIQQEL	5.19	0.5	1.9	1.19
$ApoB_{3070-3078}$	FLNNYALFL	4.75	0.3	1.86	1.18
$ApoB_{4531-4539}$	FLIYITELL	4.76	0.4	1.8	1.12

To establish the binding of the ApoB100 peptides to HLA-A2 *in vitro*, T2 cells were incubated overnight with ascending amounts of ApoB100 peptides and thereafter the expression of HLA-A2 on the T2 cells with flow cytometry was assessed (Supplementary Fig. S1). Because T2 cells are deficient for TAP1 and TAP2 expression, cytosolic peptide transport into the ER is reduced in T2 cells, reducing endogenous peptide loading onto MHC-I in the ER. MHC-I not complexed with a peptide is inefficiently transported to the cell membrane and is less stable, therefore leading to lowered expression of HLA-A2 on the cell membrane of T2 cells. Exogenous addition of peptides, forming MHC-I/peptide complexes on the cell surface, enhances MHC-I stability on the plasma membrane which increases expression of HLA-A2 on the T2 cell surface *(25)*. All 6 ApoB100 peptides increased HLA-A2 expression in a concentration dependent manner (Fig. 1A), confirming that these peptides bind to HLA-A2.

To test the immunogenicity of the peptides we vaccinated HLA-A2 transgenic animals (HHD mice) *(36)* with HHD bone marrow derived DCs ($2*10^6$ cells), which were overnight stimulated with LPS (100 ng/ml) and pulsed with a single peptide (10 µM). One week after vaccination,

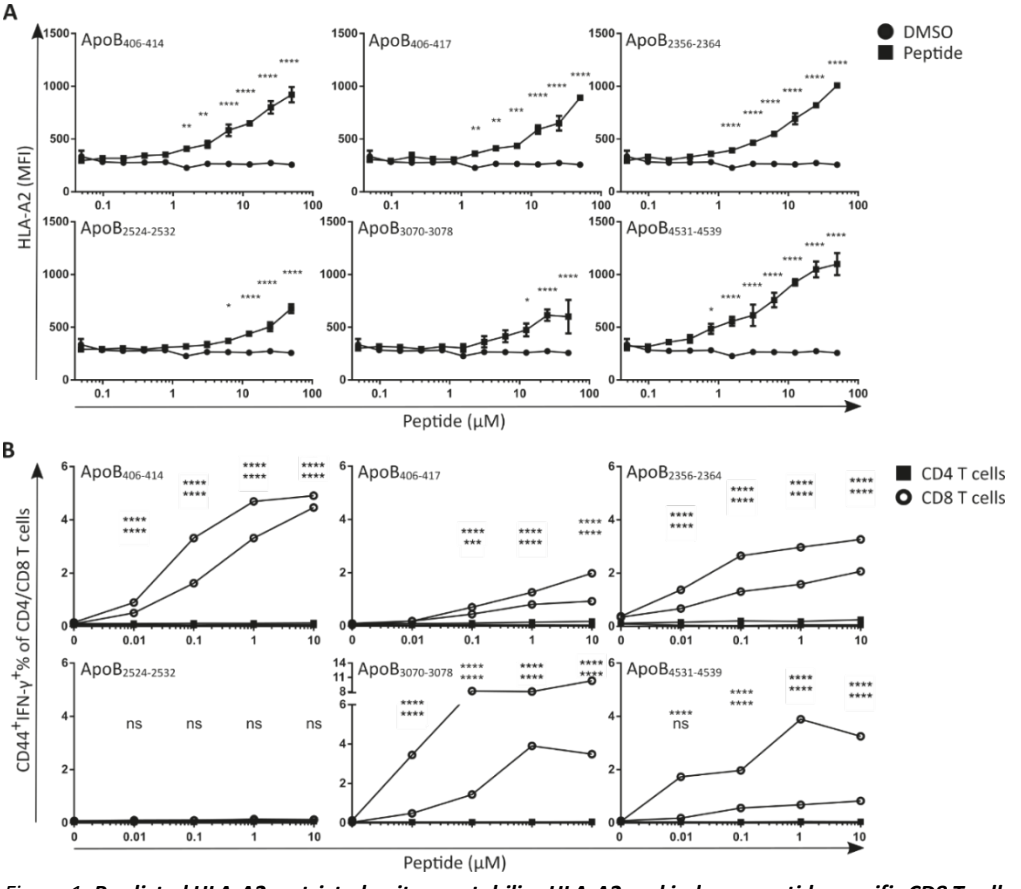

Figure 1: ***Predicted HLA-A2 restricted epitopes stabilize HLA-A2 and induces peptide specific CD8 T cell responses after DC vaccination. (A****) Binding of in silico predicted CD8 T cell epitopes was assessed using T2 cells. T2 cell cultures (n=3 / peptide) were incubated overnight with a range of peptide concentrations or vehicle control (DMSO) and assessed for HLA-A2 expression by flow cytometry. (****B****) Next we assessed whether these ApoB100 derived peptides could induce an HLA-A2 restricted CD8 T cell response. HLA-A2tg mice (n=2 per peptide), not expressing human ApoB100, were vaccinated with 2*10^6 HLA-A2tg bone marrow derived DCs pulsed with a single peptide (30uM). A week after vaccination splenocytes were ex vivo incubated with the peptide against they were vaccinated. Graphs of peptide specific T cell responses a measured by flow cytometry through gating for CD44 and IFN-γ double positive T cells. Statistical analysis of A was performed with 2-way ANOVA and Bonferroni posttest, displayed as mean with SEM. For B, samples stimulated with peptide were compared to the unstimulated control of the same animal with contingency chi-square tests and Bonferroni posttest (corrected for 96 pairwise comparisons), individual samples are plotted. * p < 0.05, ** p< 0.01, *** p < 0.001, **** p < 0.0001.*

we assessed the induction of peptide-specific CTLs through flow cytometric measurement of IFN-γ positive antigen experienced (CD44$^+$) CD4 and CD8 T cells (Supplementary Fig. S1), in splenocyte cultures which were incubated for 4h with escalating concentrations of the

ApoB100 peptide against which the particular animal was vaccinated, and Brefeldin A. For all peptides, except for $ApoB_{2524-2532}$**,** the percentage of IFN-γ positive antigen experienced CD8 T cells was increased over no peptide control, while the vaccinations did not affect CD4 T cell IFN-γ production (Fig. 1B). This indicates that 5 of 6 peptides can induce HLA-A2 restricted CD8 T cell responses *in vivo*.

Peptide vaccination induces peptide responsive CD8 T cells in spleen and mediastinal lymph nodes

A suitable experimental model to assess the role of human ApoB100 specific T cells in the context of atherosclerosis was generated by crossbreeding HHD mice, transgenic for HLA-A2 and deficient in expression of murine MHC-I molecules *(36)*, with HuBL mice, expressing human ApoB100 and deficient for the LDLr *(37, 38)*. HLA-A2 and human ApoB100 transgenic mice, which were LDLr deficient to allow atherosclerosis development, and either with normal expression (HuBL-$A2^{m+}$) or devoid of murine MHC-I expression (HuBL-$A2^{m-}$). Because immunization with the ApoB100 peptides in both mouse strains yielded similar immunological results and effect on plaque parameters in the atherosclerosis studies, only the results obtained with HuBL-$A2^{m-}$are shown.

HuBL-$A2^{m-}$ mice received western type diet for 9 weeks starting from 15 weeks of age. Mice of the treatment group were i.v. vaccinated with HHD DCs ($2*10^6$ cells) pulsed with a mixture of the 6 ApoB100 derived peptides (10 µM / peptide) at the start of western type diet. Mice were i.v. boosted a week later with peptides (30 µM/peptide) adjuvanted with anti-CD40 (50 µg) and poly(I:C) (50 µg), previously shown to induce long lasting high levels of antigen-specific memory CD8 T cells *(39–41)*. Control animals received unpulsed control DCs ($2*10^6$ cells) at WTD initiation and a week later adjuvant without peptides (vehicle group), or received two PBS injections (PBS group). At the end of the experiment we determined whether vaccination induced peptide specific CD8 T cells. To that end, CD8 T cell recall responses were assessed with flow cytometry in cell cultures of the spleen ($2*10^6$ cells) and mediastinal lymph nodes ($1*10^6$ cells), which drain from the atherosclerosis prone aortic arch, incubated for 4h with the ApoB100 derived peptides (10 µM/peptide) and Brefeldin A. The CD8 T cell percentage was significantly higher in the peptide-treated group compared to the PBS group and trended to be higher compared to the unpulsed DC/vehicle group in spleen (Fig. 2A) and blood (Supplementary Fig. S2) but not in mediastinal lymph nodes (Fig. 2G). Incubation of splenocytes with $ApoB_{406-414}$, $ApoB_{3070-3078}$ or $ApoB_{4531-4539}$ enhanced the percentage of IFN-γ and TNF-α double positive CD8 T cells in the splenocytes cultures of the peptide-treated group, indicative of successful long-term induction of memory CD8 T cells for these peptides (Fig. 2B,C). In cell cultures from the mediastinal lymph nodes, incubated with a combination of all peptides, also a higher percentage of IFN-γ^+ and TNF-α^+ double positive CD8 T cells was observed in the peptide vaccinated group (Fig. 2E,F), although at a much lower percentage than in the spleen. In circulation, a strong increase in effector memory CD8 T cells and a reduction in naïve CD8 T cells were observed in the peptide-treated group

compared to both control groups (Supplementary Fig. S2), suggesting that vaccination also resulted in peptide specific CD8 T-cells in circulation.

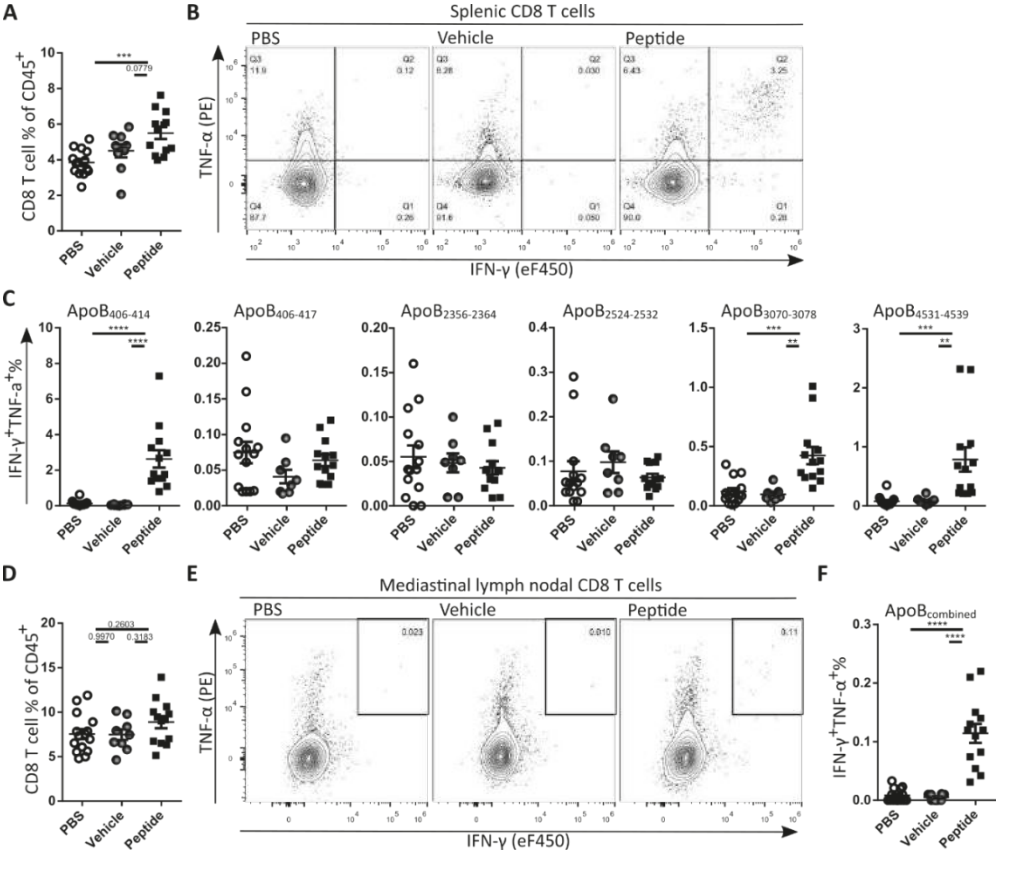

Figure 2 ***Peptide vaccination induces peptide responsive CD8 T cells. (A)*** *Quantification of splenic CD8 T cell percentage. (**B**) Representative flow cytometry plots of splenic CD8 T cell activation by $ApoB_{406-414}$ in the different treatment groups. (**C**) Quantification of IFN-γ and TNF-α double positive cells % from CD8 T cells. (**D**) Quantification of CD8 T cell percentage after ex vivo peptide stimulation mediastinal lymph nodes. (**E**) Representative flow cytometry plots of mediastinal lymph node CD8 T cell activation and (**F**) quantification of the IFN-γ and TNF-α double positive cell % from CD8 T cells after combined peptide stimulation. Statistical analysis was performed with 1-way ANOVA and Tukey's multiple comparisons test. Depicted as mean with SEM, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

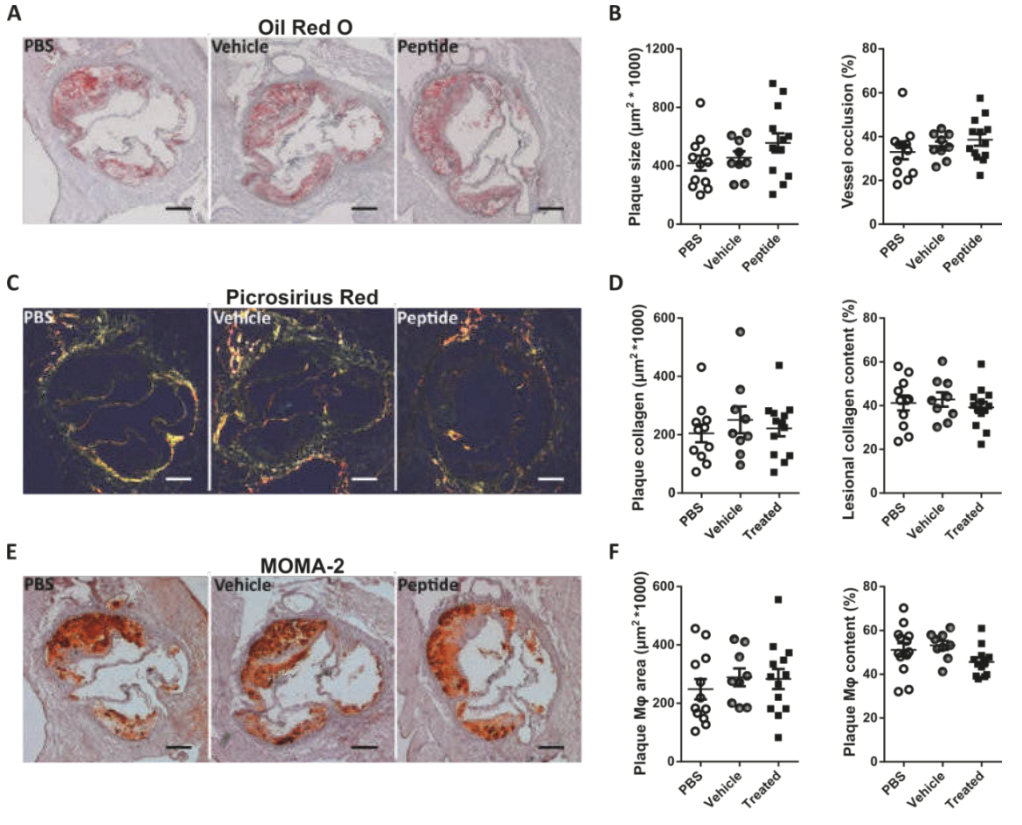

Figure 3: Vaccination with ApoB100 derived CD8 epitopes does not affect lesion size and composition *(**A**) Representative microscopic images of ORO staining of aortic root tissue sections. (**B**) Quantification of average plaque size and average vessel occlusion. (**C**) Plaque stability was assessed by collagen content determination through analysis of Sirius red stained tissue slides. Representative microscopic images of aortic root tissue sections stained with Sirius red. (**D**) Quantification of collagen content of atherosclerotic lesions in the aortic root. (**E**) Representative microscopic images of tissue slides stained with MOMA-2 staining (**F**) and quantification of MOMA-2 surface area and plaque content. Statistical analysis was performed with 1-way ANOVA and Tukey's multiple comparisons test. Plotted as mean with SEM.*

Vaccination with ApoB100 derived CD8 epitopes does not affect atherosclerotic lesion size and composition

To assess the effect of ApoB100 specific CD8 T cell induction on atherosclerotic lesion development, we stained neutral lipids in aortic root sections with ORO (Fig. 3A), and quantified plaque size (control $4.18\pm1.79*10^5$, vehicle $4.55\pm1.32*10^5$, treated $5.57\pm2.35*10^5$) and vessel occlusion (Fig. 3B). To assess the stability of the plaques we stained aortic root sections with Masson's Trichrome (Fig. 3C), and quantified the absolute area of plaque collagen and collagen content of the plaques (Fig. 3D). Finally, with immunohistochemical

staining with MOMA-2 antibody (Fig. 3E), we quantified the absolute macrophage area and macrophage content of aortic root atherosclerotic plaques (Fig. 3F). None of the mentioned plaque parameters were significantly changed by induction of ApoB100 specific CD8 T cells.

ApoB100 peptide vaccination enhances aortic root CD8 T cell content but not peptide reactivity or other aortic root cell populations

Because the induced ApoB100 peptide specific CD8 T cells were hypothesized to reduce atherosclerosis through killing of plaque phagocytes cross-presenting the ApoB100 peptides, we assessed the number of peptide specific CD8 T cells in the aortic arch with flow cytometry (Supplementary Fig. S3). Aorta's from three mice from the same treatment group were pooled, digested, and cells were isolated and cultured for 4h with all peptides combined (10 µM / peptide) and Brefeldin A. The percentage of CD8 T cells was significantly increased in the peptide treated group over PBS treated, and a trend towards increase (p = 0.0573) was observed compared to the vehicle treated (Fig. 4A). In contrast to the observed recall responses in the spleens and mediastinal lymph nodes from mice vaccinated with the ApoB100 peptides, we did not observe a recall response in the cell cultures from the aortic arch of the mice vaccinated with ApoB100 peptides (Fig. 4B,C). Besides the CD8 T cell population, we assessed multiple myeloid and lymphoid cell populations in the aortic arch cultures (Supplementary Fig. S3A). In line with a lack of CD8 T cell responsiveness towards ApoB100 peptides in the cultures of the aortic arch, no differences were observed between treatment groups in percentage of other immune populations (Fig. S3B). Thus, our data suggest that the atherosclerotic plaque environment reduces antigen specific CD8 T cell responses, probably rendering induction of ApoB100 specific CD8 T cells incapable of affecting atherosclerosis.

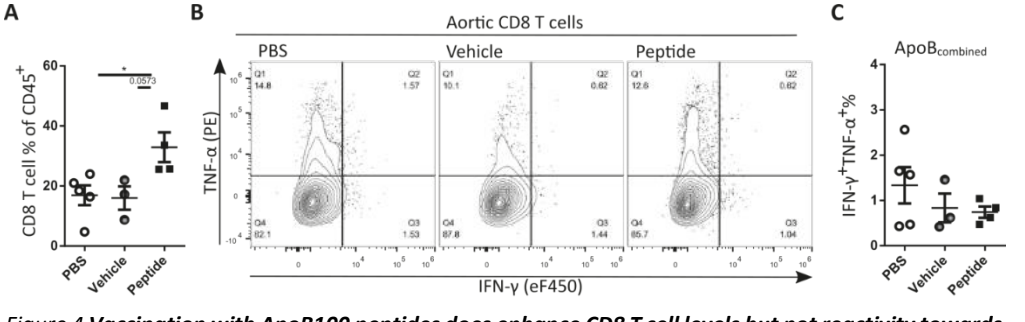

Figure 4 ***Vaccination with ApoB100 peptides does enhance CD8 T cell levels but not reactivity towards ApoB100 peptides in the aortic root (A****) Quantification of CD8 T cell percentage in aortic cultures. (****B****) Representative flow cytometry plots of aortic CD8 T cell activation and (****C****) quantification of IFN-γ and TNF-α double positive cell % from CD8 T cells after restimulation with a mix of all peptides. Statistical analysis was performed with 1-way ANOVA and Tukey's multiple comparisons test. Depicted as mean with SEM, * P < 0.05.*

Discussion

The role of CD8 T cells in atherosclerosis is complex, with different roles for different CD8 T cell subsets and CD8 T cells with different antigen specificities. Recent studies suggest an overall pathogenic role for CTLs through secretion of pro-inflammatory cytokines *(4, 5)*, which seems to correlate with observations of increased activated and cytokine producing CD8 T cells in peripheral blood of patients with coronary artery disease *(42–44)*. However, depending on target antigen, atherogenic and also atheroprotective *(12–14)* antigen specific CD8 T cells responses have been reported. Because the suppression of macrophage apoptosis enhanced atherosclerosis *(17, 18)* and the absence of MHCI molecules aggravates atherosclerosis *(19)*, we reasoned that promoting CD8 T cell mediated killing of macrophages could act atheroprotective. As the inflammatory plaque environment is likely to promote cross-presentation of plaque antigens by lesional macrophages *(20–23)*, we hypothesized that we could specifically target CD8 T cells to kill lesional macrophages through vaccination with ApoB100 derived CD8 T cell epitopes, which was proposed to be the atheroprotective mechanism behind vaccination with ApoB100 derived peptide p210 *(13, 15)*.

To be applicable for vaccination in men, the CD8 epitopes have to be capable of binding human MHC-I. Therefore we predicted human HLA-A2 binding affinity and immunoproteasomal processing of peptide sequences present within ApoB100 with in silico prediction tools *(26–35)*. In line with a high accuracy of MHC-I binding models at predict MHC binders and non-binders *(45)*, all 6 predicted peptides were found to bind HLA-A2 in T2 cell binding assays. Subsequently we assessed the immunogenicity of the peptides in HLA-A2 transgenic HHD mice *(36)*. Vaccination of HHD mice with HHD DCs pulsed with a single ApoB100 derived peptide, led to robust peptide specific recall responses a week after vaccination for all peptides except for $ApoB_{2524\text{-}2532}$, confirming immunogenicity for all ApoB100 derived peptides except $ApoB_{2524\text{-}2532}$. Through utilization of mice and DCs without murine MHC-I expression, the observed CD8 T cell responses had to be restricted to HLA-A2 *(36)*. To induce long lasting peptide specific CTLs in the atherosclerosis studies, HuBL-$A2^{m+/-}$ mice were primed with peptide pulsed HHD DCs, and then boosted after a week with peptide adjuvanted with poly (I:C). In experimental cancer models this vaccination approach induces CTLs that are effective in penetrating and killing tumor cells, indicating that this vaccination approach yields migratory and functional CTLs *(39–41)*. Moreover multivalent vaccination enhanced the anti-tumor effect of this immunization approach *(41)*. For $ApoB_{406\text{-}414}$, $ApoB_{3070\text{-}3078}$, and $ApoB_{4531\text{-}4539}$, robust recall responses were detected in the spleens of ApoB100 peptide vaccinated HuBL-$A2^{m+/-}$ mice, indicating successful vaccination. The vast majority of the ApoB100 specific CD8 T-cells made more than one cytokine (IFN-γ and TNF-α) suggesting they are functional CTLs *(46)*. Interestingly no CD8 T cell recall response could be detected for $ApoB_{406\text{-}417}$ and $ApoB_{2356\text{-}2364}$, suggesting thymic negative selection or peripheral tolerance induction towards $ApoB_{406\text{-}417}$ and $ApoB_{2356\text{-}2364}$ in the human ApoB100 expressing HuBL-$A2^{m-/+}$ mice. Besides enhanced levels of ApoB100 peptide specific CD8 T cells in the spleen, we

observed peptide specific CD8 T cells in the mediastinal lymph nodes, more effector CD8 T cells in the circulation and increased CD8 T cell levels in the aorta of ApoB100 vaccinated mice.

Despite induction of this relatively large antigen specific CD8 T cell response compared to studies in which p210 coupled to cationic BSA (p210-cBSA) was reported to reduce atherosclerosis through CD8 T cell induction *(13, 15)*, we did not observe an atheroprotective effect of ApoB100 specific CD8 T cell induction. Although *in silico* prediction models suggest that p210 does not harbor CD8 T cell epitopes (murine H2Db and H2Kb MHC-I alleles), CD8 T cell involvement in the atheroprotective effect of p210-cBSA was shown by the transfer of atheroprotection by CD8 T cell transfer from p210-cBSA vaccinated donors to recipients, not observed in recipients from CD8 T cells of vehicle treated donors *(13)*. In our hands however, various vaccination protocols including vaccination of alum adjuvanted p210 coupled to PADRE (Pan-DR epitope), resulted in antibodies against p210 but did not affect the CD8 T cell population in human ApoB100 transgenic LDLr$^{-/-}$ (unpublished results). As FITC-p210 was more effectively taken up by DCs than unconjugated FITC *(13)*, this suggests that p210 possesses adjuvant properties. The LDLr binding sites of ApoB100, including p210, were also used in a construct with CD8 T cell epitope SIINFEKL to promote cross-presentation of SIINFEKL and induce SIINFEKL specific CD8 T cell activation *(47)*. As the antigen specificity of CD8 T cells after cBSA-p210 immunization was not assessed, this could imply that p210 acted as adjuvant for cBSA and enhanced uptake and (cross-) presentation of cationic BSA. As immunization with cationic BSA was previously reported to reduce atherosclerosis *(48)*, enhancing the immune response against cBSA could underlie the atheroprotective effect of cBSA-p210 vaccination.

As we observed strong recall responses towards 3 ApoB100 derived peptides, it is unlikely that the quality and quantity of the induced CD8 T cell response was insufficient to modulate atherosclerosis. Although TLR induction and engulfment of apoptotic bodies induce cross presentation *(23, 49)*, other lesional factors could have hampered cross-presentation, such as oxidized lipids which cause disturbance of lipid bodies *(50)* which were found essential for cross presentation in DCs *(51, 52)*. The observation that profoundly reduced cross priming capacity in batf3$^{-/-}$ chimeras does not affect atherosclerosis, suggests that cross presentation is not important in atherosclerosis *(53)*. In our restimulation assays however, cross presentation is circumvented by exogenous addition of peptide which externally bind MHC-I. Despite enhanced CD8 T cell levels in the aortas of ApoB peptide treated mice, we did not observe peptide responsiveness in the cultures with cells derived from the aortic root, suggesting that the atherosclerotic environment reduces the ability of CD8 T cells to respond to TCR stimuli. It is possible that this lack in responsiveness is CD8 T cell intrinsic, e.g. due to chronic antigen exposure in the plaque leading to CD8 T cell exhaustion *(54, 55)*. In line with CD8 T cell exhaustion in atherosclerosis, upregulation of the co-inhibitory PD-1 expression was observed in atherosclerosis patients *(56)*. On the other hand, plaque cells could inhibit CD8 T cell activation, e.g. PD-L1 was found upregulated on macrophages in human lesions

(Watanabe et al., 2017), which could provide a co-inhibitory signal to plaque CD8 T cells. Moreover it was recently shown that CD8 T-cells in the atherosclerotic lesion significantly downregulate cytokine production as a result of local adenosine signaling *(57)*. Therefore it is possible that impaired antigen specific activation of plaque CD8 T cells, could have rendered induction of ApoB100 specific CD8 T cells ineffective.

MHC-I epitope elution from plaque material and single cell TCR sequencing of lesional CD8 T cells could greatly enhance understanding of CTL biology in atherosclerosis, especially since new bioinformatics avenues are opening up that allow linking of TCR sequences to antigens *(58–60)*. Identification of these antigens could help unravel atherogenic and atheroprotective CTL mediated immune responses, uncovering treatment options of atherosclerosis through vaccination of antigen specific CLTs.

In conclusion, here we assessed the effect of vaccination with ApoB100 derived CD8 T cell epitopes on atherosclerosis development. We could generate and maintain a very robust CD8 T cell response against 3 epitopes over 8 weeks, however in contrast to other studies which report CD8 T cell mediated protection from atherosclerosis resulting from vaccination with ApoB100 derived peptides *(13, 15)*, boosting CD8 T cell immunity towards ApoB100 did not reduce atherosclerosis.

Materials and Methods

In silico HLA-A2 restricted human ApoB100 CD8 T cell epitope prediction

With use of the immune epitope database and analysis resource (iedb.org), putative human ApoB100 derived HLA-A2 restricted CD8 T cell epitopes were predicted. First top 1% predicted binders were selected using NetMHCpan *(29)* which was reported to be the best prediction model for HLA-A2*(61)*. Thereafter the Proteasomal cleavage/TAP transport/MHC class I combined predictor was used to rank the remaining peptides based on proteasomal cleavage and TAP transport *(26, 27)* and a consensus binding prediction*(28)* combining Artificial neural network (ANN) *(31–34)*, Stabilized matrix method (SMM) *(30)*, and Scoring Matrices derived from Combinatorial Peptide Libraries (CombLib) models *(35)*. The 6 top peptides were synthesized (IHB, Leiden University Medical Centre).

HLA-A2 binding assay

10 mM peptide stocks were prepared in DMSO because of poor water solubility of the peptides and further diluted into complete IMDM. T2 cells, a kind gift from dr. Heemskerk (Leiden University Medical Center, Leiden) were incubated with peptide concentrations ranging from 0.01 - 50uM overnight and 3µg/ml β-2 microglobulin (Sigma-Aldrich), and HLA-A2 expression was assessed with flow cytometry.

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Human HLA-A2 transgenic (HHD), H-2D$^{b-/-}$ β2m$^{-/-}$ C57BL/6 mice *(36)*, a kind gift from dr. Lemonnier (Institut Pasteur, Paris), were crossbred with human ApoB100 transgenic LDLr$^{-/-}$ C57BL/6 mice *(37, 38)*, a kind gift from dr. Nilsson (Lund University, Lund), to generate human ApoB100 and HLA-A2 transgenic H-2D$^{b-/-}$ β2m$^{-/-}$ LDLr$^{-/-}$ (without murine MHC-I expression, HuBL-A2^{m-}) and hApoB100 and HLA-A2 transgenic H-2D$^{b+/+-}$ β2m$^{+/+-}$ LDLr$^{-/-}$ mice (with murine MHC-I expression, HuBL-A2^{m+}). Expression of ApoB100 in blood plasma, was determined with the ApoB100 ELISA developer kit (Mabtech) according to manufacturer's protocol. Expression of HLA-A2, H2Db and H2Kb on peripheral white blood cells was assessed with flow cytometry using HLA-A2-APC (BB7.2, Biolegend), H2Db-FITC (28-14-8, ThermoFisher) and H2Kb-BV421 (AF6-88.5, BD Biosciences) antibodies respectively. Blood for fenotyping purposes was obtained by lateral tail cut and collected in EDTA coated capillary tubes (Microvette®, Sarstedt). LDLr deficiency was assessed with PCR (Primers: forward-common; CAGTGCTCCTCATCTGACTTG, reverse-WT; CATCTCCCCGCAGTTTGTGT, reverse-KO; CGCCTTCTTGACGAGTTCTTCTG). All mice were kept in individual ventilated cages with aspen bedding, in groups of 2–4 mice per cage and were fed a regular chow diet or a 'Western-type' diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). All mice used in experiments were 12–20 weeks of age. Animals were randomized based on age, weight, plasma Apob100 levels, HLA-A2, H2Db and H2Kb expression. Diet and water were available ad libitum. At sacrifice, mice were anesthetized by a subcutaneous injection (120 µl) of a cocktail containing ketamine (40 mg/ml), atropine (50 µg/ml) and sedazine (6.25 mg/ml). Subsequently, the mice were euthanized and exsanguinated by femoral artery transection and perfusion with PBS through the left cardiac ventricle.

Immunization

Briefly, bone marrow was harvested from femurs and tibia from HHD mice. Bone marrow cells were cultured in complete IMDM (Lonza), IMDM supplemented with 8% FCS (GE Healthcare), 100 U/ml penicillin/streptomycin (Lonza), 2 mM Glutamax (Invitrogen) supplemented with 20ng/ml GM-CSF (ImmunoTools) in non-culture treated petri dishes at a concentration of 0.8*10^6 cells/ml (10 ml per plate). After 3 days 10 ml fresh complete IMDM with 20ng/ml GM-CSF was added to the petridishes. On day 6, 10 ml medium was carefully aspirated avoiding removal of cells, and replaced with complete IMDM with 20ng/ml GM-CSF. At day 9 non/slightly adherent DCs were harvested and cultured overnight with 25µg/ml high molecular weight poly (I:C) (Invivogen) in complete IMDM in non-culture treated petri dishes. DCs were harvested and pulsed with peptides (10 µM) or vehicle (DMSO) for 2 hours at 37°C in complete IMDM in 50 ml falcon tubes (10*10^6 cells/ml) under constant agitation

to avoid sticking to the plastic. DCs were washed twice with PBS and $2*10^6$ cells were intravenously infused through the lateral tail vein.

In the atherosclerosis studies mice received an i.v. booster vaccination consisting of PBS (control group), or PBS with 5% DMSO and 50µg high molecular weight Poly (I:C) (VacciGrade™, Invivogen) and 50µg αCD40 (FGK4.5, BioXcell) (vehicle group), or PBS with 5% DMSO and 30 µM peptide (approximately 67 µg/peptide) and 50 ug high molecular weight Poly (I:C) and 50 ug αCD40 (vehicle group), in a total volume of 200µl, a week after DC vaccination.

Primary cell preparation

For restimulation culture and flow cytometric analysis of lesional immune cells, aorta's were isolated from just above the heart until at the height of the heart apex and cleaned from adipose tissue. Aorta's were minced using scissors and digested in 0.5ml PBS containing 400 U/ml collagenase type I (Sigma-Aldrich) and 120 U/ml collagenase type XI (Sigma-Aldrich) from *Clostridium histolyticum*, 60 U/ml type I-s hyaluronidase from bovine testes (Sigma-Aldrich) and 60 U/ml DNase 1 (Sigma-Aldrich) for 30 minutes at 37^{o}C under constant agitation. Aorta digests of 3 mice were pooled and mashed over 70µm strainers in RPMI to obtain single cell suspensions. Spleens and mediastinal lymph nodes were harvested and mashed over 70µm strainers to obtain single cell suspensions. At sacrifice, blood was obtained through terminal retro-orbital bleeding. Red blood cells from blood were lysed twice and splenocytes suspensions once, through incubation with 1 ml ACK lysis buffer (NH_4Cl 150 mM, $KHCO_3$ 10mM, Na_2EDTA 0.1 mM) for 30 seconds at room temperature.

Evaluation of peptide specific immune responses

For measurement of antigen-specific CD8+ T cell responses, approximately $2.5*10^6$ splenocytes were incubated with indicated concentrations of individual peptides in U bottom plates. For assessment of specific CD8 T cells in mediastinal lymph nodes, approximately $1.5*10^6$ cells were incubated with 10µM of all peptides combined in U bottom plates. For measurement of peptide specific CD8 T cell responses in aorta,cells from 3 aorta digests were combined, approximately $20*10^5$ viable $CD45^+$ cells (flow cytometric analysis), and incubated with with 10µM of all peptides combined in V-bottom plates. Cells were incubated with peptides for 4h together with Brefeldin A (BD Biosciences) and Monensin (BD Biosciences) in RPMI (Lonza), supplemented with 8% FCS, 100 U/ml penicillin/streptomycin and 2 mM Glutamax.

Flow Cytometry

Extracellular staining of single cell suspensions was performed in PBS with 2% FCS and αCD16/32 antibody (93, Biolegend) and eBioscience™ Fixable Viability Dye eFluor™ 780 (ThermoFisher) to discriminate between living and dead cells at 4^{o}C for 30 minutes. For intracellular cytokine staining, cells were incubated in Cytofix/Cytoperm™ Buffer (BD Biosciences) for 15 minutes at 4^{o}C after extracellular staining, then washed twice with

Perm/Wash Buffer (BD Biosciences) and stained in Perm/Wash Buffer for 45 minutes at 4°C. The following antibodies were purchased at BD biosciences: IL-17-FITC (TC11-18H10), F4/80-BV421 (BM8), CD4-V500 (RM-4-5), CD19 PE-Cy7 (1D3). CD3 V500 (500A2). The following antibodies were purchased at Biolegend: CD8-BV510 (53-6.7), NK1.1-BV650 (PK136), CD4-PerCP (RM4-5), CD45-AF700 (30-F11), CD11b-PE/Dazzle 594 (M1/70), Thy1.2-PE-Cy7 (53-2.1), TNF-α-PE (MP6-XT22), CD11c-FITC (N418), Ly6G-PerCP (1A8), Ly6C-APC (HK1.4). The following antibodies were purchases at ThermoFisher: IFN-Y-eFluor 450 (XMG1.2), IL-10-APC (JES5-16E3), CD11b –eVolve 605 (M1/70), MHCII-eVolve 655 (M5/114.15.2), CD19-PE (eBio1D3), CD8-PE-TR (5H10), CD62L-PerCP/Cy5.5 (MEL-14), CD44-APC (IM7). Compensation measurements were performed using UltraComp eBeads (ThermoFisher) and ArC Amine-Reactive Compensation Beads (ThermoFisher). Cells were analyzed with a Cytoflex S flow cytometer (Beckman Coulter) with Cytexpert 2.0 software (Beckman Coulter) and further analyzed using FlowJo software (Tree Star, inc.).

Histology

Hearts were cut in half and incubated in OCT medium for 30 minutes. After 30 minutes hearts were fast frozen on dry ice, and stored at -80°C before cryosections (10 µm) of the aortic root were collected on Superfrost Plus™ Adhesion Microscope Slides (ThermoFisher) at 70 µm intervals (7 slides/mice). Neutral fats were stained with Oil Red O to assess lesion size in five subsequent sections of the heart within the three aortic valve area. Lesion collagen content was determined with Masson trichrome staining (Sigma-Aldrich). Corresponding sections analyzed for plaque area and collagen content were immunohistochemically stained for macrophages with MOMA-2 antibody (Sanbio, 1:1000 dilution). Slides were blocked with 5% milk powder before primary antibody was added for 2h at RT, after which primary antibody was incubated overnight at 4°C. Endogenous peroxidase activity was blocked by incubating slides in 0.3% Hydrogen peroxide for 30 minutes at RT. Then slides were incubated for 1h at RT with a polyclonal Rabbit Anti-Rat Ig HRP (DAKO), after which VECTASTAIN ABC HRP Kit (Vector Laboratories) was used. Stained with NovaRed (Vector Laboratories).

Author contributions

FHS and **JK** devised the study, and designed the experiments. **JWD** synthesized and purified the peptides. **FHS**, **JA**, **HD**, **MJK**, **BAS**, **GHMvP**, **IB**, and **ACF**, carried out the experiments. **FHS** analyzed the data and wrote the manuscript. **JWD**, **BAS**, **JK** contributed to revisions of the manuscript.

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Competing interests

The author (s) declare no competing interests

Data Availability

The data generated and analyzed during the current study are available from the corresponding author on reasonable request.

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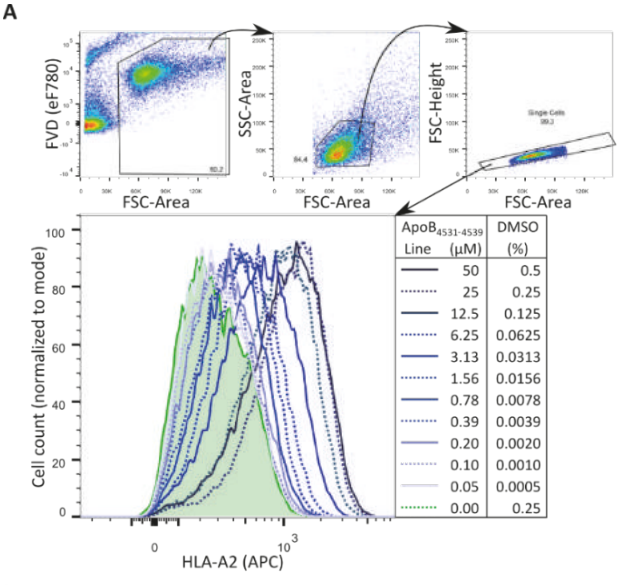
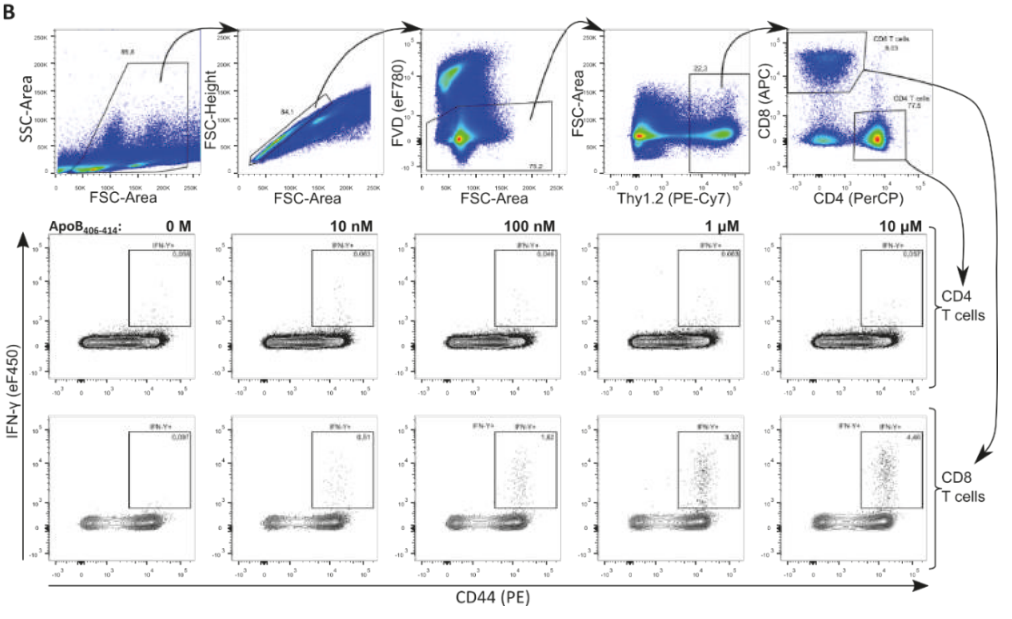
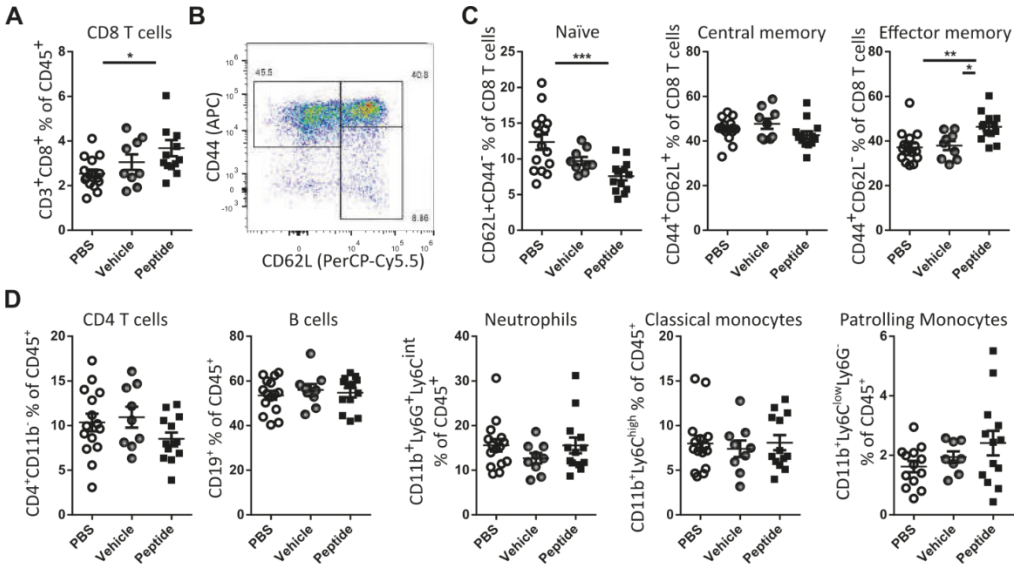


Supplementary Fig. S1 ***Gating strategy for T2 stabilization assay and ex vivo splenocyte peptide restimulation.*** *(**A**) Representative flow cytometry plots of the T2 stabilization assay gating strategy, exemplified by ApoB$_{4531-4539}$ induced HLA-A2 stabilization on T2 cells. First living cells were gated based on FSC and fixable viability dye (FVD) staining. Then T2 cells were gated based on FSC and SSC after which singlets were gated based on FSC-A and FSC-H. The median fluorescent intensity in the APC channel in the living, singlet T2 cell population, was then determined.*



*(**B**) Representative flow cytometry plots of the gating strategy of ex vivo CD4 and CD8 T cell stimulation as exemplified by ApoB406-414 splenocyte stimulation. First small debris and large cell aggregates were gated out based on FSC-A and SSC-A. Next single cells were gated based on FSC-A and FSC-H, after which T cells were gated based on Thy1.2 expression. Subsequently CD4 and CD8 T cells were gated. Peptide specific T cell responses were assessed by gating for CD44 and IFN-γ double positive cells.*



Supplementary Fig. S2: Peptide vaccination enhances effector memory CD8 T cells in circulation. ***(A)*** *CD8 T cells were gated based on expression of CD3 and CD8 with flow cytometry and quantified.* ***(B)*** *Representative flow cytometry plot of the CD8 T cell population for the gating of $CD44^{+}CD62L^{-}$ effector memory CD8 T cells, $CD44^{+}CD62L^{+}$ central memory CD8 T cells, and $CD62L^{+}CD44$ naïve^{-} CD8 T cells.* ***(C)*** *Quantification of memory and naïve CD8 T cell populations. Statistical analysis was performed with 1-way ANOVA and Tukey's multiple comparisons test. Depicted as mean with SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$..*

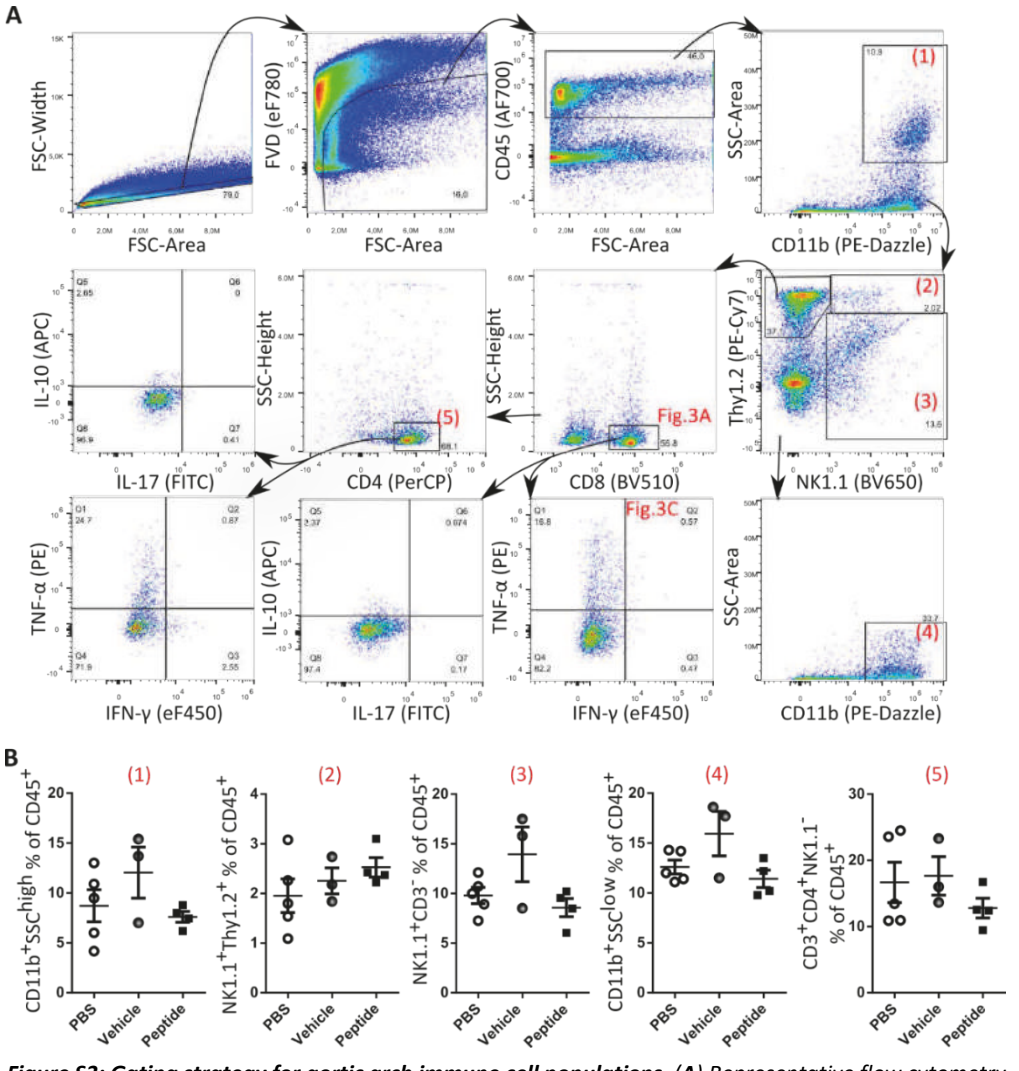


Figure S3: Gating strategy for aortic arch immune cell populations. (**A**) *Representative flow cytometry plots of the gating of aortic cell populations. Cell populations in peptide stimulation cultures of mesenteric lymph nodes and spleen were gated similarly. Singlets were gated based on FSC-W and FSC-A. Viable cells were gate based on size (FSC-A) and low fluorescent viability dye (FVD) fluorescence. Immune cells were selected through gating on CD45$^+$ cells. (1) A CD11b$^+$SSC^{high} was gated. From the CD11b$^+$SSC^{high} negative a Thy1.2$^+$NK1.1$^+$ (2), a Thy1.2$^-$NK1.1$^+$ (3) and Thy1.2$^+$NK1.1$^-$ were gated. From the Thy1.2$^-$NK1.1$^-$ population CD11b$^+$ cells were gated (4), and from the Thy1.2$^+$NK1.1$^-$ population CD8$^+$SSC^{low} cells were gated (CD8 T cells, Fig. 3A). From the CD8$^+$SSC^{low} negative population CD4$^+$SSC^{low} cells (5) were gated, being CD4 T cells. The CD4 T cell and CD8 T cell populations were then assessed for cytokine expression in IFN-γ vs TNF-α, and IL-17 vs IL-10 plots. (**B**) Quantifications of cell populations in cell cultures of the aortic arch as assessed by flow cytometry. Statistical analysis of B was performed with 1-way ANOVA and Tukey's multiple comparisons test, plotted as mean with SEM.*

Immunoproteasomal inhibition with ONX-0914 attenuates atherosclerosis and reduces white adipose tissue mass and metabolic syndrome

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Abstract

Atherosclerosis is the major underlying pathology of cardiovascular disease, and is driven by hyperlipidemia and inflammation. Cells of hematopoietic origin, including immune cells, express high levels of the immunoproteasome. Inhibition of immunoproteasomal catalytic subunits LMP7 and LMP2 with ONX-0914 has been shown to have immunosuppressive effects and was effective in treating various autoimmune diseases in preclinical models, while its effects on atherosclerosis have not been studied yet. We found that intraperitoneal ONX-0914 treatment reduced atherosclerosis and dendritic cell levels and activation, and levels of antigen experienced T cells in various immune organs. Additionally, ONX-0914 reduced white adipose tissue (WAT) mass, which surprisingly coincided with neutrophil and macrophage accumulation in WAT. We found that primary mature murine adipocytes express immunoproteasomal subunits and upregulate CCL2 after incubation with ONX-0914, providing a mechanism for ONX-0914 induced immune infiltration. ONX-0914 reduced intestinal triglyceride uptake, which was likely the primary cause of the reduction in WAT mass, as ONX-0914 did not increase energy expenditure or reduce food intake. Intraperitoneal treatment with clodronate liposomes to deplete macrophages abolished the inhibitory effect of ONX-0914 on intestinal lipid uptake, indicating involvement of intraperitoneal macrophages in ONX-0914 mediated lipid uptake reduction. Concomitant with a reduction in WAT mass upon ONX-0914 treatment, we observed improvements in markers of metabolic syndrome, including lowered blood triglyceride levels, insulin levels, and fasting blood glucose. These data indicate that immunoproteasomal inhibition could be a useful tool to treat atherosclerosis, obesity, and metabolic syndrome, major health issues in the developed world.

Introduction

Cardiovascular disease is the most common cause of death in the Western world with atherosclerosis as the most common underlying pathology *(1)*. Atherosclerosis is characterized by lipid deposition in the intima of medium to large sized arteries, which evokes immune infiltration in the vessel wall and inflammation. The inflammatory response in atherosclerosis is characterized by a pro-inflammatory and pathogenic Th1 immune response *(2)*. Current treatment of atherosclerosis has however mainly been focused on treating dyslipidemia in patients, which has led to a decrease in cardiovascular events *(1)* . However, as evidenced by the CANTOS trial, immune modulating treatment combined with lipid management can lead to an additional lowering of the risk for major cardiovascular events *(3, 4)*.

An interesting immune regulator is the immunoproteasome, a proteasome variant mainly basally expressed in cells of hematopoietic origin *(5)* and a variant of the constitutive proteasome which is basally expressed in all cells. Proteasomes are responsible for the degradation of the vast majority of cellular proteins and are involved in regulation of cellular processes via targeted degradation of polyubiquitinated proteins *(5)*. Structurally, proteasomes are comprised of a large barrel-like protein complex, the 20S proteasome, which is formed through axial stacking of two heptameric outer α-rings and two heptameric inner β-rings. Regulatory protein subunits can bind to the α-rings and are responsible for binding and unfolding of client proteins and open up the α-ring, which is otherwise almost completely closed restricting untargeted protein entry into the 20S proteasome *(5)*. Client proteins that enter the proteasome are proteolytically cleaved by the lumen facing active sites of the 3 distinct catalytically active β-subunits present per β-ring, which differ between the different proteasome variants. These catalytic subunits exert caspase-like, trypsin-like, and chymotrypsin-like activities which are executed by the β1, β2 and β5 subunit in the constitutive proteasome respectively, and β1i (LMP2), β2i (MECL-1) and β5i (LMP7) subunit in the immunoproteasome respectively *(6)*.

The constitutive- and immunoproteaosomal catalytic subunits diverge in the amino acids after which they proteolytically cleave proteins, resulting in different protein cleavage products *(7)*. Through these divergent protein cleavage patterns, immunoproteasomes were found superior in the generation of MHC-I epitopes, important for the induction of CD8 T cell responses. IFN-γ promotes the expression of the immunoproteasomal subunits, causing inducible immunoproteaome expression also in cells of non-hematopoietic origin. In addition to the generation of MHC-I epitopes, more recent studies suggest that the immunoproteasome also has a more general pro-inflammatory role, inducing pro-inflammatory gene expression in DCs *(8)*. How immunoproteasomal activity induces this pro-

inflammatory gene expression is still subject of speculation. Several studies using the immunoproteasomal LMP7 and LMP2 specific inhibitor ONX-0914 (formerly known as PR-957) *(9, 10)* have confirmed the pro-inflammatory role of the immunoproteasome and have demonstrated the involvement of the immunoproteasome in regulation of multiple inflammatory processes in different immune in cells *in vitro* and *in vivo*. As such, LMP7 inhibition reduced inflammatory cytokine secretion in PBMCs and T cells *(9, 11)*. Furthermore ONX-0914 was found to impact T cell differentiation, as ONX-0914 inhibited CD4 T cell differentiation towards the pro-inflammatory Th1 and Th17 helper T cells, and promoted differentiation towards Tregs *in vitro (12)*. LMP7 inhibition was also found to affect DC functionality, inhibiting the capacity of DCs to prime T cells *(8)*. In various experimental murine models of auto-immune diseases, such as experimental autoimmune encephalomyelitis (EAE), and arthritis, LMP7 inhibition with ONX-0914 reduced disease severity *(9–17)*.

Since the effects of LMP7 inhibition described thus far are potentially beneficial for the outcome of cardiovascular disease, we studied the effect of ONX-0914 on atherosclerosis in LDLr deficient animals. Our results show that on top of reducing atherosclerosis by altering the innate and adaptive immune response, ONX-0914 treatment reduced intestinal lipid uptake, reduced white adipose tissue mass, and improved markers of metabolic syndrome. Our data indicate that immunoproteasomal inhibition using specific inhibitors may be a highly useful to combat atherosclerosis, obesity, and metabolic syndrome.

Results

ONX-0914 treatment reduces atherosclerosis

Pharmacological inhibition of the immunoproteasomal active subunit LMP7 has reduced inflammation in experimental models of autoimmune diseases, thereby reducing disease severity *(9–17)*. To assess the effect of LMP7 inhibition on atherosclerosis development, 12-14 week old female LDLr$^{-/-}$ mice were treated 3 times weekly with ONX-0914 (10 mg/kg intraperitoneally) for 7 weeks while being fed a Western-type diet (WTD). We analyzed the atherosclerotic lesions in the aortic root (Fig. 1A) and observed a 28.4% reduction (p=0.0054) in lesion size in the ONX-0914 treated group (2.8±0.5 x10^5 µm^2) compared to the vehicle treated group (3.8±0.7 x10^5 µm^2), and a trend towards reduced vessel occlusion (p=0.0975) (Fig. 1B). In addition we analyzed the accumulation of macrophages in the atherosclerotic lesions by immunohistochemistry (Fig. 1C) and found that the macrophage area in the lesions was reduced by 26.6%, but the relative macrophage area in the plaque was not affected upon LMP7 inhibition (Fig. 1D). Furthermore we assessed collagen buildup in the plaque by Masson's trichrome staining (Fig. 1E), and found a reduced absolute collagen area and a trend towards relative collagen content of the plaque (p=0.0538) in the ONX-0914 treated group

(Fig. 1F), which indicates a less progressed plaque phenotype in the ONX-0914 treated group than in the control group.

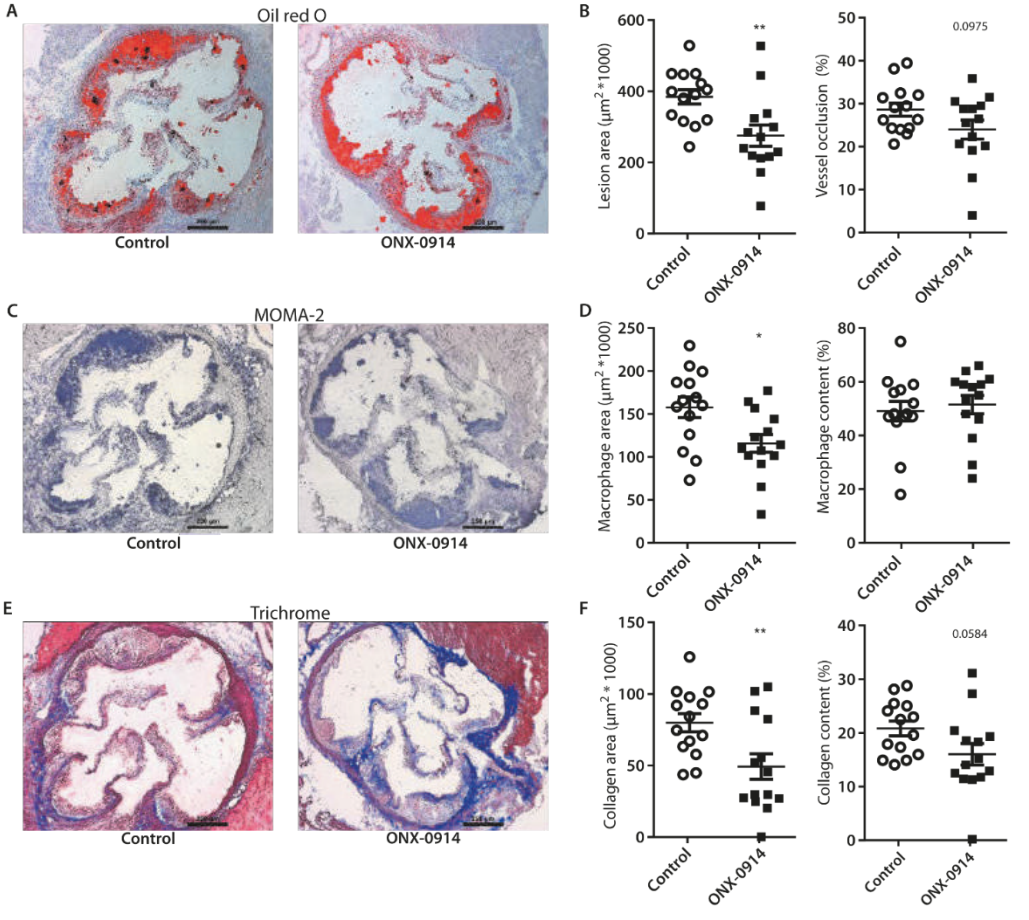

Fig. 1 ***ONX-0914 treatment reduces atherosclerosis in aortic root.*** *12-14 week old Female LDLr$^{-/-}$mice (n=15) were fed WTD for 7 weeks during which ip injections with ONX-0914 (10 mg/kg) or control injections were administered three times weekly.* ***A****) Lipids in the aortic root were stained with oil red O* ***B****) to quantify atherosclerotic lesion area and vessel occlusion.* ***C****) Aortic root sections were immunohistochemically stained using MOMA-2 antibody* ***D****) to determine macrophage area and macrophage content of the plaque.* ***E****) Masson's Trichrome staining was used to assess* ***F****) collagen area and collagen content of the plaque. Expressed as mean ± SEM, unpaired two tailed t-test, *p < 0.05, **p < 0.01.*

ONX-0914 reduces dendritic cell levels and activation

DCs have a pivotal role in atherosclerosis and enhanced activation of dendritic cells induces atherogenesis and mediates CD4 and CD8 T cell activation *(18)*, whereas vaccination with

ApoB100 loaded DCs is atheroprotective and inhibits Th1 and Th2 immunity to ApoB100 *(19, 20)*. It was previously reported that immunoproteasomal deficiency hampers DC functionality *(8)*. Therefore, we assessed the effect of LMP7 inhibition on dendritic cell levels and their activation status with flow cytometry (Fig. 2A) in the spleen, cervical lymph nodes draining from the plaque *(21)* and disease unrelated mesenteric lymph nodes (MLN). ONX-0914 treatment significantly reduced the numbers of conventional CD11c^{+}MHC-II^{+} DCs in all three lymphoid organs (Fig. 2B). Activation of conventional DCs was unaltered in the spleen (Fig. 2C) but slightly lowered in the CLNs (Fig. 2D) and MLNs (Fig. 2E) upon LMP7 inhibition, as judged by the expression of CD86.

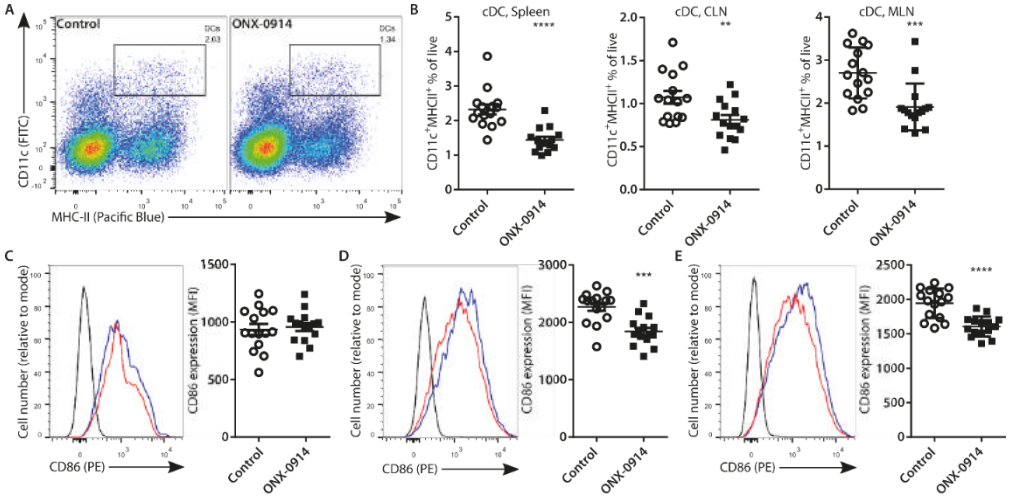

Fig. 2 ***ONX-0914 lowers conventional DC levels and activation in lymphoid organs. A****) Representative flow cytometry plots from spleen, with gating depicted of cDCs based on expression of CD11c and MHC-II.* ***B****) Quantification of cDC levels in spleen, CLN, and MLN. Representative histograms of expression and quantification of median fluorescent intensity (MFI) of CD86 on cDCs in* ***C****) spleen,* ***D****) CLN, and* ***E****) MLN. Expressed as mean ± SEM, unpaired two tailed t-test, **p < 0.01, ***p < 0.001, **** p < 0.0001.*

To assess the direct effect of ONX-0914 on DCs, bone marrow derived DCs (BMDCs) were exposed overnight to ONX-0914 (200 nM) and assessed by flow cytometry (Fig. S1A). ONX-0914 incubation reduced DC viability (Fig. S1B), which could have accounted for the lowered DC levels that were observed in vivo. We did not observe a significant decrease in CD86 expression on DCs *in vitro*, probably due to a low sample size (Fig. S1B). Strikingly, stimulation of BMDCs with LPS reduced cell death caused by ONX-0914 treatment (50-100nM) (Fig. S1C). The observed upregulation in gene expression of constitutive and immunoproteasomal catalytic subunits in LPS-stimulated BMDCs compared to unstimulated DCs (Fig. S1D) likely rendered LPS-stimulated BMDCs less susceptible to ONX-0914 induced cell death. Furthermore, *in vitro* exposure to ONX-0914 (50-100 nM) upregulated expression of the

proteasome subunit β5 in BMDCs (Fig. S1D), much like the previously reported upregulation of the constitutive proteasomal subunit β5 subunit in T cells and B cells treated with ONX-0914 *(22)*. Concomitant with the upregulation of the constitutive β5 subunit, the subunits β1 and β2 were upregulated (Fig. S1D). In contrast to the upregulation of constitutive proteasomal subunits, all immunoproteasomal catalytic subunits and PA28α and PA28β, subunits from the immunoproteasomal PA28αβ regulator, were downregulated by incubation with both concentrations of ONX-0914 in the presence of LPS (Fig. S1D). Since immunoproteasomal deficiency was found to reduce inflammatory gene expression in DCs and DC functionality *(8)*, enhanced expression of constitutive proteasomes over immunoproteasomes in ONX-0914 exposed DCs might explain the suppressive effect of ONX-0914 on DCs. Since pro-inflammatory DCs promote atherogenesis, the observed reduction in DC levels and activation are likely to have contributed to reduced atherosclerosis.

ONX-0914 treatment reduces memory T cell levels

Because atherosclerosis is marked by a pathogenic Th response *(2)*, we assessed whether ONX-0914 treatment skewed T helper differentiation away from Th1 cells towards atheroprotective Tregs, conform previously described *in vitro* and *in vivo* data *(12)*. To assess whether Tregs were induced by ONX-0914 treatment we assessed $CD4^{+}FoxP3^{+}CD25^{+/-}$ cells by flow cytometry (Fig. S2A) in the spleen (Fig. S2B), blood (Fig. S2C), cervical lymph nodes (Fig. S2D) and mesenteric lymph nodes (Fig. S2E), however no enhanced Treg levels were found. To assess whether proliferative capacity of T cells and T cell differentiation were altered by ONX-0914 treatment, splenocyte cultures were stimulated with αCD3 and αCD28, and thymidine incorporation and the cytokine profile in supernatant were assessed. ONX-0914 did not affect thymidine incorporation (Fig. S3A) or IFN-γ, IL-4, IL-17a and IL-10 levels in culture supernatant (Fig. S3B), indicating that splenic T cell proliferation and splenic T cell differentiation towards Th1, Th2, Th17 and Treg were unaffected by ONX-0914 treatment.

In line with these data we did not observe changes in CD4 (Fig. S3C) and CD8 T cell (Fig. S3E) levels, and central memory (Tcm), effector memory (Tem) and naïve (Th0/Tc0) T cell levels after ONX-0914 treatment in the spleen (Fig. S3D/F). Similarly, no differences in the CD4 population were observed in the cervical lymph nodes (Fig. S4A/B). The overall CD8 T cell percentage was, however, significantly increased in the CLN of ONX-0914 treated mice (Fig. S4C) but did not coincide with increases in any of the memory CD8 T cell subpopulations (Fig. S4D). These data suggest that enhanced CD8 T cell levels in the CLN were not the result of enhanced priming of CD8 T cells in the CLN. In mesenteric lymph nodes, however, ONX-0914 treatment caused an overall reduction of CD4 T cell levels (Fig. 3A) and a trend ($p=0.14$) towards reduction in CD4 Tem cells (Fig. 3B). Furthermore LMP7 inhibition reduced CD8 Tem and Tcm cells in the mesenteric lymph nodes (Fig. 3D) while overall CD8 T cell levels were unchanged (Fig. 3C). In the blood overall CD4 and CD8 T cell levels were not significantly

altered (Fig. 3E,G) but CD4 Th0 cell levels were increased (Fig. 3F), and CD8 Tc0 cells were increased coinciding with lowered CD8 Tem cells (Fig. 3H). These data indicate that even though ONX-0914 did not induce Tregs, induction of memory T cells was inhibited by ONX-0914 treatment. This is in line with the previously reported suppressive effect of ONX-0914 on T cells *(12, 22)*. ONX-0914 treatment reduced DC levels and activation, and locally reduced memory CD4 and CD8 T cell levels, which are likely to have contributed to the lowered atherosclerotic lesion size observed upon LMP7 inhibition.

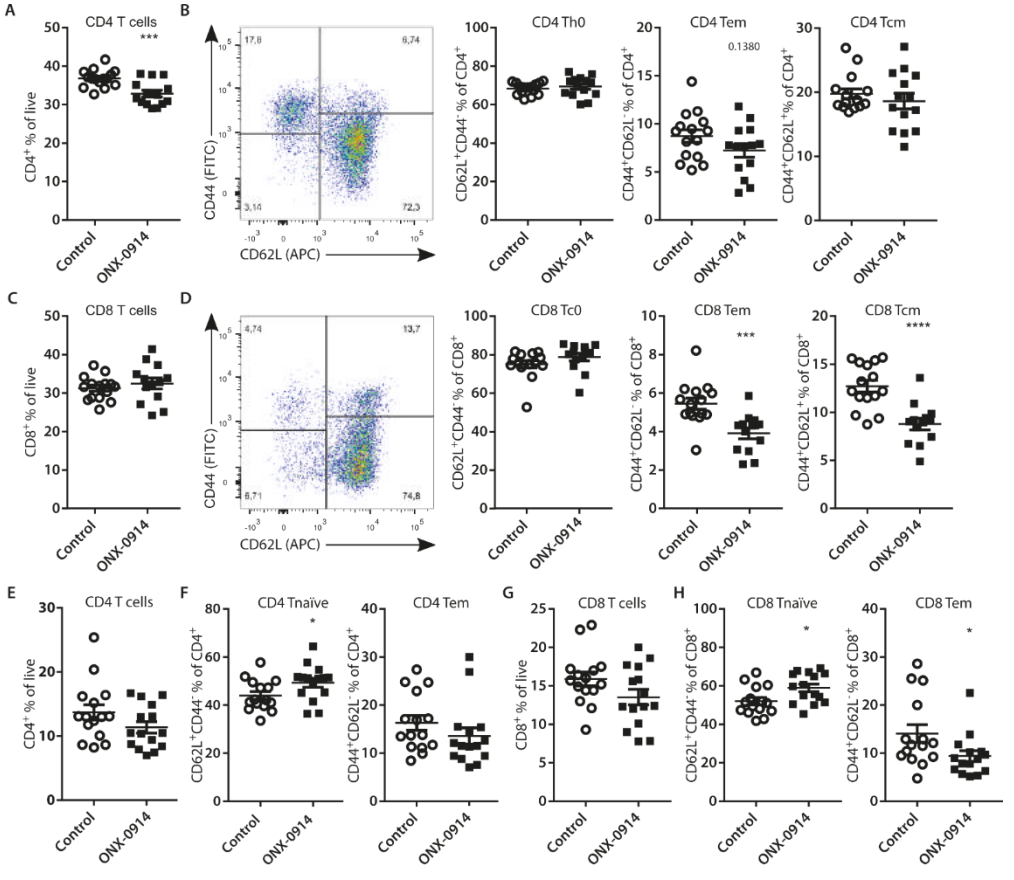

Fig. 3 ***ONX-0914 treatment attenuates maturation of T cells in vivo.*** *Quantification of flow cytometric analysis of* ***A****) CD4 T cell and (C) CD8 T cell content of MLN. Representative flow cytometry plots and quantification of CD62L⁺CD44⁻ naïve, CD44⁺CD62L⁻ effector memory, and CD44⁺CD62L⁺ central memory* ***B****) CD4 T cells and* ***D****) CD8 T cells from MLN. Quantification of* ***E****) CD4 T cells and* ***G****) CD8 T cells and naïve and effector memory* ***F****) CD4 T cells and* ***H****) CD8 T cells in circulation based on flow cytometric analysis. Expressed as mean ± SEM, unpaired two tailed t-test, *p < 0.05, ***p < 0.001, **** p < 0.0001.*

ONX-0914 reduces markers of metabolic syndrome while inducing innate immune activation

Since ONX-0914 was reported to suppress pro-inflammatory cytokine secretion/production *(11, 12)*, a yet additional beneficial effect in the context of atherosclerosis, we assessed the serum cytokine profile in serum of ONX-treated mice using multiplex ELISA. Surprisingly, elevated levels of IL-6 and TNF-α, and a trend towards increased IL-1β levels were detected in serum of ONX-0914 treated mice (Fig. 4A, Fig. S5A for remainder of assessed cytokines). We observed a profound increase in neutrophil levels in blood (Fig. 4B), indicative of innate inflammation, but circulating classical monocyte levels remained stable, and patrolling monocyte populations were reduced (Fig. 4B). Furthermore ONX-0914 treatment reduced body weight (Fig. 4C), virtually depleting gonadal white adipose tissue (gWAT, visual observation), and lowered triglyceride (TG) levels but not cholesterol levels in blood plasma (Fig. 4D).

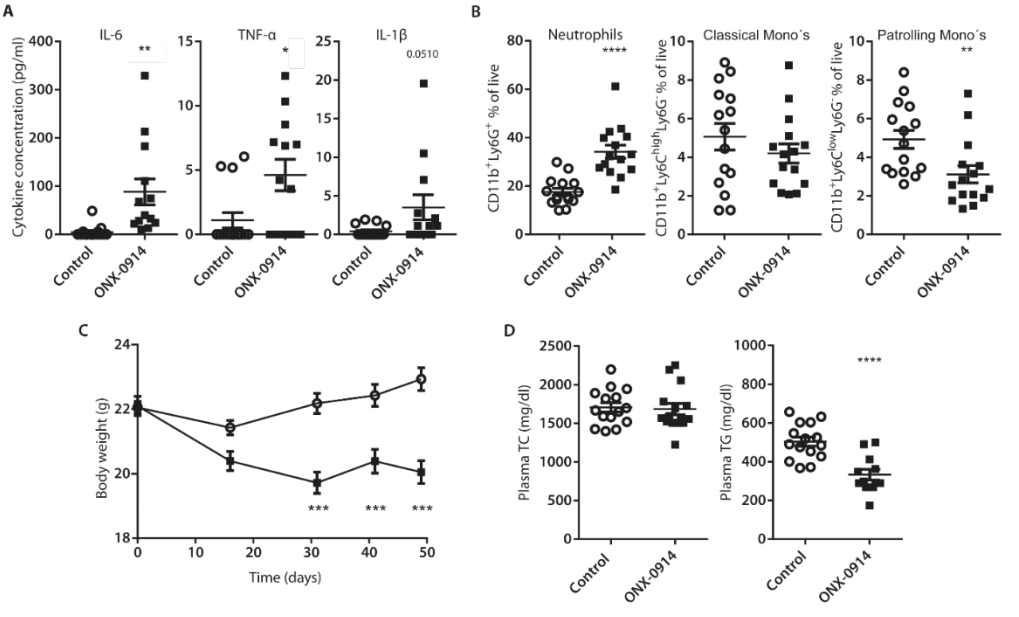

Fig. 4 ***ONX-0914 induces unexpected beneficial metabolic effects and signs of innate inflammation.*** ***A****) Quantification of cytokine levels in blood plasma and* ***B****) quantification of most abundant myeloid cell populations in circulation based on flow cytometric analysis at sacrifice at sacrifice.* ***C****) Body weight over the course of the experiment.* ***D****) Total cholesterol and triglyceride levels in blood plasma at sacrifice. Expressed as mean ± SEM, ABD unpaired two tailed t-test, C two-way repeated measures ANOVA with Holm-Sidak posttest, *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.*

Lowered body weight and TG levels as a result of ONX-0914 treatment have not been described previously, therefore we further investigated the mechanisms through which ONX-0914 improved these markers of metabolic syndrome. LMP7 deficiency was previously found to reduce preadipocyte differentiation into mature adipocytes *(23, 24)*. Since ONX-0914 treatment virtually depleted gonadal WAT and reduced body weight, we suspected that ONX-0914 besides possibly affecting preadipocytes, also affected preexistent fat mass. To assess the effect of ONX-0914 on preexisting fat mass, male LDLr$^{-/-}$ mice were fed a WTD for 4 weeks, after which mice were randomized for body weight and age. At this point the baseline group was sacrificed, while the remaining animals were vehicle treated or ONX-0914 treated for 7 weeks.

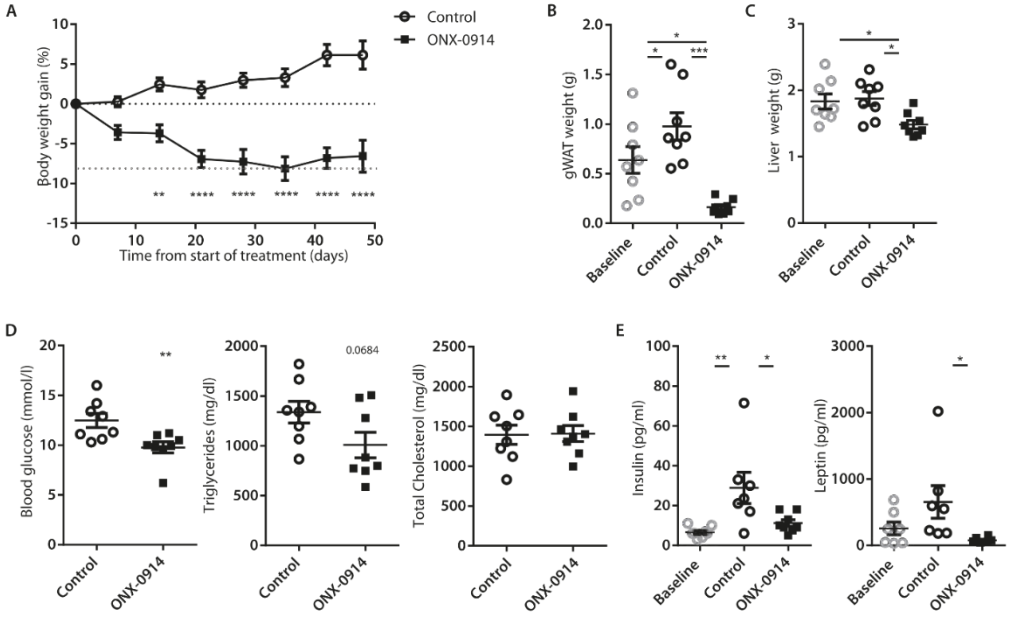

Fig. 5 ***ONX-0914 treatment decreases pre-existing white adipose tissue and improves metabolic parameters.*** *To assess the effect of ONX-0914 treatment on pre-existing fat mass, male 12-14 week old LDLrKO males (n=8) were fed a WTD for 4 weeks. At this point the baseline group was sacrificed and treatment with ONX-0914 (10 mg/kg, three times weekly) or control was commenced and continued for 7 weeks after which the control and treated groups were sacrificed.* ***A****) Gain in body weight of the control and ONX-0914 treated group over the course of treatment.* ***B****) gWAT and* ***C****) Liver weight at time of sacrifice.* ***D****) Blood glucose, triglyceride and total cholesterol levels in blood plasma after 4h fasting after 3.5 weeks of treatment. (****E****) Insulin and leptin concentrations in serum at sacrifice determined with multiplex ELISA. Expressed as mean ± SEM,* ***A****) two-way repeated measures ANOVA with Holm-Sidak posttest* ***BCE****) one-way ANOVA with Holm-Sidak posttest,* ***D****) two tailed t-test, *p < 0.05, **p < 0.01, **** p < 0.0001.*

ONX-0914 treatment resulted in an immune phenotype similar to the previous study in respect to DCs (Fig. S6A-D), CD4 and CD8 T cells (Fig. S7) and neutrophil levels (Fig. S8A/B) compared to vehicle treated mice. Body weight declined from the start of ONX-0914 treatment and stabilized after 4-5 weeks of treatment (Fig. 5A). Consistent with the body weight gain, gWAT weight increased compared to baseline in the vehicle group, and declined nearly 4 fold in ONX-0914 treated animals compared to baseline (Fig. 5B). Liver weight was reduced in ONX-0914 treated animals (Fig. 5C), indicative of less steatosis *(25)*. After 4 weeks of treatment and 40h after ONX-0914 administration, mice were fasted for 4h after which blood glucose, TG and cholesterol levels were determined. Blood glucose was significantly lowered, and a trend towards reduced TG levels was found in the ONX-0914 treated group compared to the vehicle treated group while total cholesterol levels remained stable (Fig. 5D). Furthermore multiplex analysis of metabolism related hormone and cytokine levels in serum after 7 weeks of treatment, revealed increased insulin levels in the vehicle treated group, indicative of insulin resistance, while insulin levels were similar to baseline in ONX-0914 treated animals (Fig. 5E). Leptin levels, correlating with gWAT weight, were lowered in ONX-0914 treated animals compared to vehcicle control group (Fig. 5E). To confirm that weight loss was not due to an off target (hepato)toxic effect of ONX-0914 treatment, we determined the activity of the liver-derived enzymes alanine aminotransaminase (ALAT) and aspartate aminotransferase (ASAT) in blood serum, and hepatic gene expression of Cyp3A11, as readout for PXR activation. We did not observe differences in ALAT and ASAT activities (Fig. S9A) nor in expression of Cyp3A11 (Fig. S9B) between the vehicle and treated group, indicating that ONX-0914 treatment did not induce liver toxicity or PXR activation.

ONX-0914 mediated reduction of white adipose tissue mass is accompanied by neutrophil and macrophage accumulation in gWAT

In humans a homozygous missense mutation in PSMB8, encoding for LMP7, causes lipodystrophy, adipose inflammation and enhanced IL-6 levels *(23)*. Since the phenotype observed in individuals with dysfunctional LMP7 is remarkably similar to the reduced WAT mass and enhanced inflammatory markers we observed when LMP7 (and LMP2) activity in mice was inhibited with ONX-0914, we assessed whether reduced WAT mass was accompanied by immune infiltration in gWAT. To that end we treated mice, which had been fed WTD for 6 weeks already to induce adiposity, with ONX-0914 for 1 week (4 ONX-0914 injections) or 1 day (3 PBS injections followed by 1 ONX-0914 injection), or with vehicle injections (4 PBS injections) to assess the immune response in WAT. 18h after final ip injection, mice were sacrificed and gWAT was isolated. Besides mature adipocytes, WAT is comprised of blood vessels, immune cells, and precursors of mature adipocytes, namely adipose stem cells and preadipocytes, forming the stromal vascular fraction (SVF). To assess the composition of gWAT, gWAT was digested and its SVF was isolated and analyzed by flow

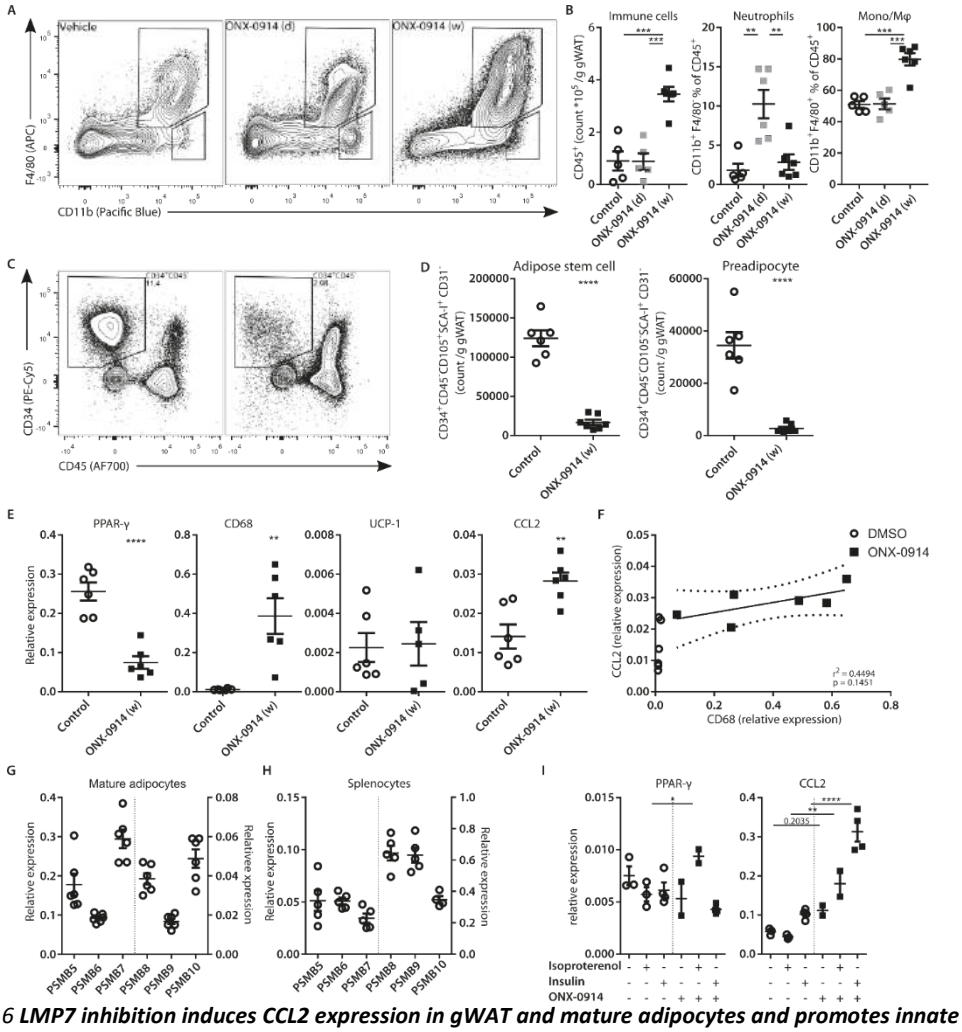

Fig. 6 ***LMP7 inhibition induces CCL2 expression in gWAT and mature adipocytes and promotes innate immune infiltrate. A****) Representative flow cytometry plots of the CD45⁺ immune fraction of the SVF from gWAT, showing the gating for CD11b⁺F4/80⁺ macrophages and CD11b⁺F4/80⁻ neutrophils.* ***B****) Quantification of total immune cells, neutrophils and macrophages in SVF from gWAT.* ***C****) Representative flow cytometry plots for the gating of CD34⁺CD45⁺ cells, containing* ***D****) the SCA-I⁺CD105⁺CD31⁻ adipose stem cells, and SCA-I⁺CD105⁻CD31⁻ preadipocytes.* ***E****) Gene expression of the floating cell fraction from digested gWAT.* ***F****) Correlation analysis between CD68 and CCL2 expression of the floating cell fraction of the ONX-0914 treated group.* ***G****) Gene expression of proteasomal active subunits in freshly isolated mature adipocytes and* ***H****) splenocytes from control treated mice.* ***I****) Gene expression of mature adipocytes isolated from gWAT, ex vivo cultured without or with ONX-0914 (200nM) for 24h under basal, lipolytic (10 µM isoproterenol), and lipogenic (100 nM insulin) conditions. Expressed as mean ± SEM,* ***BI****) 1-way ANOVA with Holm-Sidak posttest,* ***C****) unpaired two tailed t-test,* ***F****) Pearson correlation, *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.*

cytometry (Fig. 6A). A single injection of ONX-0914 already led to infiltration of $CD11b^{+}F4/80^{-}$ cells, most likely neutrophils (Fig. 6B). A week of ONX-0914 treatment led to increased overall immune cell counts ($CD45^{+}CD34^{-}$) and accumulation of $CD11b^{+}F4/80^{+}$ monocytes/macrophages (Fig. 6B), confirming the infiltration of immune cells upon ONX-0914 treatment. Furthermore, treatment with ONX-0914 for a week massively reduced the number of adipose stem cells and preadipocytes in gWAT SVF (Fig. 6CD).

To assess in more detail whether ONX-0914 affected the mature adipocyte population and how ONX-0914 caused the innate immune infiltrate in gWAT and reduction in gWAT mass, RNA was isolated from the adipocyte cell fraction after 1 week of treatment with ONX-0914. UCP-1 expression was not increased (Fig. 6E), suggesting that beiging of white adipose tissue due to ONX-0914 treatment did not underlie a reduction in body weight in ONX-0914 treated mice. Surprisingly PPAR-γ expression, a marker for mature adipocytes, was decreased approximately 3-fold with a concomitant increase in CCL-2 and CD68 expression (Fig. 6E), indicating the presence of macrophages in the adipocyte cell fraction. Correlation analysis of CCL2 expression and CD68 of the ONX-0914 treated group, suggests that CCL2 expression was unlikely to be derived from solely macrophages ($r2 = 0.4494$, $p = 0.1451$) (Fig. 6F). Therefore we determined whether mature adipocytes could have been a source of CCL2 expression in the adipocyte fraction of ONX-0914 treated mice. To assess this, mature adipocytes were isolated from gWAT of 40 week old female $ApoE^{-/-}$ animals on chow diet, and we determined whether immunoproteasomal subunits were expressed in mature adipocytes by qPCR. Freshly isolated mature adipocytes did express immunoproteasomal PSMB8 (LMP7), PSMB9 (LMP-2) and PSMB10 (MECL-1) (Fig. 6G), although expression of immunoproteasomal subunits was low compared to expression of proteasomal subunits PSMB5 (β5), PSMB6 (β1) PSMB7 (β2), and expression of immunoproteasomal subunits in splenocytes (Fig. 6H). Exposure of mature adipocytes overnight to ONX-0914 (200 nM) did not reduce the expression of PPAR-γ, but significantly induced CCL-2 expression in insulin and isoproterenol treated adipocytes (Fig. 6I), indicating that ONX-0914 treatment can directly affect mature adipocytes, causing CCL2 induction, which could lead to WAT inflammation.

ONX-0914 reduces intestinal lipid uptake through a macrophage dependent mechanism

To further investigate the mechanism of ONX-0914 mediated reduction of gWAT mass, we assessed whether LMP7 inhibition influenced energy expenditure using metabolic cages. Since TG levels were lowered by LMP7 inhibition we also studied the effect of ONX-0914 on clearance of iv injected VLDL-like particles with radiolabeled [^{14}C]cholesteryl oleate and glycerol tri[^{3}H]oleate *(26)*. Because our initial studies in LDLr deficient mice may have prevented a cholesterol-lowering effect due to the absence of a functional LDLr *(27)*, we

opted to use APOE*3-Leiden.CETP mice in this experiment. After 3 weeks of WTD feeding, ONX-0914 treatment was started for 2.5 weeks (10 mg/kg, 3 times weekly).

Two weeks of treatment of APOE*3-Leiden.CETP mice with ONX-0914 led to reduced body weight gain compared to control treatment (Fig. 7A). Similar to the studies using LDLr KO mice, ONX-0914 reduced fat mass but not lean mass as assessed by EchoMRI (Fig. 7B), while overall food intake throughout the study was similar (Fig. 7C). Unexpectedly, locomotor activity (Fig. 7E) and energy expenditure (Fig. 7F) were decreased during the dark phase after treatment. Carbohydrate oxidation decreased directly after ONX-0914 treatment (Fig. 7G) and was accompanied by increased fat oxidation after treatment (Fig. 7H). The night after treatment (29-41h after treatment) locomotor activity normalized (Fig. 7E) and fat oxidation decreased (Fig. 7G) whilst carbohydrate oxidation tended to increase (Fig. 7H). Because locomotor activity was lowered and fat combustion was increased after treatment, we hypothesized that food intake could be reduced directly after treatment, and was compensated for during the 24h after that. Because this should be reflected in the fecal output, feces was collected the first 18h after treatment and the subsequent 24h. ONX-0914 treatment reduced total fecal weight in the first 18h and increased in the 18h-42h timeframe (Fig. 7D), suggesting that food intake may indeed be inhibited directly upon ONX-0914 treatment. TG and total nonHDL-cholesterol levels were lowered by LMP7 inhibition (Fig. S10A/B). Clearance of [^{14}C]cholesteryl oleate and glycerol tri[^{3}H]oleate levels from iv injected VLDL-like particles was slightly slower in ONX-0914 treated animals (Fig. S10C), indicating that lowered TG and TC levels were not due to a faster uptake from the blood.

Similar to the results in LDLr$^{-/-}$ mice, macrophage accumulation was detected in gWAT, marked by enhanced CD68 gene expression upon ONX-0914 treatment (Fig. S11A). M1 macrophage markers SOCS3, TNF-α, and IL-6, and M2 macrophage markers Arginase-I and IL-10 gene expression were increased to a similar extent upon LMP7 inhibition (Fig. S11A), indicating that the accumulated macrophages were not skewed towards a particular phenotype (Fig. S11B). Unlike in the previous experiments in which we assessed immune infiltrate in gWAT after shorter periods of ONX-0914 treatment, we did not find neutrophil accumulation in gWAT after 2.5 weeks of ONX-0914 treatment (Fig. S11C), and like previous experiments we did not find T cell accumulation in gWAT (Fig. S11C). No accumulation of macrophages was observed in interscapular brown adipose tissue tissue (iBAT) (Fig. S11D/E) or liver (Fig. S11F), suggesting that macrophage accumulation was WAT specific.

Because it was previously reported that intestinal lipid uptake was reduced in LMP7 deficient mice *(28)* we wondered whether lipid uptake was affected in ONX-0914 treated mice, and whether macrophages, consistently found to accumulate in gWAT after ONX-0914 treatment, were involved in this process. To assess involvement of the accumulated

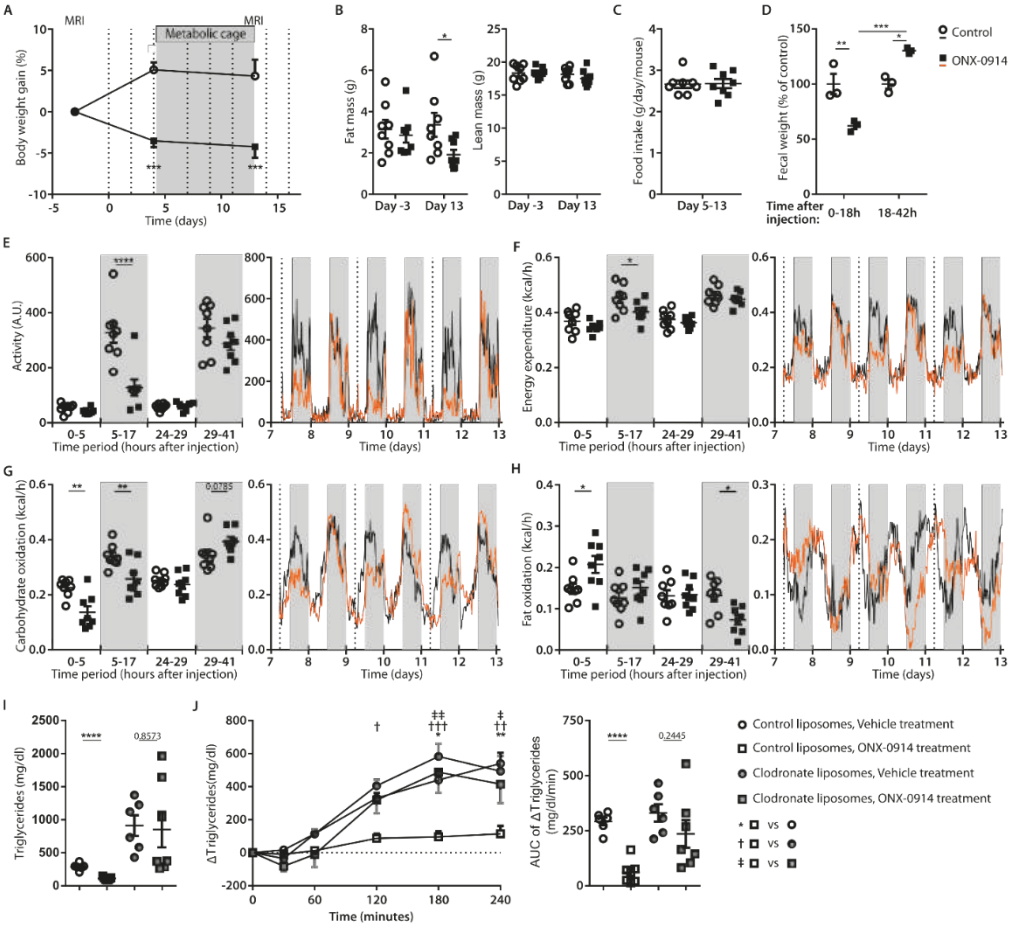

Fig. 7 ONX-0914 mediated weight loss is mediated by reduced intestinal lipid uptake A*) Body weight change over the course of the the metabolic cage study and its experimental setup. Dotted lines indicate control and ONX-0914 injections.* ***B****) At day -3 and 13 body composition was analyzed by EchoMRI. From day 5-13 animals were single housed in metabolic cages in which* ***C****) food intake,* ***E****) activity,* ***F****) energy expenditure and* ***H****) glucose and* ***G****) lipid utilization were determined.* ***D****) Feces was collected from the bedding of group housed animals at day 15 (0-18h after treatment) and day 16 (18-42h after treatment), and weighed.* ***I****) Baseline TG levels in blood plasma, obtained just before oral gavage with olive oil (200 µL).* ***(J)*** *Change in blood plasma TG levels at several timepoints after oral gavage with olive oil (left panel), and area under the curve of the change in TG levels (panel right) . Expressed as mean ± SEM,* ***A,D-H, J****) (left panel) two-way paired ANOVA with Holm-Sidak posttest,* ***B****) (day 13) and* ***C****) two-tailed T-test,* ***I,J****) (right panel) multiple two-tailed T-test with Holm-Sidak posttest, *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.*

macrophages, intraperitoneal macrophages were depleted with ip injections of clodronate

liposomes a day prior to control or ONX-0914 treatment and compared to mice injected with empty liposomes. ONX-0914 treatment (10 mg/kg, 4 injections every other day) enhanced macrophage and neutrophil numbers in gWAT like previously after a week of ONX-0914 treatment (Fig. S12A-D). Clodronate liposome treatment successfully reduced $CD11b^{+}F4/80^{+}CD206^{+}$ adipose tissue resident macrophage levels (Fig. S12A/B), and reduced accumulation of $CD206^{-}$ macrophages (Fig. S12A/B) and neutrophils (Fig. S12C/D) after ONX-0914 treatment. After three ONX-0914 injections mice were fasted overnight after which blood was collected, and mice received an oral bolus of 200 µl olive oil after which blood was collected at several time points to assess TG levels. Macrophage depletion increased baseline TG levels (Fig. 7G) in line with previous data *(29)*, whilst only in the control liposome/ONX-0914 treated group triglyceride uptake was decreased (Fig. 7H), suggesting that ONX-0914 acted through a macrophage population to reduce intestinal lipid uptake. In LMP7 deficient animals, reduced intestinal lipid uptake was believed to be the result of a lower expression of fat digesting lipases, pancreatic lipase (Pnlip) and pancreatic lipase-related protein 2-like (Pnliprp2) *(28)*, however we did not observe this (Fig. S13A) nor did we observe reduced expression of proteins involved in intestinal lipid uptake (Fig. S13B).

To further investigate the mechanism through which macrophages are involved in ONX-0914 induced weight loss, we performed a literature search for macrophage derived factors known to temporarily reduce food intake and activity, as seen in our metabolic cage study, and known to reduce body weight. From this literature search, we identified GDF15 which was recently described to induce similar metabolic profile as we observed with LMP7 inhibition *(30)*. GDF15 levels were indeed increased in blood plasma from ONX-0914 treated mice from multiple studies, shown for the initial atherosclerosis study (Fig. S14A). Therefore, we assessed whether induction of GDF15 upon treatment with ONX-0914 was the cause of the reduced body weight and treated $GDF15^{-/-}$ mice with the LMP7 inhibitor. Since already after a week of treatment a strong trend towards weight loss was observed in $GDF15^{-/-}$ mice treated with ONX-0914 (Fig. S14B), it is unlikely that GDF15 mediated ONX-0914-induced weight loss.

Besides reducing atherosclerosis through its immunosuppressive properties, ONX-0914 thus reduces white adipose tissue weight but not lean mass through reduced intestinal lipid uptake, and causes macrophage accumulation in gWAT. Macrophages appear to be involved in ONX-0914 mediated reduction in intestinal lipid uptake, however the exact mechanism in which intestinal lipid uptake is affected by LMP7 inhibition remains to be elucidated.

Discussion

ONX-0914 mediated LMP7 and LMP2 inhibition *(10)* has proven to be effective in reducing disease severity in various auto-immune animal models through immune inhibition, but had not yet been tested in the context of atherosclerosis. In this study we show that ONX-0914 treatment reduced atherosclerosis in female LDLr$^{-/-}$ mice fed a WTD. Unexpectedly LMP7/LMP2 inhibition also reduced body weight by reducing WAT and substantially improved markers of metabolic syndrome.

Atherosclerosis is characterized by lipid accumulation in vessel wall and subsequent chronic immune activation of the vessel wall, aggravating the disease. Oxidation and aggregation of LDL in the subendothelial space promotes uptake of LDL by macrophages and other APCs and results in activation of autoreactive (ox)LDL specific T cells. The inflammatory response in atherosclerosis is marked by a Th1 response *(2, 31)*, resulting in production of the atherogenic Th1 cytokines IFN-γ *(32–36)* and TNFα *(37, 38)*. Because of the detrimental effects of the Th1 response in atherosclerosis *(2, 39, 40)* we aimed to inhibit this response. Inhibition of the immunoproteasomal LMP7 subunit with ONX-0914 was previously described to inhibit Th1 and Th17 development *(9, 11)*, reduce secretion of pro-inflammatory cytokines *(9, 41)*, and reduce various auto-immune diseases in experimental models. Therefore, we were interested to study the effect of ONX-0914 on atherosclerosis.

Treatment with ONX-0914 reduced atherosclerosis in LDLr$^{-/-}$ mice fed WTD for 7 weeks. At this time point we did not observe reductions in Th1 cytokines in serum and in supernatant of αCD3/αCD28 stimulated splenocytes, but did observe an overall decrease in CD4 and CD8 Tem and Tcm cell populations in different lymphoid compartments, confirming the inhibitory effect of LMP7/LMP2 inhibition on T cell function. Circulating effector memory T cells were previously found to correlate with carotid intima-media thickness independent of classical CVD risk factors in human, and were increased in patients with stable angina and acute myocardial infarction compared to controls *(42)*, indicating that is likely that ONX-0914 mediated reduction in circulating effector T cells helped reducing atherosclerosis.

Besides direct inhibition of T cells, ONX-0914 treatment also reduced the cDC content in the spleen and reduced cDC content and activation in mesenteric lymph nodes and cervical lymph nodes which drain the peritoneal cavity and intestines (atherosclerosis unrelated) *(42)* and drain the aorta and supra-aortic arteries (atherosclerosis related) *(43)* respectively, providing an indirect way to inhibit T cell activation. This is in line with the finding that immunoproteasome deficient DCs are inferior at priming T cell responses compared to wild type DCs *(8)*. Enhanced activation of dendritic cells previously proofed to be atherogenic and led to increased CD4 and CD8 T cell activation *(18)*, therefore the reduced DC numbers and activation are likely to have contributed to the smaller lesions observed after ONX-0914

treatment. We found that overnight incubation of bone marrow derived DCs with ONX-0914 reduced DC viability and led to a trend towards reduced BMDC activation, indicating that reduced DC levels and activation in vivo were most likely a direct result of ONX-0914 treatment.

The exact molecular mechanisms by which immune cells are inhibited by proteasomal inhibition are largely unknown, however have been proposed to involve the unfolded protein response (UPR) pathway *(17)* which is activated in response to accumulation of unfolded and misfolded proteins in the ER, inducing ER stress *(44)*. Presumably the primary task of the UPR is regulating the expression of numerous genes aimed to regain homeostasis in the ER or induce apoptosis if ER stress remains unresolved *(44)*. An important secondary task of the UPR in immune cells may also be the inhibition of activation of immune cells which cannot accurately process incoming signals due to accumulation of ubiquitinated proteins, preventing uncontrolled tissue damage. In light of such a protective mechanism, it is fitting that Nrf2 (*n*uclear factor erythroid-derived 2-*r*elated *f*actor *2*), which under normal circumstances is very rapidly degraded by the proteasome but stabilized upon proteasome inhibition *(45)*, upregulates gene expression of proteasomal subunits to regain healthy protein homeostasis *(46)*, and is known to suppress the immune system *(47–53)*.

Next to developing atherosclerosis, LDLr$^{-/-}$ mice on WTD develop obesity, obesity associated metabolic syndrome, and insulin resistance *(54, 55)*. In addition to reducing atherosclerosis, ONX-0914 consistently reduced body weight in WTD fed LDLr$^{-/-}$ mice and APOE*3-Leiden.CETP mice, markedly reducing WAT mass while preserving lean mass, and inducing improved metabolic parameters such as lowered insulin levels, lowered fasting blood glucose, lowered TG levels, and in APOE*3-Leiden.CETP mice, also lowered cholesterol levels. To ensure that weight loss in ONX-0914 treated mice was not caused by (hepato)toxicity, we assessed the activity of liver enzymes ALAT and ASAT in the blood, and Cyp3A11 expression in the liver. ALAT and ASAT activity, and Cyp3A11 expression were not elevated, indicating no toxic side-effects of ONX-0914 treatment. To our knowledge this is the first study to show that LMP7 inhibition using the LMP7 specific inhibitor ONX -0914 has metabolic effects in addition to its immunomodulatory effects, which is likely the consequence of the use of a WTD in our study, leading to the substantial accumulation of WAT mass and development of metabolic syndrome, versus the use of lean mice on chow diet in previous studies applying ONX-0914 *(9–17)*. In addition, disease-related weight loss due to induction of colitis *(12)*, EAE *(11)* or arthritis *(9)*, may have masked the metabolic effects of ONX-0914 in these disease models.

We found that ONX-0914 reduced WAT mass, not through increased energy expenditure or reduced overall food intake, but through reduced intestinal uptake of lipids, similar to

findings in LMP7 deficient animals *(28)*. In the study of Kimura *et al.* reduced intestinal lipid uptake in LMP7 deficient mice was attributed to lowered expression of pancreatic lipases Pnlip and Pnlrp2 *(28)*, however pharmacological inhibition of LMP7/LMP2 did not lead to lowered expression of Pnlip and Pnlrp2 in our study. Indeed, intestinal uptake of lipids is minimally affected in Pnlip *(56)* or Pnlrp2 deficient mice *(57)*. Instead, we found that ONX-0914 initially induced neutrophil infiltration and subsequently macrophage accumulation in gWAT. We found that ONX-0914 mediated inhibition of lipid absorption was peritoneal macrophage dependent, as intestinal lipid uptake was not affected in animals treated with ONX-0914 intraperitoneally administered clodronate liposomes. From a literature search, we found GDF15 as a macrophage derived factor which is known to (temporarily) reduce food intake and induce weight loss *(30)*. ONX-0914 treatment upregulated GDF15 levels in the blood, but as GDF15$^{-/-}$ mice also lost weight when treated with ONX-0914, GDF15 did not appear to mediate ONX-0914 induced weight loss in ONX-0914 treated mice. More research is needed to assess the exact mechanism in which ONX-0914 induces loss of WAT mass. Other macrophage derived products, including IL-1β *(58)* and IL-15 *(59)*, were previously found to reduce intestinal lipid uptake.

Moreover, the innate inflammatory response in gWAT is most likely responsible for the enhanced TNF-α, IL-1β and IL-6 levels in blood upon ONX-0914 treatment. Interestingly, patients with a loss of function mutation in PSMB8, coding for LMP7, present themselves with similar symptoms including elevated serum IL-6 levels, white adipose tissue inflammation, and lipodystrophy *(23)*. These effects of dysfunctional LMP7 were attributed to dependency of preadipocytes on LMP7 for differentiation towards mature adipocytes. Moreover, Psmb8$^{-/-}$ mice have lowered preadipocytes and adipose stem cell numbers *(23, 24)*. In line with these findings, we found that ONX-0914 treatment severely reduced preadipocyte and adipose stem cell numbers in gWAT. Since we found that isolated mature adipocytes express immunoproteasomal catalytic subunits and upregulate CCL2 in response to overnight exposure to ONX-0914, this provides a direct mechanism for ONX-0914 induced innate immune infiltrate in gWAT. Similar to LMP7/LMP2 inhibition in immune cells, ONX-0914 is likely to have affected proteostasis in adipocytes. UPR activation in multiple cell types of non-hematopoietic origin was found to induce CCL2 expression *(60–62)*, which may explain CCL2 expression in adipocytes and gWAT in response to LMP7 inhibition. The presence of macrophages in the buoyant adipocyte fraction after gWAT digestion in the ONX-0914 treated group, indicates either tight interaction between macrophages and adipocytes, or enhanced uptake of lipids by gWAT macrophages through uptake of free fatty acids or efferocytosis of apoptotic adipocytes which could have contributed to the lowered TG levels in blood plasma of ONX-0914 treated mice.

In contrast to our studies with ONX-0914, no immune infiltrate was observed in gWAT from LMP7 deficient mice *(28)*, and no effect of LMP7 deficiency was found on body weight and atherosclerosis in LDLr$^{-/-}$ animals on WTD for 6 weeks and 24 weeks *(63)*. It was argued that in the LMP7/LDLr dKO mice the lowered expression of the immunoproteasome constituents LMP2 and MECL-I was effectively compensated by enhanced constitutive proteasome expression leading to similar overall caspase-like, trypsin like, and chymotrypsin-like proteolytic activities in BMDM cell lysates from LMP7 deficient and sufficient animals *(63)*. In line with the discrepancy between LMP7 deficiency *(63)* and ONX-0914 treatment in our study with respect to atherosclerosis, similar data were observed in experimental autoimmune EAE, where LMP7 deficiency did not affect disease whereas treatment with ONX-0914 did reduce disease severity *(11)*. These data seem to correlate with the recent discovery that ONX-0914 acts through inhibition of not only LMP7, but also through inhibition of LMP2 inhibition, and that inhibition of both subunits was needed to ameliorate experimental colitis and EAE *(10)*.

Treatment of mice with ONX-0914 reduces atherosclerosis and considerably reduces WAT mass in obese mice fed a WTD, concomitantly improving parameters of metabolic syndrome. Because atherosclerosis is still the primary cause of death, and the obesity epidemic is feeding metabolic syndrome related diseases worldwide, immunoproteasomal inhibition could be a valuable therapeutic tool for the western world to combat both. Identification of the mechanisms behind ONX-0914 reduced weight loss could allow more specific treatment options.

Materials and Methods

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Mice were housed in individual ventilated cages with aspen bedding, in groups of 2–4 mice per cage except for the duration of the metabolic cage measurements, during which mice were single housed. Mice were fed a regular chow diet prior to initiation of the *in vivo* experiments after which LDLr$^{-/-}$ (Jackson Laboratory; original purchase, further bred in house), and GDF15$^{-/-}$ (a generous gift from dr. ing. S. de Jager, University Medical Center Utrecht) were fed a WTD containing 0.25% cholesterol 15% cocoa butter (Special Diet Services, Witham, Essex, UK), and APOE*3-Leiden.CETP mice *(64)* were fed a WTD containing 0.1% cholesterol, 15% cocoa butter, and 1% corn oil (HopeFarms, Woerden, The Netherlands) for indicated durations. In the week prior to initiation of treatment mice were randomized based on age and weight. APOE*3-Leiden.CETP mice were additionally randomized based on lean and fat mass, and TC

and TG levels. Mice were intraperitoneally treated with ONX-0914 *(65)* (synthesized by the Leiden Institute of Chemistry) at a concentration of 10 mg/kg, 3 times weekly for the indicated durations. ONX-0914 was solubilized in DMSO after which it was diluted in PBS (37°C) to prevent precipitation of ONX-0194 (4% DMSO). 4% DMSO in PBS served as control treatment. Macrophages were depleted in the indicated experiment with 110 mg/kg clodronate liposomes or treated with an equivalent amount of empty liposomes (both purchased at clodronateliposomes.org, Vrije University, Netherlands). At the end-point of the studies using $LDLr^{-/-}$ and $GDF15^{-/-}$ mice, mice were anesthetized by subcutaneous injection with a mix of ketamine (100 mg/mL), sedazine (25 mg/mL) and atropine (0.5 mg/mL) and retro-orbitally exsanguinated, and perfused with PBS. Directly after the final blood withdrawal after the kinetic study with radiolabeled VLDL-like particles in APOE*3-Leiden.CETP mice, mice were sacrificed by cervical dislocation and perfused with PBS to remove radiolabels that had not been taken up by organs.

Indirect calorimetry

After 3 days of acclimatization, O_2 consumption, CO_2 production, and food intake were measured for 6 consecutive days in fully automatic metabolic cages (LabMaster System, TSE Systems, Bad Homburg, Germany). Total EE was estimated from the VO_2 and resting energy requirement. Carbohydrate oxidation was calculated using the formula $((4.585*VCO_2)-(3.226*VO_2))*4$, in which the 4 represents the conversion from mass per time unit to kcal per time unit *(66)*. Similarly, fat oxidation was calculated using the formula $((1.695*VO_2)-(1.701*VCO_2))*9$. Physical activity was monitored using infrared sensor frames. Metabolic cage data from the first 5 h directly after injection at 1 PM with ONX-0914 or vehicle ('Day of treatment', light phase) were analyzed and compared to the same 5-h period 24 h later ('Day after treatment', light phase). For calculations of averages during the dark phase (night on 'Day of/after treatment'), 12 h periods of time were used for analysis.

Preparation of VLDL-like TG-rich emulsion particles and clearance assay

VLDL-like TG-rich emulsion particles (80 nm) containing radiolabeled glycerol tri[^{3}H]oleate (TO) and [^{14}C]cholesteryl oleate (CO) were synthesized like previously *(67)*. In brief, emulsion particles were obtained by sonicating a mixture of TO (70 mg), egg yolk phosphatidylcholine (22.7 mg), CO (3.0 mg), lysophosphatidylcholine (2.3 mg), and cholesterol (2.0 mg), containing [^{3}H]TO (100 µCi) and [^{14}C]CO (10 µCi) tracers, at 54°C using a Soniprep 150 (MSE Scientific Instruments, UK) set at 10 µm output. VLDL-like particles were obtained through density gradient ultracentrifugation using a Beckman SW 40 Ti rotor. First, chylomicron-like particles were discarded by removing the top fraction after centrifugation (20,000 r.p.m., 27 min, 20°C), thereafter the remainder was centrifuged (40,000 r.p.m., 27 min, 20 °C) and VLDL-like particles were isolated and stored at 4 °C under argon and used within 5 days.

To study the *in vivo* clearance of cholesteryl oleate and glycerol trioleate, APOE*3-Leiden.CETP mice treated with ONX-0914 or vehicle for 2.5 weeks were fasted for 4 h directly after a final ONX-0914 injection after which a baseline blood sample was drawn, and 200 µL VLDL-like emulsion particles (1.0 mg TG per mouse) were administered iv. Blood samples were obtained 2, 5, 10, and 15 minutes after administration of the emulsion particles by tail bleeding into paraoxon (Sigma)-coated heparinized capillary tubes (Hawksley, Sussex, England). ^{3}H- and ^{14}C -activity was determined in plasma. Total plasma volume was estimated by multiplying body weight (g) with 0.04706 *(26, 68)*. Half-life times were derived from the half-life constant which was calculated using the log values for ^{3}H and ^{14}C measurements for t = 2, 5, and 10 minutes.

Oral TG loading test, plasma lipoprotein analysis

Mice were fasted overnight, after which a baseline blood sample was drawn. Mice received an oral bolus of 200µl olive oil (Bertolli), and blood was drawn 30, 60, 120, 180, and 240 minutes after that. Blood was collected in Microvette CB300 Lithium-Heparin coated capillary tubes (Sarstedt).

TG and TC levels in blood plasma from the oral TG loading test and other experiments were assessed with commercially available kits (Roche). HDL-cholesterol (HDL-C) levels were determined in supernatant from blood plasma of APOE*3-Leiden mice, upon precipitation of ApoB-containing lipoproteins by addition of 20% polyethylene glycol in 200 mM glycine buffer (pH 10) and measurement of TC in the supernatant. The activity of Aspartate Aminotransferase and Alanine Aminotransferase in blood plasma were assessed with activity assay kits (Sigma, MAK055 and MAK052, respectively) according to manufacturer's protocols.

Thymidine incorporation assay

Splenocytes (200,000 cells/well) were stimulated with anti-CD3e (1 µg/mL) and anti-CD28 (0.5 µg/mL) (both from Thermo Fischer) for 72 h and incubated with 0.5 µCi/well ^{3}H-thymidine (Perkin Elmer) for the last 16 h, or remained unstimulated. Cells were thoroughly washed with PBS and thereafter lysed with natriumhydroxide and taken up in Emulsifier-Safe™ (Perkin Elmer). (^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as the mean disintegrations per minute (dpm). The stimulation index (s.i.) was defined by dividing the dpm under activated conditions by the dpm under non-activated conditions per mouse.

gWAT digestion, SVF and primary adipocyte isolation, and primary adipocyte culture

After perfusion with PBS, gonadal white adipose tissue was excised, and minced in DPBS (Lonza) supplemented with 0.5% BSA (Sigma). Thereafter minced adipose tissue was

incubated in DPBS with 10 mM $CaCl_2$, and 4 mg/mL collagenase type II derived from Clostridium Histolyticum (Sigma) at 37°C, while gently agitated in a rotational shaker. Adipose tissue homogenates were passed over a 300-µm mesh (Elko, Fisher Scientific) and thereafter centrifuged at 150 x g for 10 min at RT. Infranatant was removed, saved for assessment of stromal vascular fraction (SVF) cell populations with flow cytometry or discarded, and remaining floating adipocytes were washed twice with DPBS (Gibco) supplemented with 0.5% BSA. Adipocytes were either directly taken up in GTC and stored at -80°C for gene expression analysis, or cultured overnight in DMEM (Lonza) supplemented with 10% FCS (GE Healthcare Life Sciences), 100 U/ml penicillin/streptomycin (GE Healthcare Life Sciences) and 2mM L-glutamine (Thermo fisher Scientific).

Histology

Hearts were transversally cut in half and incubated in OCT medium for 30 minutes. After 30 minutes hearts were fast frozen on dry ice, and stored at -80oC before cryosections (10 µm) of the aortic root were collected on Superfrost Plus™ Adhesion Microscope Slides (ThermoFisher) and analyzed at 70 µm intervals (7 slides/mice). Neutral fats were stained with Oil Red O to determine the average lesion size of five subsequent sections of the aortic root containing 3 valvular leaflets, as a measure of atherosclerotic lesion size. Lesion collagen content was determined with Masson trichrome staining (Sigma-Aldrich) for 3 subsequent sections in 3 sections containing the aortic root valvular leaflets. Corresponding sections were immunohistochemically stained for macrophages with MOMA-2 antibody (Sanbio, 1:1000 dilution). Slides were blocked with 5% milk powder before primary antibody was added for 2h at RT, after which primary antibody was incubated overnight at 4oC. Then slides were incubated with Goat anti-rat Ig alkaline phosphatase (A8438, Sigma-Aldrich) for 1h at RT, after which BCIP/NBT Substrate (DAKO) was used to stain macrophages blue. Blinded histological analysis was performed using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, Cambridge, UK).

Flow Cytometry

Extracellular staining (ECS) of single cell suspensions was performed in PBS with 2% FCS and αCD16/32 antibody (93, Biolegend) and eBioscience™ Fixable Viability Dye eFluor™ 780 (ThermoFisher) to discriminate between living and dead cells for 30 minutes at 4°C. For intracellular transcription factor staining after ECS, cells were fixed and permeabilized with the FoxP3 transcription factor buffer set (Thermofisher/eBioscience) according to manufacturer's instructions, and incubated with flow cytometry antibodies for 45 minutes at 4°C. Spleen and lymph nodes were mashed over a 70 µm cell strainer (Greiner) to obtain single cell suspensions and red blood cells were lysed with ACK lysis buffer if necessary.

The following antibodies were purchased at BD biosciences: The following antibodies were purchases at BD Biosciences: CD4-PerCP (RM4-5), CD105-PE-CF594 (MJ7/18), CD31-BV421 (Mec13,3), CD45-FITC (30-F11), Ly6G-FITC (1A8); Biolegend: CD11b-eFluor 450 (M1/70), CD11c-FITC (N418), CD4-BV421 (IM7), CD44-FITC (IM7), CD45-Alexa Fluor 700 (30-F11), CD8-BV510 (53-6.7), CD8-APC (53-6.7), CD206-PE/Cy7 (C068C2), CD34-PE/Cy5 (MEC14.7); eBioscience/Thermofisher scientific: CD115-PE (AFS98), CD11c-PE (N418), CD19-Pacific Blue (eBio1D3), CD19-PE/Cy7 (eBio1D3), CD25-APC (PC61.5), CD25-FITC (PC61.5), CD3e-PE/Cy5 (145-2C11), CD34-PB (RAM34), CD44-APC (IM7), CD62L-APC (MEL-14), CD62L-PB (MEL-14), CD8-PE (16-10A1), CD86-PE (GL1), F4/80-APC (BM8), Foxp3-Pacific Blue (FJK-16s), FoxP3-PE (NRRF-30), GATA-3-PE (TWAJ), Ly6C-PE (HK1.4), Ly6C-PerCP/Cy5.5 (HK1.4), NK1.1-APC (PK136), T-bet-PE/Cy7 (eBio4B10), CD11b-eVolve 605 (M1/70), CD161-PE (HP-3G10), CD86-PE/Cy5 (GL1), CD8a-PE/Texas Red (5H10), SCA-1-APC (CT-6A/6E), MHC-II-PerCP/eFluor 710 (M5/114.15.2), MHC-II-eVolve 655 (M5/114.15.2), SCA-1-PE (D7). Compensation measurements were performed using UltraComp eBeads (ThermoFisher) and ArC Amine-Reactive Compensation Beads (ThermoFisher). Cells were measured with a FACSCanto II (BD Biosciences) and a Cytoflex S flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star, inc.).

Real-time quantitative PCR

RNA was extracted from adipocytes, and mechanically disrupted gWAT, interscapular brown adipose tissue (iBAT), liver, and spleen, using Trizol reagent following manufacturer's instructions (Invitrogen). Thereafter cDNA was generated using RevertAid M-MuLV reverse transcriptase according to manufacturer’s protocol (Thermo Scientific). Quantitative gene expression was measured using Power SYBR Green Master Mix (Thermo Fisher Scientific) on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to housekeeping genes ACTB and RPLP0. A list of all primers can be found in the supplementary table (Table S1).

Multiplex ELISA

Inflammatory cytokines in blood plasma, and culture supernatant of splenocytes stimulated overnight with anti-CD3e (1 µg/mL) and anti-CD28 (0.5 µg/mL) were assessed with a T cell differentiation 17-plex Luminex bead-based assay (eBioscience, EPX170-26087-901) according to manufacturer’s instructions. Metabolic syndrome related biomarkers were assessed in blood plasma with a diabetes 8-plex Luminex bead-based assay (BioRad, 171F7001M) following manufacturer’s protocol. Luminex assays were measured on a MAGPIX System (Luminex).

Statistical Analysis

Statistical analysis was performed using Graphpad Prism (version 7.01). Outliers were removed from analysis by the ROUT method (Q = 1%). For statistical comparison of 2 groups an unpaired two-tailed T-test was used. For comparison of more than 2 groups with one variable a one-way ANOVA was used. For statistical comparison of 2 treatment groups with multiple timepoints a two-way repeated measures ANOVA was used. For the comparison of multiple groups defined by two variables, a two-way ANOVA was used. After multiple comparisons, the Holm-Sidak posttest was used to calculate multiplicity adjusted p-values of individual comparisons. Differences between groups were considered statistically significant at $p < 0.05$.

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Author contributions

JK, devised the main outlines of the project and supervised the project. **GdB** synthesized ONX-0914 under supervision of **BF** and **HO,** and together advised how to handle ONX-0914. The metabolic study in ApoE*3-Leiden mice was designed, executed and analyzed by **AvD** and **PCNR**. **FHS** designed, executed, analyzed the remainder of the experiments, and wrote the manuscript. **ACF**, **IB**, **MK**, **HD**, **JA**, **PJvS** and **GHMvP** helped carrying out most of the experiments and together with **BF**, **HO**, **AvD**, **PCNR**, and **JK**, gave valuable feedback to improve the studies. **BF**, **HO**, **AvD**, **PCNR**, and **JK** commented on the manuscript.

Competing interests

None.

Data and materials availability

All data associated with this study are available in the main text or the supplementary materials**.**

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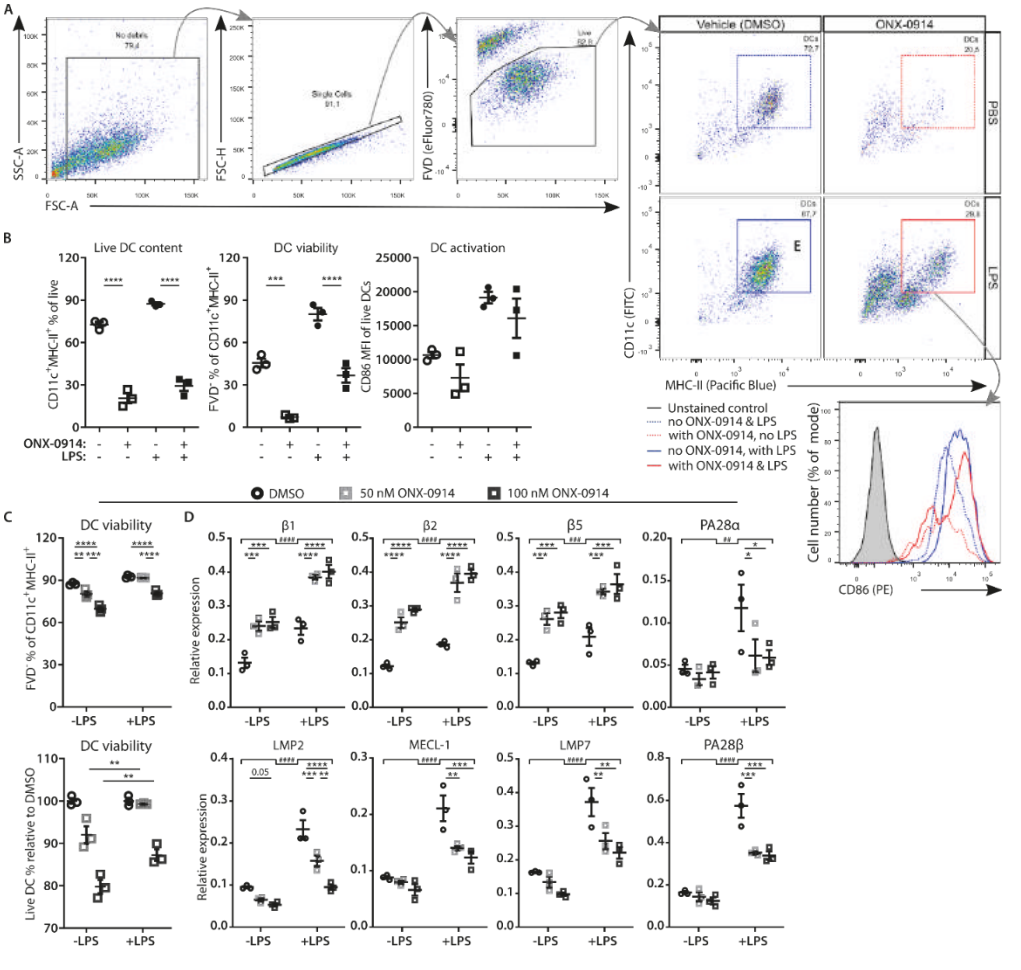
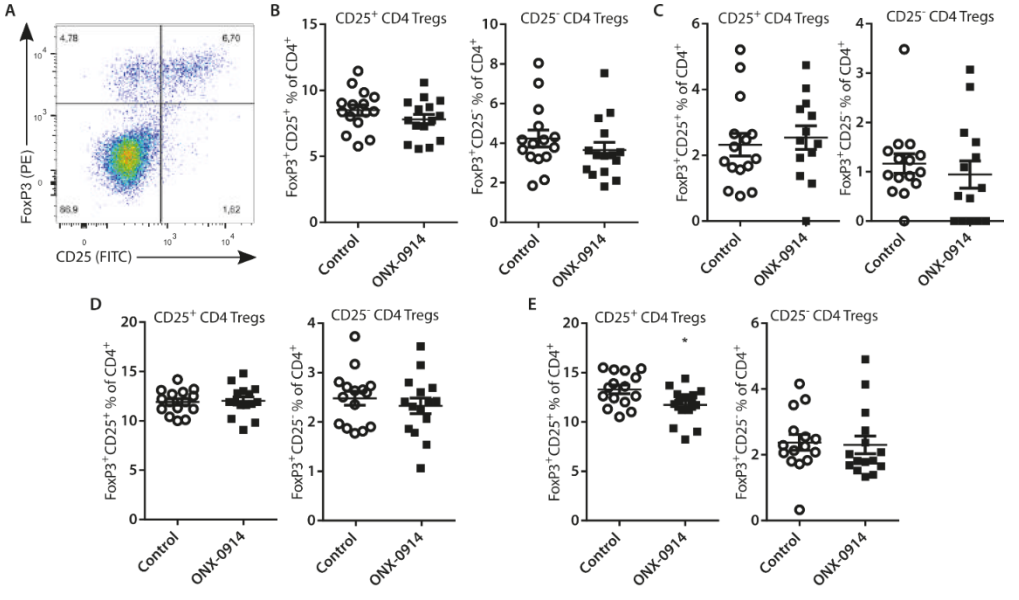
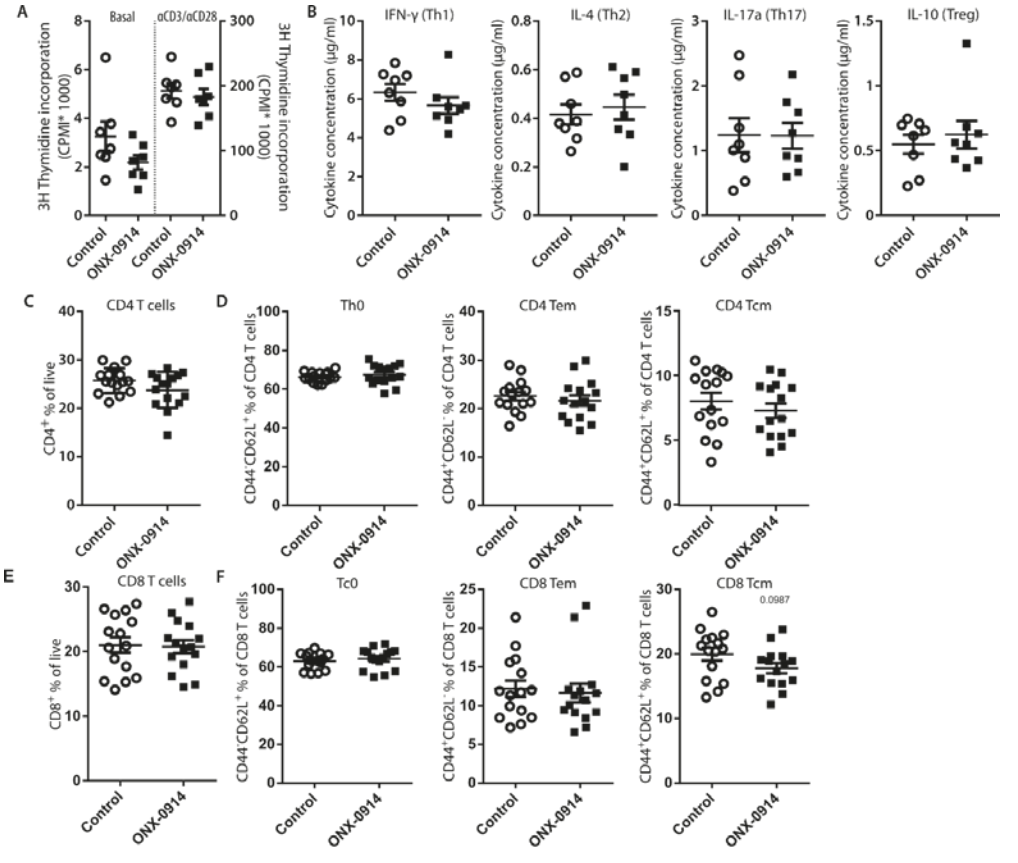
Supplementary figures



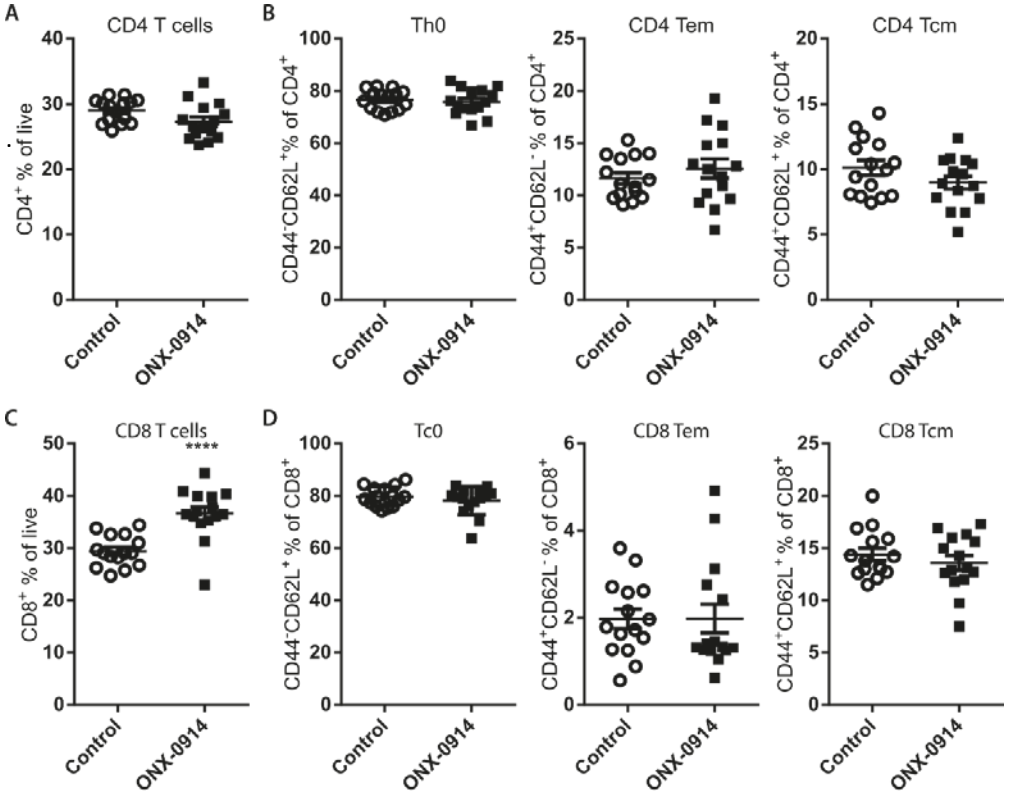
Supplementary Fig. S1 ***LMP7 inhibition directly inhibits DC activation and causes upregulation of constitutive proteasomal active subunits. A****) Bone marrow derived DCs were incubated with ONX-0914 (0-200 nM) overnight in absence or presence of LPS (100 ng/ml) and assessed by flow cytometry.* ***B****) Cell culture viability was assessed by gating as shown in '****A****'. For quantification of DC viability single cells were gated like in '****A****', but directly thereafter CD11c⁺MHC-II⁺ cells were gated after which live DCs were gated. CD86 median fluorescent intensity of the live MHC-II⁺CD11c⁺ population was assessed as a measure for DC activation.* ***C****) Bone marrow derived DCs were incubated with ONX-0914 (0-100 nM) overnight in absence or presence of LPS (100 ng/ml) after which DC viability was determined by flow cytometry like explained in '****B****', and* ***D****) expression of catalytic constitutive and immuno- proteasomal subunits, and immunoproteasomal activators were determined by qPCR. Expressed as mean ± SEM, two-way ANOVA (#) with Holm-Sidak posttest, *p < 0.05, **p < 0.01, *** p < 0.001 **** p < 0.0001.*



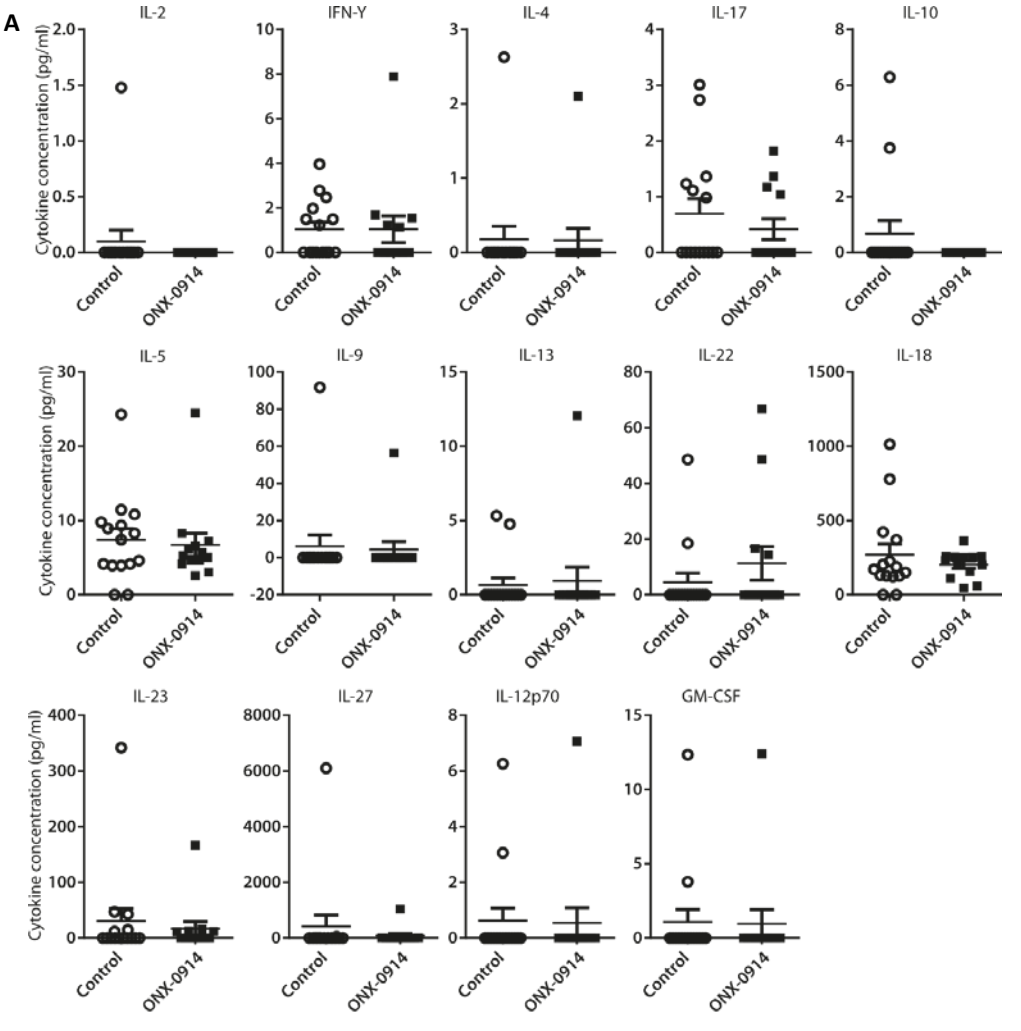
Supplementary Fig. S2 ***Tregs are not increased by ONX-0914 treatment in early atherosclerosis. A****) After sacrifice CD4 T cell FoxP3⁺CD25⁺ and FoxP3⁺CD25⁻populations were determined with flow cytometry, gating shown for the CD4 T cell population in the spleen. Quantification of CD4 Treg populations in* ***B****) spleen,* ***C****) blood,* ***D****) cervical lymph nodes, and* ***E****) mesenteric lymph nodes. Expressed as mean ± SEM, unpaired two tailed t-test, *p < 0.05.*



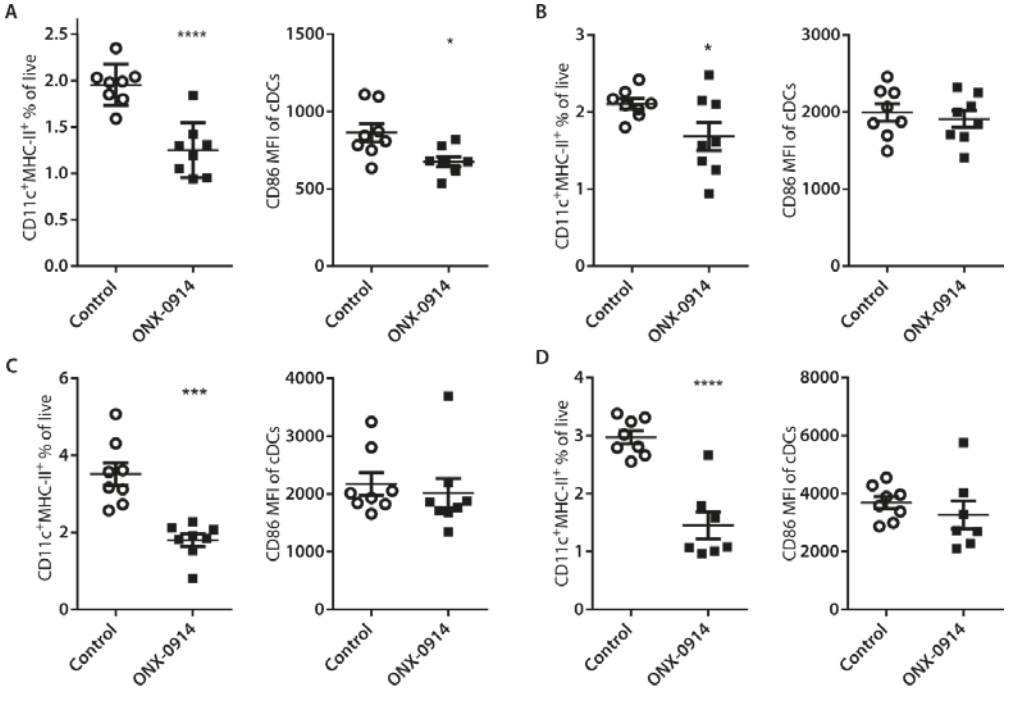
Supplementary Fig. S3 ***LMP7 inhibition does not impact splenic T cell differentiation in early atherosclerosis.*** *(**A**) T cell proliferation in unstimulated or αCD3/αCD28 splenocyte cultures as measured by thymidine incorporation. **B**) Cytokine levels in supernatant of overnight αCD3/αCD28 stimulated splenocyte cultures. C) Overall CD4 T cell levels, **D**) CD4 Tem, Tcm and Th0 levels, and (**C**) overall CD8 T cell levels, and **D**) CD8 Tem, Tcm and Tc0 levels, as determined by flow cytometry. Expressed as mean ± SEM, unpaired two tailed t-test, ***p < 0.001.*



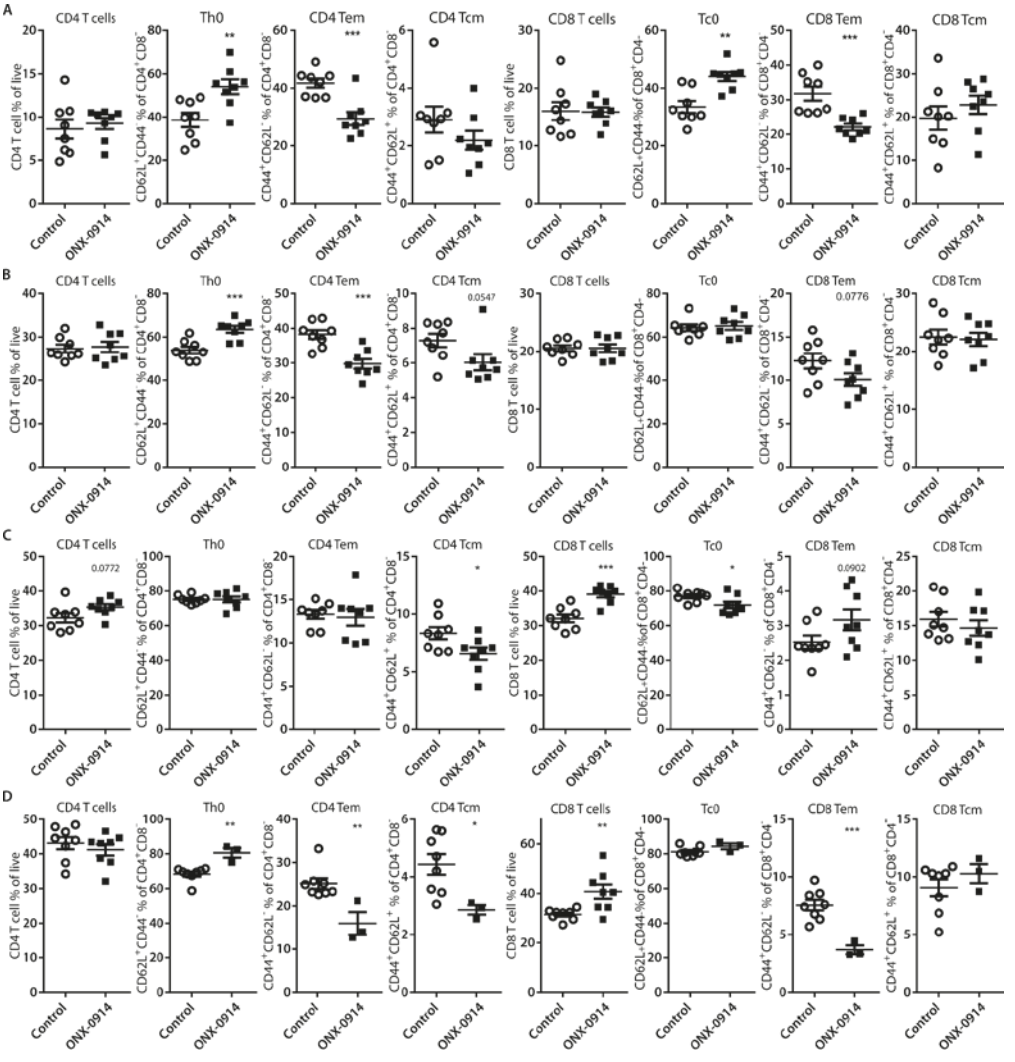
Supplementary Fig. S4 ***Memory and naïve T cell effector populations in cervical lymph nodes.*** *Quantification of flow cytometric analysis of* ***A****) CD4 T cell content and* ***B****) memory and naïve CD4 T cell populations, (****C****) CD8 T cells and* ***D****) memory and naïve CD8 T cell populations in CLN. Expressed as mean ± SEM, unpaired two tailed t-test, ****p < 0.0001.*



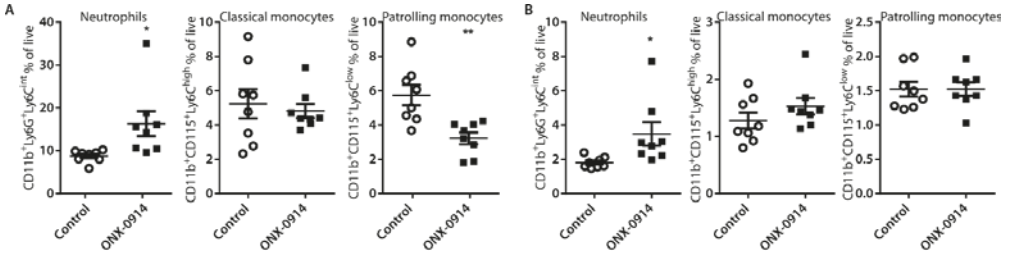
Supplementary Fig. S5 ***Cytokine levels not significantly altered by ONX-0914 treatment.*** ***A****) Quantification of remaining cytokine levels in blood plasma, measured with multiplex ELISA.*



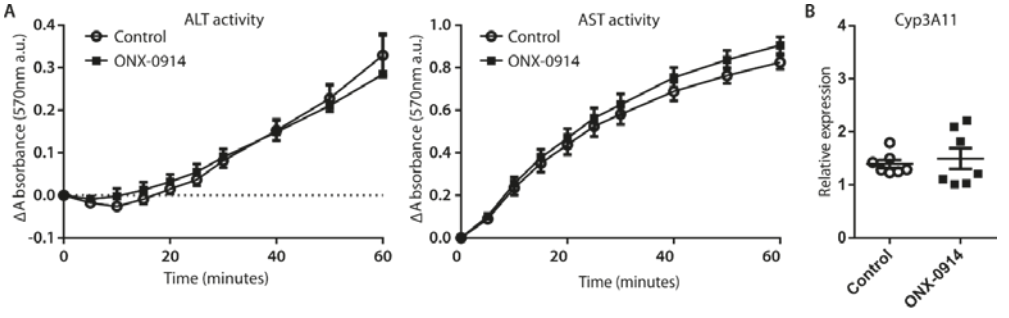
Supplementary Fig. S6 ***Conventional DC levels are reduced by ONX-0914 treatment.*** *Conventional DCs were gated by selection of CD11c⁺MHC-II⁺ cells whilst activation of cDCs was assessed through expression of CD86, as determined by flow cytometric analysis of cells from* ***A****) spleen,* ***B****) cervical lymph nodes,* ***C****) mediastinal lymph nodes, and* ***D****) mesenteric lymph nodes. Expressed as mean ± SEM, unpaired two tailed t-test, *p < 0.05, ***p < 0.001, **** p < 0.0001.*



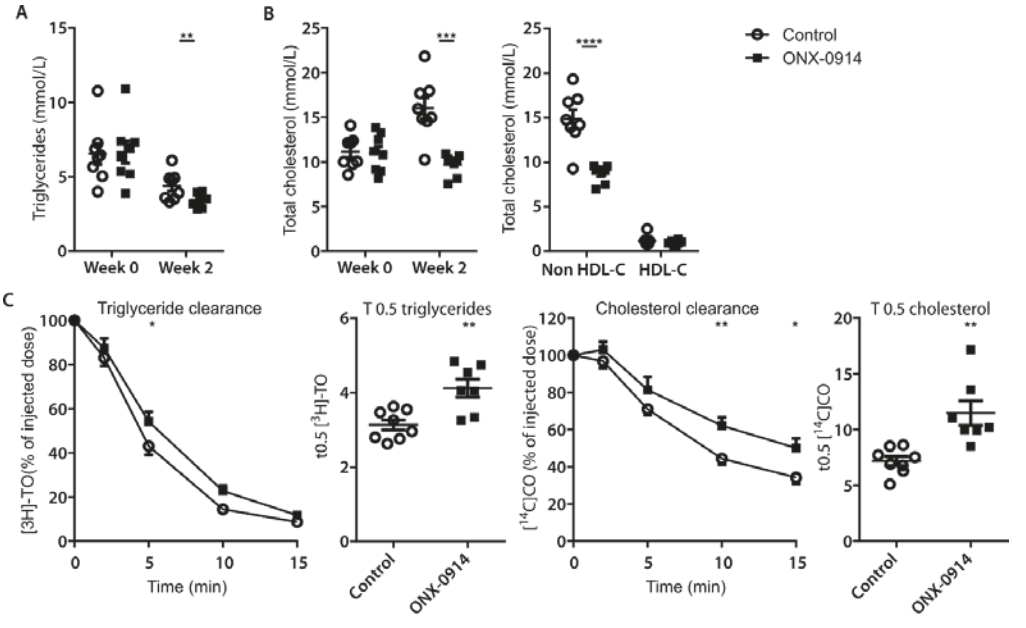
Supplementary Fig. S7 ***ONX-0914 treatment reduces memory T cell levels in various immune compartments.*** *CD4 and CD8 T cells were gated based on expression of CD4 and CD8 respectively. Thereafter, naïve and memory populations were gated based on expression of CD62L and CD44 (Naïve T cells, $CD62L^{+}CD44^{-}$; Effector Memory T cells, $CD44^{+}CD62L^{-}$; Central Memory T cells ($CD44^{+}CD62L^{+}$). Quantification of CD4 and CD8 T cell populations in* ***A****) blood,* ***B****) spleen,* ***C****) cervical lymph nodes, and* ***D****) mesenteric lymph nodes. Expressed as mean ± SEM, unpaired two tailed t-test, *p < 0.05, **p < 0.01, ***p < 0.001.*



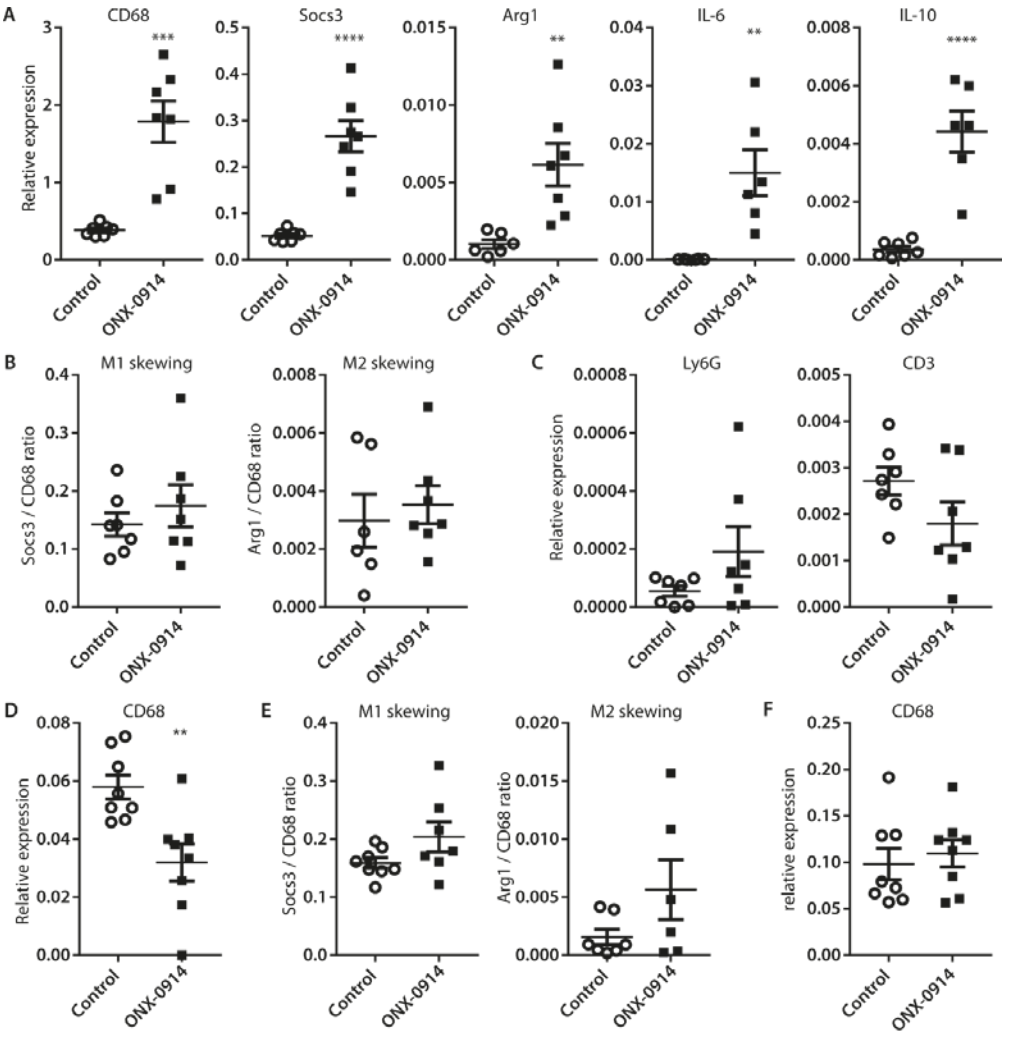
Supplementary Fig. S8 ***ONX-0914 increases neutrophil levels in blood and spleen.*** *Quantification of flow cytometric analysis of neutrophils, classical monocytes and patrolling monocytes in* ***A****) blood and* ***B****) spleen. Expressed as mean ± SEM, unpaired two tailed t-test, *p < 0.05, **p < 0.01.*



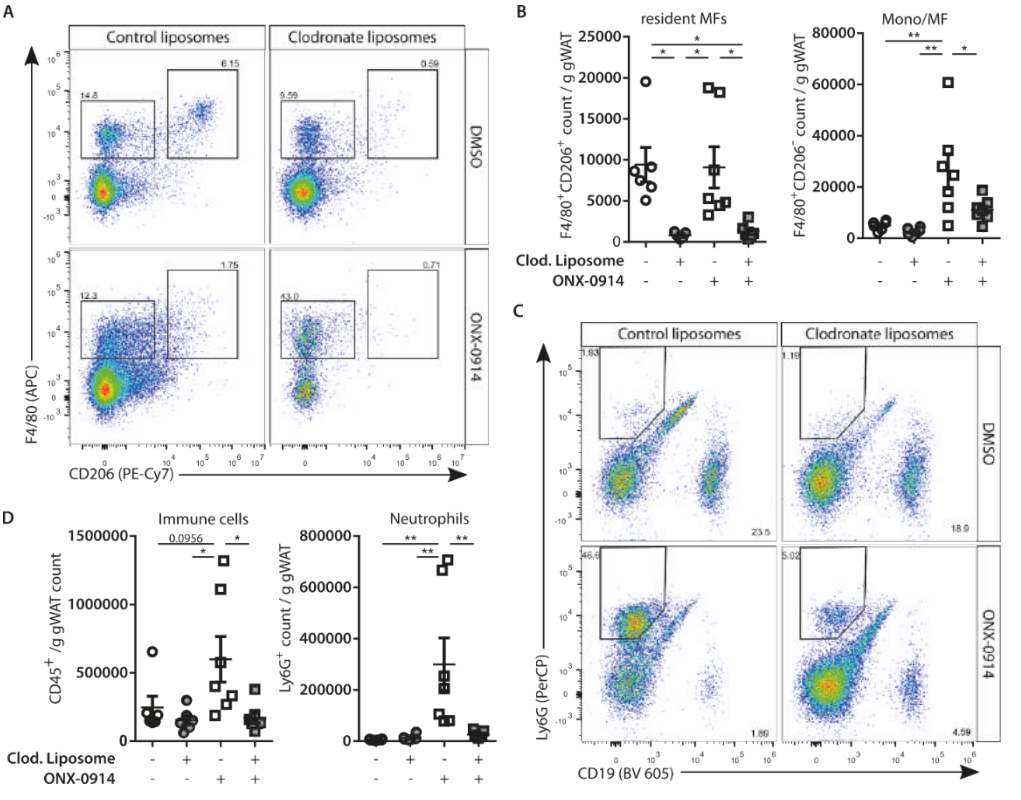
Supplementary Fig. S9 ***Treatment with ONX-0914 does not lead to hepatotoxicity. A****) ALT and AST activity were assessed on blood plasma derived from blood collected at sacrifice after 7 weeks of control or ONX-0914 treatment.* ***B****) Hepatic Cyp3A11 expression. Expressed as mean ± SEM,* ***A****) 2-way repeated measures ANOVA with Holm-Sidak posttest,* ***B****) two tailed t-test, no significant differences.*



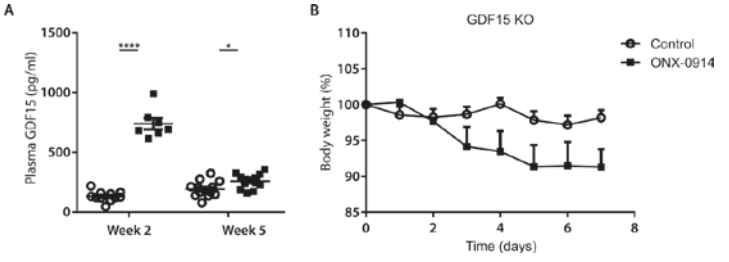
Supplementary Fig. S10 ***TG and TC clearance from the blood is not enhanced by ONX-0914 treatment.*** *After 2.5 weeks of treatment, APOE*3-Leiden.CETP mice received a final control or ONX-0914 injection and were fasted for 4h.* ***A****) Plasma triglyceride levels and* ***B****) cholesterol levels in plasma after 4h starvation.* ***C****) To determine clearance of TG and TC from the blood, VLDL like particles containing radiolabeled [^{14}C]cholesteryl oleate and glycerol tri[^{3}H]oleate were iv administered. Radioactivity in plasma was assessed in blood drawn at indicated time points after VLDL like particle administration, and half life time was approximated. Expressed as mean ± SEM* ***A,B)*** *(left panel),* ***C*** *(1st and 3rd panel from the right), two tailed T-test,* ***B)*** *(right panel) One-way ANOVA Holm-Sidak posttest,* ***C)*** *(1st and 3rd panel from the left) two way ANOVA with Holm-Sidak posttest, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001*



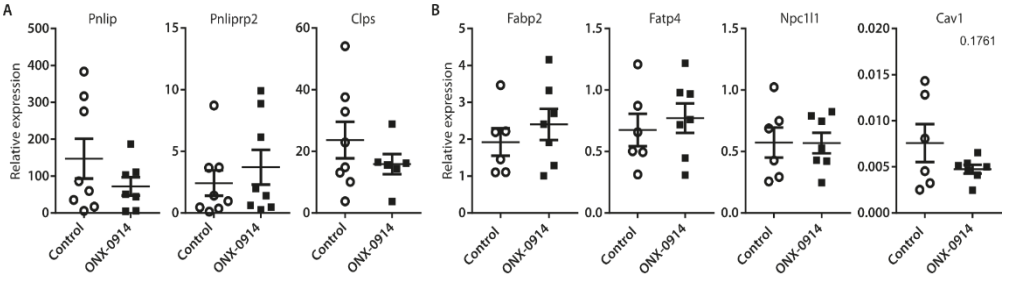
Supplementary Fig. S11 ***Macrophage infiltrate in gWAT is WAT specific and unskewed. A****) Gene expression of macrophage related genes in gWAT.* ***B****) Ratios of gene expression of M1 marker suppressor of cytokine signaling 3 (Socs3) and M2 marker arginase-1 (Arg-1) with macrophage marker CD68 in gWAT and* ***E****) interscapular BAT (iBAT). (****C****) Gene expression of Ly6G (neutrophils), and CD3 (T cells) in gWAT. CD68 gene expression in* ***D****) iBAT and* ***F****) liver. Expressed as mean ± SEM, two tailed t-test, **p < 0.01, ***p < 0.001, **** p < 0.0001*



Supplementary Fig. S12 ***Depletion of peritoneal macrophages prevents macrophage and neutrophil infiltrate in gWAT.*** *gWAT from mice on WTD for 24 weeks and treated only in the final week was excised, digested and the SVF was isolated for flow cytometric analysis.* ***A****) Representative flow cytometry plot of live single CD45⁺ cells gated for F480⁺CD206⁺ resident macrophages and F480⁺CD206⁻ infiltrating macrophages* ***B****) Quantification of resident and infiltrating macrophage levels.* ***C****) Flow cytometric gating of Ly6G⁺ neutrophils in SVF, and (****D****) quantification of overall CD45⁺ immune cells and neutrophils in SVF of gWAT. Expressed as mean ± SEM, One-way ANOVA with Holm-Sidak posttest, *p < 0.05, **p < 0.01.*



Supplementary fig. S14 ***GDF15 is induced by ONX-0914 but is not the cause of ONX-0914 induced weight loss. A****) GDF15 levels in blood plasma from two time points of the initial atherosclerosis study as assessed by ELISA.* ***B****) GDF15$^{-/-}$ mice (n=3/group) were fed WTD for 4 weeks after which mice were treated with ONX-0914 (10mg/kg, 3 times weekly) or control for a week, during which body weight was monitored. Expressed as mean ± SEM, One-way ANOVA with Holm-Sidak posttest, *p < 0.05, ****p < 0.0001.*



Supplementary Fig. S13 ***ONX-0914 does not affect expression of pancreatic lipases or receptors involved in intestinal lipid uptake. A****) Expression of pancreatic lipase (Pnlip), pancreatic lipase-related protein 2-like (Pnliprp2), and colipase (Clps) in pancreatic tissue isolated from female E3L.CETP mice on WTD for 5 weeks, treated with ONX-0914 for the final 2 weeks (10 mg/kg , 3 times weekly).* ***B****) Gene expression of the lipid transporters fatty acid binding protein-2 (Fabp2), fatty acid transfer protein 4 (Fatp4), caveolin 1 (Cav1), and NPC1-like intracellular cholesterol transporter 1 (Npc1l1), in intestines from female LDLr$^{-/-}$ females on WTD for 6 months, ip treated with ONX-0914 (10 mg/kg, 3 times weekly) in the final week. Expressed as mean ± SEM, unpaired two-tailed t-test, no significant data.*

Table S1**. Primers used for gene expression analysis.** Gene expression was normalized to housekeeping genes Actb and Rplp0.

Gene	Protein	Fw primer	Rev primer
Arg1	Arginase-1	tggcagaggtccagaagaatgg	gtgagcatccacccaaatgacac
Cav1	Caveolin-1	actgagaagcaagtgtatgacgcgc	cagatgccgtcgaaactgtgtgtcc
Ccl2	Chemokine (C-C motif) ligand 2	ctgaagccagctctctcttcctc	ggtgaatgagtagcagcaggtga
CD3e	T-cell surface glycoprotein CD3 epsilon chain	tctgctacacaccagcctcaaa	atgaccatcagcaagcccaga
CD68	CD68	tgcctgacaagggacacttcggg	gcgggtgatgcagaaggcgatg
Clps	Colipase	ttgcacacacaaggccatggagaa	catagttggtgttggtgatggcgc
Cyp3a11	Cytochrome P450 3A11	cccacattcaccagtggaaaactcaag	cttgcctttctttgccttctgcctc
Fabp2	Fatty acid-binding protein	cacgtgtagacaatggaaaggagctga	aatcgcttggcctcaactccttcat
Il10	Interleukin-10	gggtgagaagctgaagaccctc	tggccttgtagacaccttggtc
Il6	Interleukin-6	tgagactggggatgtctgtagctcat	gttgtcaccagcatcagtcccaaga
Ly6G	Lymphocyte antigen 6G	gatggattttgcgttgctctgga	gagtagtggggcagatgggaag
Npc1l1	NPC1-like intracellular cholesterol transporter 1	ctacacggcctggtcttcct	aaggggtactgtgggcaag
Pnlip	Pancreatic triacylglycerol lipase	tgggagcatcaaggatcacagtgga	gagacagtgtgagcaggacgtcttc
Pnliprp2	Pancreatic lipase-related protein 2	atgggagcttcacaaatcacagtgca	acgtttattatgggaagaggcacggg
Pparg	peroxisome proliferator activated receptor gamma	agggcgatcttgacaggaaagacaac	aaaattcggatggccacctctttgc
Psmb10	20S proteasome subunit beta-2i	cgtctgccctttactgcccttgg	tgatcacacaggcatccacattgcc
Psmb5	20S proteasome subunit beta-5	gcctccaaactgctcgctaacatgg	gatcctgttcccctcgctgtctacg
Psmb6	20S proteasome subunit beta-1	gaatcatcattgcaggctgggaccc	tagccatagatgtacgagctcccgg
Psmb7	20S proteasome subunit beta-2	actttcttcgtccattctcagtgccc	aaccacccacagcaccattcacg
Psmb8	20S proteasome subunit beta-5i	ttccaacatgatgctgcagtaccgg	gtggaaaacatctgtcccgagagcc
Psmb9	20S proteasome subunit beta-1i	gtgccggcgtttcaccacagat	agaattttggcagctcatctcccagg
Psme1	PA28a	tttcgctttcccttcccgct	cttgctacacaggtcttcacgga
Psme2	PA28b	ggccttgctcgctttggttaag	actctctccagcaccttctcct
Slc27a4	Long-chain fatty acid transport protein 4	ctggagagctttgcacagaccttga	ctccttccgcaactctgtcttctgg
Socs3	Suppressor of cytokine signaling 3	cccaaggccggagatttcgctt	gcgggaaacttgctgtgggtga
Ucp-1	Uncoupling protein 1	ccaagctgtgcgatgtccatgtaca	aaacatgatgacgttccaggacccg
Actb	Actin beta	cttctttgcagctccttcgttgccg	aatacagcccggggagcatcgtc
RPLP0	60S acidic ribosomal protein P0	ctgagtacaccttcccacttactga	cgactcttcctttgcttcagcttt

General Discussion

Background

Cardiovascular diseases form the most common cause of death in the western world with atherosclerosis as the most common etiology *(1)*. Atherosclerosis is characterized by lipid deposition in the intima of medium to large-sized arteries, evoking pathogenic immune infiltration in the vessel wall causing low grade inflammation of the vessel wall *(2–4)*. Development of atherosclerotic lesions spans decades and can lead to the formation of large plaques which occlude the coronary vessel lumen to such an extent that during e.g. exercise, myocardial tissue is deprived of sufficient oxygen supply leading to symptoms such as shortness of breath, nausea and fatigue, and pressure and tightness of the chest *(5, 6)*. Although this situation of stable angina is just weakly correlated with cardiovascular death, it is associated with heart failure, increased hospitalizations, and reduced quality of life *(5)*. Besides stable angina, atherosclerosis can also lead to the formation of vulnerable lesions, which due to plaque rupture or plaque erosion induce sudden formation of a thrombus *(7, 8)*. The formed thrombus can restrict blood flow in situ, or can break away from the site of thrombus formation and clog an artery in the narrowing arterial tree *(7, 8)*. This can lead to acute oxygen deprivation and injury of downstream tissues and underlies the life-threatening conditions of myocardial infarction and ischemic stroke *(7, 8)*. Treatment of an acute thrombotic occlusion is aimed at restoring blood flow as quickly as possible to prevent ischemia mediated tissue damage *(7)*, by fibrinolytic treatment or surgical removal of the clot *(9)*. Vulnerable or occlusive atherosclerotic plaques can be surgically removed through endarterectomy *(10)*. Impaired blood flow due to narrowed artery lumen can be restored through angioplasty and stent placement opening up the vessel lumen, or by bypass surgery redirecting blood flow *(10)*. Restenosis is a frequently occurring phenomenon after cardiovascular surgery, making repeated interventions sometimes necessary *(11)*.

Current treatment regimens to prevent progression of atherosclerosis and (re)occurrence of major cardiovascular events are predominantly aimed at normalizing lipid levels through adopting a healthy lifestyle, and pharmacologically by use of lipid lowering drugs such as statins *(12)* and PCSK9 inhibitors *(13)*. Lowering lipid levels reduces the risk of a cardiovascular event, however in many patients statins are not well tolerated, or despite successful reduction in lipid levels, still a residual risk for a cardiovascular event is present due to unresolved inflammation *(14)*. The recent success of the CANTOS trial, reducing major cardiovascular events through administration of a monoclonal antibody neutralizing the pro-inflammatory cytokine IL-1β (Canakinumab) *(14–16)*, although not approved for the treatment of atherosclerosis by the FDA, implies that modulation of the immune system is also a feasible way of treating atherosclerosis and reducing cardiovascular risk in human. Following the paradigm that lowering inflammation can reduce atherosclerosis and prevent major cardiovascular events, clinical trials with low doses of the immunosuppressant methotrexate *(17)* and colchicine *(18)* were commenced. The results from treatment with low doses of the methotrexate were rather disappointing, as cardiovascular deaths were not

inhibited by methotrexate while negative side effects, including increased liver enzymes in circulation, a higher incidence of non-basal-cell skin cancers, and more prevalent mouth sores and oral pain, were also found in the methotrexate treated group *(19)*. Similarly, side effects for treatment with Canakinumab and colchicine are commonly reported. In experimental models of atherosclerosis, modulation of the antigen specific immune response towards plaque constituents, including albumin *(20)*, oxidized LDL *(21, 22)*, Apolipoprotein-B100 (ApoB100) *(23–28)*, collagen type VI *(29)*, and heat shock proteins *(30)*, have been capable of reducing atherosclerosis. These studies indicate that vaccination-based approaches against plaque antigens could be interesting to further explore for use in humans, and would likely result in fewer side effects than general immunosuppressants.

In this thesis

In this thesis we aimed to beneficially modulate the immune response to treat atherosclerosis. Hyperlipidemia and inflammation are driving factors behind atherosclerosis, the interactions between lipids and immune system are therefore reviewed in **Chapter 2**. A pivotal step in atherogenesis is the attraction and activation of macrophages in the subendothelial space due to lipid retention. The Oxidized and aggregated lipoproteins induce TLR4 mediated activation of macrophages, and in combination with signals derived from antigen specific Th1 cells promote macrophages to adapt a pro-inflammatory M1 phenotype *(31, 32)*. Due to secretion of pro-inflammatory cytokines and chemokines, and high MHC-II and co-stimulatory molecule expression, M1 macrophages attract other immune cells to the atherosclerotic lesion, sustain inflammation, and are capable of interacting with Th1 CD4 T cells and hereby have a detrimental effect in the context of atherosclerosis *(33, 34)*. During atherosclerosis the natural immune tolerance present to self-derived plaque antigens is broken, as was elegantly shown for collagen type V *(35)*. The ensuing autoimmune response against plaque antigens is considered to be mainly pro-atherogenic *(2, 3)* and is skewed towards a Th1 response *(3, 4)*, resulting in high IFN-γ and TNF-α levels in the atherosclerotic lesion *(36)*. Since immune responses against (ox)LDL in the context of atherosclerosis are well documented *(37, 38)*, we aimed to modulate the immune response against LDL in **Chapter 3-5** to treat atherosclerosis. In **Chapter 6** we aimed to inhibit the pathogenic Th1 response as a whole through immunoproteasomal inhibition, which surprisingly also affected lipid homeostasis and improved parameters of metabolic syndrome.

As atherosclerosis is marked by accumulation of (ox)LDL in the vessel wall leading to induction of auto-reactive (ox)LDL specific CD4 T cells *(37, 38)*, an attractive treatment option would be reinstating natural tolerance to LDL, reducing pathogenic inflammation. Regulatory CD4 T cells (Tregs) are pivotal for keeping immune tolerance and were found capable of keeping autoimmune Th1 immune reactions in check *(39, 40)*. Although Tregs can confer bystander immunosuppression, the immunosuppressive effect of antigen specific Tregs was found greater than that of polyclonal Tregs *(41)*. A means to induce antigen specific Tregs is

the administration of antigens through oral administration, which leads to antigen presentation by the intestinal tolerogenic $CD11C^{+}CD103^{+}$ DC population, which favors the induction of antigen specific immune suppressive regulatory B cells (Bregs), Th3, Tr1 and inducible Tregs (iTregs) *(42)*. Oral administration of oxLDL was previously found to reduce atherosclerosis through induction of oxLDL specific Tregs *(21)*, however induced Treg numbers quickly decline to baseline levels *(21, 43)*. To improve the therapeutic effect of oral oxLDL administration we hypothesized that we could first induce oxLDL specific Tregs through oral administration of oxLDL and then maintain the levels of oxLDL specific Tregs through expansion of the total Treg population. To specifically expand the Treg population IL-2 complexed to antibody clone JES6-1A12 was administered, allowing high affinity IL-2 receptor mediated growth and expansion of Tregs, but inhibiting binding to moderate and low affinity IL-2 receptors *(44)*. Also this clonal induction of Tregs was previously found to confer atheroprotection in our lab *(45)*.

Despite establishing elevated levels of Tregs after IL-2c treatment, coincident with lowered numbers of circulating immune cells indicative of immune suppression, only separate oxLDL treatment significantly reduced atherosclerosis. Separate IL-2c treatment and IL-2c treatment preceded by oxLDL treatment led to a trend towards atherosclerosis reduction compared to the control group, but appeared to be less effective than separate oxLDL treatment. Tregs are known to adapt to environmental cues to effectively inhibit different types of immune responses *(46–48)*. Moreover Tregs with distinct developmental origins, being thymus derived (nTregs) or the peripherally induced iTregs, have been described to have complementary and so, distinct functions. It is possible that differential spacial localization, or intrinsic differences between Treg populations cause differential expansion of Treg populations in response to IL-2c treatment. Moreover adaptive transfer of nTregs was found to reduce iTreg numbers *(49)*, implicating the existence of a feedback mechanism between the Treg populations, through which polyclonal expansion of non-oxLDL specific Tregs could have reduced oxLDL specific Tregs instead of expanding them. Lack of reliable markers for the different Treg populations *(50)* and difficulties in distinguishing antigen specific T cells make it very difficult to assess the effect of IL-2c treatment on the different Treg populations *in vivo*. It would be interesting to study the impact of the different Treg populations on atherosclerosis, and whether skewing of the Treg phenotype could impact atherogenesis.

The heterogeneous nature of LDL particles native LDL is not suitable for use in vaccinations. Therefore, several studies have been dedicated to finding immunogenic epitopes in ApoB100. A peptide library spanning the full ApoB100 protein was screened with human blood plasma for antibody binding to identify antibody epitopes in ApoB100 *(51)*. One of the peptides recognized by human serum derived antibodies is p210 (KTTKQSFDLSVKAQYKKNKH, 3163-3182), named after the peptide number in the peptide library *(51)*. Several vaccination strategies centered around p210 have been employed, successfully reducing atherosclerosis,

however the proposed mechanisms of action of p210 are divergent. P210 induced atheroprotection has been dedicated to induction of p210 antibodies *(27)*, Bregs *(52)*, CD4 Tregs *(25, 52)*, and CD8 T cells *(24, 53)*. To be therapeutically applicable, and to optimize the vaccine formulation and administration, it is important to identify the mechanism of action of vaccination with p210. Because p210 is a human ApoB100 derived sequence with 90% homology with the corresponding murine sequence we used a preclinical model with endogenous expression of human ApoB100, to allow normal thymic selection of T cell clones specific for human apoB100 and p210, and in vivo presentation of the cognate antigen for p210 specific T cell clones. Furthermore in all but one study *(54)* regarding p210 immunization in atherosclerosis, ApoE deficient mice were utilized. Since p210 is part of the LDL receptor binding site A in ApoB100 *(55, 56)*, we inquired whether the protective effect of p210 vaccination still was observed when no LDLr was present. Therefore we used LDLr deficient and human $ApoB^{100/100}$ transgenic (HuBL) mice *(56, 57)* in the experiments described in this paper.

As shown previously *(21)* and confirmed in **chapter 3**, induction of tolerance towards oxLDL reduces atherosclerosis. Therefore, we first aimed to induce a tolerogenic response against p210 through oral administration *(42)* of p210 coupled to cholera toxin B (CTB), known to promote mucosal uptake and tolerance *(58)*. As intranasal vaccination with CTB-p210 was previously reported to mediate atheroprotection through induction of regulatory B cells (Bregs) *(52)* and regulatory T cells *(25)*, we assessed the induction of Tregs and Bregs by flow cytometry. We indeed observed an increase in IL-10 producing Bregs in PMA and Ionomycins stimulated splenocyte cultures of CTB-p210 treated HuBL mice, but did not find increased Treg levels in various immune organs. The absence of Treg induction by oral CTB-p210 administration could be caused by the absence of a CD4 T cell epitope in p210, supported by lack of p210 binding to MHC-II ($I\text{-}A^b$) *(59)* and in line with in silico models of MHC-II binding *(59)*. CTB-p210 treatment did increase p210 IgG, which also had been described in previous studies *(25, 52)*. Despite induction of Bregs and p210 antibodies we did not observe a reduction in aortic root and brachiocephalic artery lesion size. In the only other published study in HuBL mice studying p210, intranasal CTB-p210 treatment was reported to lead to a trend towards reduced plaque formation measured by en face ORO staining of the aorta ($p = 0.059$) *(54)*. However, since the median lesion area of the control group was below 1% it is difficult to interpret these results.

Since the only effect of oral CTB-p210 administration we observed was induction of p210 IgG, we wanted to ensure that the absence of an effect on atherosclerosis by CTB-p210 vaccination was not due to an insufficient induction of p210 IgG levels. Therefore we performed another atherosclerosis study in which we first induced high levels of p210 IgG through vaccination with p210 coupled to pan DR epitope (PADRE) and adjuvanted with alum, and then switched the HuBL mice to a western type diet (WTD). With ELISA for p210,

we confirmed that p210 antibody levels remained high until sacrifice. As alum adjuvanted vaccination with p210, with cBSA as carrier protein, was used in a similar scheme reducing atherosclerosis in ApoE$^{-/-}$ mice *(24, 60, 61)*, reportedly via CD4 and CD8 T cells, we also assessed the induction of CD4 T cell and CD8 T cell populations upon PADRE-p210 vaccination. In accordance with binding predictions for p210 binding to murine MHC-I (H-2Kb and H-2Db) and MHC-II *(59)*, we did not observe T cell activation or induction of regulatory T cell subsets. Although we established high p210 antibody levels over the entire course of WTD feeding, we did not observe an effect on aortic root atherosclerotic lesion size or composition of PADRE-p210 immunization.

The lack of p210 antibody induced atheroprotection in our studies could be linked to the biological function of p210 in ApoB100, as it is part of the LDLr binding site A in ApoB100 *(55, 56)*. In line with p210 antibodies meddling with binding of ApoB100 to the LDLr, LDL uptake by cultured adipoctyes was inhibited by p210 antibodies *(62)*. In macrophage cultures incubated with oxLDL, addition of p210 antibodies inhibited the formation of foam cells but did not limit uptake of oxLDL *(62)*. As upregulation of cholesterol efflux gene expression in macrophages was observed after incubation with p210 IgG in another study *(27)*, it is possible that p210 IgG can improve lipid handling of macrophages. In vivo, immunization with p210, and administration of antibodies against MDA-modified p210 reduced atherosclerosis in ApoE$^{-/-}$ mice *(27)*, indicating that at least part of the atheroprotective effect of p210 vaccination in ApoE$^{-/-}$ mice is antibody derived. Also in humans, p210 IgM and IgG levels are correlated with improved carotid intima-media thickness parameters *(63–65)*, indicating atheroprotective properties of p210 antibodies in human. Interestingly the inverse correlations between anti-p210 IgG levels and baseline composite measures of carotid intima-media thickness disappeared when adjusted for known risk factors *(63)*, suggesting that improved lipid handling induced by anti-p210 antibodies *(27, 62)* might also occur in human.

Besides induction of protective antibodies, the protective effect of vaccination with p210 formulations has been dedicated to the induction of atheroprotective T cell populations *(24, 25, 53)*. As mentioned, we did not detect induction of CD4 T cells or CD8 T cells, in line with MHC binding and prediction. Neither did we detect an atheroprotective effect which, if T cell dependent, should not have been affected by the use of an LDLr$^{-/-}$ model or ApoE$^{-/-}$ model. This suggests that T cell responses induced by p210 vaccination are likely based on an indirect mechanism. Adjuvant properties of p210 could explain the divergent immunological effects which have been described upon administration of different p210 formulations. Actually, the heparan sulfate proteoglycan and LDLr binding properties of the LDLr binding sites of ApoB100 were used to enhance uptake of the SIINFEKL peptide, promoting cross priming of CD8 T cells *(55)*. Furthermore, p210 coupling to FITC enhanced FITC uptake by DCs *(24)*, showing that p210 can promote uptake of coupled proteins. In splenocyte cultures incubated with CTB-p210 but not with p210 or CTB, we observed enhanced cell death independent of the treatment group (control, CTB, CTB-p210). As flow cytometric analysis of CTB-p210

incubated cultures did not reveal activation of cytotoxic CD4 or CD8 T cells, this suggest that p210 reinforced the known pro-apoptotic properties of CTB *(66)*. Similarly, CTB-p210 was found to induce significantly higher levels of Bregs in culture than CTB-OVA *(52)*. Many of the studies centered around vaccination with p210 have used cBSA as a carrier protein without proving antigen specificity of the T cell responses to which the observed atheroprotection was dedicated. As immunization with cBSA itself using alum was found to reduce atherosclerosis *(20)*, it is possible that p210 promoted the atheroprotection observed in combined p210 and cBSA immunization. Vaccination with cBSA, and the 70% homology between bovine albumin and murine albumin (accession M73993.1 vs accession BC049971.1), could have led to cross-reactive antigen specific responses against murine albumin, which is present in large quantities in atherosclerotic plaques *(67)*. The possibility that treatment of atherosclerosis with ApoB100 derived CD4 T cell epitopes is feasible was shown by immunization (CFA used for priming, IFA for booster) with ApoB100 derived peptides, predicted to bind I-A^b and thereby capable of inducing CD4 T cells *(68)*. The enhanced IL-10 expression in aorta's of ApoB100 peptide vaccinated mice suggest that regulatory CD4 T cells were induced by vaccination although the overall FoxP3 cell levels were not increased *(68)*. It would be interesting to assess whether this tolerogenic response towards ApoB100 peptide vaccination could still observed when vaccination was commenced in later stages of atherosclerosis development when immune tolerance towards plaque antigens like ApoB100 might have been eroded, as was observed for collagen type V *(35)*. Induction of CD4 Treg mediated tolerance towards plaque antigens seems a promising strategy to specifically inhibit the atherosclerotic immune response. To be effective in advanced stages of atherosclerosis, probably mucosal administration of human MHC-II binding epitopes of plaque antigens in combination with tolerogenic adjuvants, like CTB, would be required.

Much less is known about the relevance of CD8 T cells in the context of atherosclerosis, although CD8 T cells are present in large quantities in the atherosclerotic plaque *(69)* and have an activated phenotype *(70)*. CD8 T cells are specialized at killing of specific target cells, mediated through T cell receptor (TCR) interaction with an MHC-I/peptide complex present on the target cell. Depending on target antigen, likely reflecting which cell types were targeted, induction of antigen specific CD8 T cell responses were found to be atheroprotective *(24, 71, 72)* or atherogenic *(73)*. As ApoB100 is considered one of the main plaque atherosclerotic plaque antigens, we aimed to assess the role of ApoB100 specific CD8 T cells in atherosclerosis. Cross-presentation of plaque constituents, including ApoB100, by APCs could make plaque APCs subject to killing by ApoB100 specific CD8 T cells. Because suppressed macrophage apoptosis results in increased atherosclerosis *(74, 75)*, we hypothesized that induction of ApoB100 specific CD8 T cells, presumably promoting killing of plaque macrophages by CD8 T cells, could be therapeutically relevant. Therefore, we opted to test this hypothesis with ApoB100 derived human MHC-I (HLA-A2) restricted epitopes to be directly applicable in human, in **chapter 5**. With *in silico* prediction tools of peptide

processing and HLA-A2 binding *(76–85)*, we selected 6 ApoB100 derived peptides to be synthesized. HLA-A2 binding was confirmed for all 6 epitopes in T2 cell binding assays, and immunogenicity was confirmed for 5 peptides by vaccination with peptide pulsed HLA-A2 transgenic DCs in HLA-A2 transgenic mice (HHD mice) *(86)*, deficient for murine MHC-I and human ApoB100. For the atherosclerosis studies HHD mice and HuBL mice were crossbred to generate HLA-A2 and human ApoB100 transgenic mice, deficient for the LDLr to allow atherosclerosis development, and either with normal expression (HuBL-A2^{m+}) or devoid of murine MHC-I expression (HuBL-A2^{m-}). CD8 T cells were again primed with a mixture of peptide pulsed HHD DCs, but then boosted after a week with peptide adjuvanted with poly(I:C) and αCD40. This vaccination regimen was previously reported to induce neo-epitopes specific CTLs that were effective in penetrating and killing tumors, indicating that this vaccination approach yields migratory and functional CTLs *(87, 88)*. For ApoB$_{406\text{-}414}$, ApoB$_{3070\text{-}3078}$, and ApoB$_{4531\text{-}4539}$, recall responses were detected in the spleens of ApoB100 peptide vaccinated HuBL-A2^{m-} and HuBL-A2^{m+} mice 8-9 weeks after booster vaccination at sacrifice, indicating successful vaccination. Interestingly no CD8 T cell recall response could be detected for ApoB$_{406\text{-}417}$ and ApoB$_{2356\text{-}2364}$ in the ApoB100 peptide treated HuBL-A2^{m-} and HuBL-A2^{m+} mice, suggesting thymic negative selection or peripheral tolerance induction towards ApoB$_{406\text{-}417}$ and ApoB$_{2356\text{-}2364}$. Recall responses towards pooled peptides were also assessed in cultures from mediastinal lymph nodes and aortic arches from HuBL-A2^{m-} mice. Also, in cultures of cells from mediastinal lymph nodes recall responses were observed, however not in aortic arch derived cells. As enhanced effector CD8 T cells in the blood and CD8 T cell levels in the aortic arch cultures were found due to ApoB100 peptide vaccination HuBL-A2^{m-} mice, it is very likely that ApoB100 peptide specific CD8 T cells homed to the atherosclerotic lesion. Since the ApoB100 peptides can be externally loaded on MHC-I, so without need for cross-presentation, the absence of recall responses in the aortic arch cultures could also not be dependent on defective cross-presentation. These data therefore suggest that CD8 T cell activation in to response to TCR stimuli is reduced in the plaque environment. Reduced responsiveness could be CD8 T cell intrinsic, e.g. due to chronic antigen exposure in the plaque leading to CD8 T cell exhaustion *(89, 90)*. In line with CD8 T cell exhaustion in atherosclerosis, upregulation of the co-inhibitory PD-1 expression was observed in atherosclerosis patients *(91)*. On the other hand, plaque cells could inhibit CD8 T cell activation, e.g. PD-L1 was found upregulated on macrophages in human lesions *(92)*, which could provide a co-inhibitory signal to plaque CD8 T cells. In line with impaired CD8 T cell activation in the atherosclerotic environment ApoB100 peptide vaccination did not impact cellular content of the plaques, and did not affect plaque size and stability. As vaccination with ApoB100 derived CD8 T cell epitopes did induce CD8 T cell responses but did not affect atherosclerosis, vaccination with ApoB100 derived CD8 T cell epitopes does not seem to be a viable way of treating atherosclerosis. Moreover, from a safety perspective, inducing strong CD8 T cell responses towards endogenously expressed proteins might lead to autoimmunity and tissue damage in organs where the protein is endogenously expressed *(93)*. Therefore, induction of specific tolerance towards plaque antigens or reducing overall

inflammation are likely to sort better results than inducing antigen specific CD8 T cell responses for treatment of atherosclerosis.

Although currently no drugs aimed at general reduction of inflammation are approved for treatment of cardiovascular disease by the FDA and EMA, the LODOCO *(94)* and CANTOS *(14, 95)* trial have indicated that a reduction in cardiovascular risk can be achieved through immune inhibition in humans. As immunoproteasomal inhibition with ONX-0914, inhibiting immunoproteasomal catalytic subunit LMP7 and LMP2 *(96)*, ameliorated multiple auto-immune diseases in experimental auto-immune models *(96–104)*, we assessed the effect of ONX-0914 treatment on atherosclerosis in **chapter 6**. LDLr$^{-/-}$ were fed a WTD from 15 weeks of age for 7 weeks, while fed a WTD were ONX-0914 or vehicle treated. ONX-0914 treatment reduced cDC content in the spleen and reduced cDC content and activation in mesenteric lymph nodes and cervical lymph nodes, which drain the peritoneal cavity and intestines (atherosclerosis unrelated) *(105)* and drain the aorta and supra-aortic arteries (atherosclerosis related) *(106)* respectively. Furthermore, in several lymphoid organs decreased antigen experienced effector memory (Tem) and central memory (Tcm) CD4 and CD8 T cells were observed. As the enhanced activation of DCs proofed atherogenic *(107)*, and enhanced levels of circulating Tem cells correlated with carotid intima-media thickness and circulating Tem cells were increased in patients with stable angina and acute myocardial infarction *(105)*, it is likely that ONX-0914 induced alteration in cDC and T cell populations aided in the observed atherosclerosis reduction in the ONX-0914 treated group.

Besides developing atherosclerosis, LDLr$^{-/-}$ mice on WTD develop obesity, obesity associated metabolic syndrome, and insulin resistance *(108, 109)*. Next to ameliorating atherosclerosis, ONX-0914 reduced body weight in multiple experiments with WTD fed LDLr$^{-/-}$ mice and APOE*3-Leiden.CETP mice. White adipose tissue (WAT) mass was markedly reduced in all studies, while EchoMRI in APOE*3-Leiden.CETP mice showed that lean mass was unaffected by ONX-0914 treatment. Coincident with reduced WAT mass, improved metabolic parameters such as lowered insulin levels, lowered fasting blood glucose, lowered TG levels, and in APOE*3-Leiden.CETP mice also lowered cholesterol levels were observed. To our knowledge this is the first study to show that immunoproteasomal inhibition using the LMP7 and LMP2 specific inhibitor ONX -0914 has metabolic effects in addition to its immunomodulatory effects. This is likely the linked to the use of a WTD in our study, leading to obesity and development of metabolic syndrome, instead of using lean mice on chow diet in other studies applying ONX-0914 *(96–104)*. In addition, disease-related weight loss due to induction of e.g. colitis *(99)*, EAE *(98)* or arthritis *(97)*, may have masked the metabolic effects of ONX-0914. To make sure that reduced weight (gain) in ONX-0914 treated mice was not due to (hepato)toxicity in our studies, we measured the activity of ALAT and ASAT liver enzymes in the blood, and the expression of Cyp3A11 in the liver. No indications for toxic side-effects of ONX-0914 treatment were measured.

Metabolic cage measurements with APOE*3-Leiden.CETP mice did not reveal reduced food intake or increased energy expenditure upon ONX-0914 treatment. In an oral lipid loading test using LDLr$^{-/-}$ mice, the rise in TG levels after olive oil administration was blunted in the ONX-0914 treated group. Because clearance of cholesterol and triglycerides from the blood was not affected in ONX-0914 treated mice, as assessed by injection of VLDL like particles containing radiolabeled cholesteryl oleate and glycerol trioleate in APOE*3-Leiden.CETP mice, results of the oral lipid loading test indicated reduced intestinal lipid uptake by ONX-0914 treatment. Similarly, reduced intestinal lipid uptake was observed in LMP7$^{-/-}$ animals *(110)*. Reduced intestinal lipid uptake in LMP7$^{-/-}$ mice was dedicated to reduced pancreatic lipases Pnlip and Pnlrp2 expression *(110)*, however we did not observe this upon ONX-0914 treatment. We found that ONX-0914 treatment repeatedly induced neutrophil and macrophage accumulation in white adipose tissue, likely through the upregulation of CCL2 expression we observed in mature adipocytes which were also found to express immunoproteasomal catalytic subunits. The increased levels of neutrophils and macrophages in gWAT were likely responsible for the enhanced IL-1β and TNF-α levels observed in ONX-0914 treated mice. Interestingly, patients with a loss of function mutation in PSMB8, coding for LMP7, present themselves with similar symptoms including white adipose tissue inflammation, and lipodystrophy *(111)*.

Peritoneal macrophages (including macrophages residing in gWAT) appeared to be involved in ONX-0914 mediated reduction in intestinal lipid uptake, as clodronate liposome mediated depletion of peritoneal macrophages prior to ONX-0914 treatment led to normalization of increase in TG levels upon oral lipid loading. Therefore, we looked into literature for macrophage derived factors which were reported to induce weight loss, and identified GDF15 *(112)*. GDF15 was indeed increased in the blood by ONX-0914 treatment, however also GDF15$^{-/-}$ mice lost weight upon ONX-0914 treatment, indicating that GDF15 was not mediating ONX-0914 induced weight loss. Another macrophage derived factor that has been reported to cause weight loss and reduce intestinal lipid uptake is IL-1β *(113, 114)*. Interestingly, a common side effect listed on the on the information leaflet of the (atheroprotective) IL-1β neutralizing antibody canacinumab (Ilaris) is weight gain. Therefore, it would be interesting to investigate whether IL-1β mediates ONX-0914 induced weight loss.

Treatment of mice with ONX-0914 reduces atherosclerosis and considerably reduces WAT mass in obese mice fed a WTD, concomitantly improving parameters of metabolic syndrome. Because atherosclerosis is still the primary cause of death, and the obesity epidemic is feeding metabolic syndrome related diseases worldwide, immunoproteasomal inhibition could be a valuable therapeutic tool for the western world to combat both. Currently phase 1b trials in SLE patients with the immunoproteaomal KZR-616 are ongoing, with phase 2 clinical trials focused on treatment of lupus nephritis, dermatomyositis, polymyositis, autoimmune hemolytic anemia, and immune thrombocytopenia, on the docket. It would be interesting to see whether immunoproteasomal inhibition also leads to weight loss in human, and whether there are indications for reduced cardiovascular mortality.

Future perspectives

The recent success of the CANTOS trial, reducing major cardiovascular events through administration of a neutralizing monoclonal antibody against the pro-inflammatory cytokine IL-1β (Canakinumab) *(14–16)*, implies that modulation of the immune system is also a feasible way of treating atherosclerosis and reducing cardiovascular risk in human. In line with the paradigm that lowering inflammation can reduce atherosclerosis and prevent major cardiovascular events, a clinical trial with the immunosuppressant colchicine *(18)* is still underway, while the immunosuppressive methotrexate treatment failed to reduce atherosclerosis *(19)*. When successful in preventing major cardiovascular events, colchicine could become the first immunomodulatory treatment approved for use in atherosclerosis. Dependent on the success of clinical studies with immunoproteasomal inhibitors in the context of other auto-immune diseases, immunoproteasomal inhibition in clinical trials in context of atherosclerosis and obesity could be considered. Drawbacks of general immunosuppressants are obviously increased risk for infectious diseases *(115, 116)*, but also other side effects like gastrointestinal issues, have been reported for methotrexate and colchicine which could reduce patient compliance with drug intake.

Far less side effects are to be expected with atheroprotective vaccination approaches specifically targeting plaque antigens, and especially restoring tolerance to plaque antigens. Restoring immune tolerance to plaque antigens has been effective in treating atherosclerosis in pre-clinical atherosclerosis models *(21, 117, 118)*, however has not been studied in clinical trials. In multiple sclerosis, tolerance induction towards myelin peptides in multiple sclerosis (MS) patients resulted in a decrease in antigen-specific T cell responses in a phase 1 trial *(119)*, suggesting that tolerance induction is feasible to modulate T cell mediated autoimmunity. Currently our knowledge about the antigen specific T cell responses taking place in atherosclerosis is very limited, but is known to include CD4 T cell responses directed against LDL *(38)*, collagen type V *(35)*, and HSPs *(29)*. In **chapter 3** we were able to reduce atherosclerosis by oral administration of oxLDL, but could not achieve atheroprotection in **chapter 4** through oral administration of the p210 peptide derived from the ApoB100 protein which is present in LDL, likely due to lack of a CD4 epitope in p210. Apart from difficulties identifying suitable CD4 T cell epitopes for tolerization, variations in human MHC alleles are not likely to allow a single epitope to bind all MHC-II molecules and could thereby only beneficial in some patients. Therefore, induction of tolerance towards complete antigens, or a selection of multiple CD4 epitopes should be considered for tolerization. Moreover, Induction of tolerance towards a combination of plaque antigens against which auto-immunity is developed would likely sort better effects than targeting a single antigen, targeting more T cell clones. Better characterization of the adaptive immune response, identifying antigens and antigenic epitopes in antigens could therefore be very beneficial for the treatment of atherosclerosis. Another major hurdle for application of tolerance induction in human, is the development of most effective, and safe tolerization regimens.

Induction of CD8 T cell responses towards p210 has been reported to reduce atherosclerosis *(24, 53)*, however we did not observe protective effects of vaccination with p210 in **chapter 4** or strong CD8 epitopes derived from ApoB100 in **chapter 5**. Also, when successful at inducing an atheroprotective CD8 T cell response through targeting an endogenous plaque antigen, this would likely lead to auto-immunity related side effects through killing of cells expressing the antigen outside the plaque. Due to the high risk of side effects, the use of therapeutic induction of CD8 T cell responses against plaque antigens for treatment of atherosclerosis are likely limited from a safety perspective. Still vaccination approaches in experimental settings could provide very useful insights on the role of CD8 T cells in atherosclerosis.

Besides modulation of T cell responses, induction of antibodies (IgG) to several plaque antigens, has been capable of reducing atherosclerosis in pre-clinical studies. Also induction of antibodies against (epitopes) of (ox)LDL have been studied and appeared to be atheroprotective in multiple studies *(23)*, including antibodies directed against p210 *(27)*, and were correlated with atheroprotection *(63–65)* We could not confirm atheroprotective properties of p210 antibodies in **chapter 4,** probably due to utilization of $LDLr^{-/-}$ mice instead of $ApoE^{-/-}$ mice in our study, as p210 is part of a LDLr binding site in ApoB100 *(55)*. Besides (ox)LDL antibodies, antibodies against collagen type VI have been reported to act atheroprotective *(120)*. Mechanistically, antibodies were found to inhibit macrophage activation, and enhance expression of cholesterol efflux genes through binding to FC receptors *(23, 27, 120, 121)*. Therefore, induction of antibodies against plaque antigens could be desired to treat atherosclerosis, however pathogenic activation of CD4 T cells should be limited. Through mucosal administration or use of antibody epitope mimotopes for vaccination, protective antibodies could be induced while preventing induction of proatherogenic CD4 T cells *(62)*.

There are several viable approaches through which modulation of the immune system could be deployed to reduce atherosclerosis. General immune suppression in the form of low-dose methotrexate and colchicine are currently being tested in clinical trials for treatment of atherosclerosis and if successful will likely comprise the first immunomodulatory treatments to be available for treatment of atherosclerosis *(17, 18)*. Antigen specific modulation of the immune response for treatment of atherosclerosis is further away, however pre-clinical studies have indicated that such approaches are feasible for treatment of atherosclerosis. A combination of lipid and immune management, and promoting a healthy lifestyle will likely comprise the preventive measures of the future to combat atherosclerosis and cardiovascular disease.

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Nederlandse Samenvatting

Achtergrond

Hart- en vaatziekten vormen de meest voorkomende doodsoorzaak in de westerse wereld met atherosclerose als de dominante etiologie. Atherosclerose wordt gekenmerkt door afzetting van lipiden in de intima van middelgrote tot grote slagaders, wat leidt tot immuuncel accumulatie in de vaatwand en een pathogene chronische ontsteking in de vaatwand. De ontstekingsreactie versterkt de ophoping van vetten en stimuleert daarmee de vorming van laesies. De ontwikkeling van een atherosclerotische laesie duurt meestal tientallen jaren. De vorming van grote plaques kan het lumen van de kransslagaders zodanig afsluiten dat tijdens inspanning de zuurstoftoevoer naar onderliggend myocardweefsel onvoldoende is, wat kan leiden tot symptomen zoals kortademigheid, misselijkheid en vermoeidheid, en druk op de borst. Alhoewel deze situatie van stabiele angina maar zwak gecorreleerd is met cardiovasculaire sterfte, is stabiele angina wel geassocieerd met hartfalen, verhoogde ziekenhuisopnames, en verminderde kwaliteit van leven. Naast stabiele angina kan atherosclerose ook leiden tot de vorming van kwetsbare laesies, die ten gevolge van scheuring van de plaque of erosie van de endotheel laag op de plaque, de vorming van een trombus kunnen veroorzaken. De gevormde trombus kan de bloedstroom in situ beperken, of kan losbreken van de plaats van trombus formatie en de slagader in de vernauwende vertakkingen van de slagaders verstoppen. In beide gevallen kan een acuut zuurstoftekort in achterliggende weefsels ontstaan wat ten grondslag ligt aan de levensbedreigende situaties zoals een hartinfarct en ischemische beroerte. Behandeling van een acute verstopping van de slagaders is gericht op het zo snel mogelijk herstellen van de bloedstroom, door fibrinolytische behandeling of chirurgische verwijdering van de trombus, om weefselbeschadiging door zuurstoftekort te voorkomen. Ter voorkoming van cardiovasculaire gebeurtenissen kunnen kwetsbare of occlusieve atherosclerotische plaques operatief worden verwijderd door middel van endarterectomie. Verder kan de verminderde bloedstroom ten gevolge van een vernauwd slagaderlumen worden hersteld door te dotteren en een stent te plaatsen die het vatlumen open houdt, of door bypass-chirurgie die de bloedstroom omleidt. Restenose is een vaak voorkomend fenomeen na cardiovasculaire chirurgie, waardoor herhaalde interventies soms noodzakelijk zijn.

Huidige behandelingsregimes om progressie van atherosclerose en (her)optreden van cardiovasculaire gebeurtenissen te voorkomen zijn voornamelijk gericht op het normaliseren van lipide niveaus door een gezonde levensstijl en door het gebruik van lipide verlagende middelen zoals statines en PCSK9 remmers. Verlaging van de lipiden niveaus verlaagt het risico op een cardiovasculaire complicatie, maar bij sommige patiënten worden statines niet goed verdragen of is therapietrouw een probleem, wordt het lipide niveau niet voldoende verlaagd, of blijft er ondanks succesvolle verlaging van lipide niveaus een verhoogd risico aanwezig voor een cardiovasculaire gebeurtenis ten gevolge van een onopgeloste ontstekingsreactie. Het recente succes van de CANTOS-studie, waarin cardiovasculaire complicaties werden gereduceerd door toediening van een monoklonaal antilichaam dat het pro-inflammatoire cytokine IL-1β neutraliseeert (Canakinumab), impliceert dat modulatie

van het immuunsysteem ook een haalbare manier is om atherosclerose te behandelen, en het cardiovasculaire risico in de mens te verminderen. In navolging van het paradigma dat het verlagen van de ontsteking atherosclerose kan verminderen en zware cardiovasculaire complicaties kan voorkomen, zijn klinische onderzoeken met lage doses van de immunosuppressiva methotrexaat en colchicine in de context van atherosclerose gestart. De resultaten van de behandeling met lage doses methotrexaat waren echter teleurstellend, cardiovasculaire sterfte werd niet geremd door behandeling met methotrexaat, terwijl negatieve bijwerkingen, waaronder verhoogde leverenzymen in de bloedsomloop, een hogere incidentie van huidkanker (niet basaalcelcarcinoom) en meer voorkomende mondzweren en orale pijn, werden gevonden in de met methotrexaat behandelde groep. Ook voor canakinumab en colchicine worden veel voorkomende bijwerkingen beschreven. In experimentele modellen van atherosclerose, is de modulatie van de antigeen-specifieke immuunrespons naar plaque bestanddelen, waaronder albumine, geoxideerd LDL, Apolipoproteïne-B100 (ApoB100), collageen, en heat shock-proteïnes, in staat om atherosclerose te verminderen. Deze specifieke immuun modulatie zal waarschijnlijk resulteren in veel minder bijwerkingen dan algehele immuun suppressie, en zou daarom interessant zijn om verder te onderzoeken voor gebruik in de mens.

In dit proefschrift

In dit proefschrift hebben we ernaar gestreefd om de immuunrespons voor de behandeling van atherosclerose gunstig te moduleren. Hyperlipidemie en ontsteking zijn de drijvende factoren achter atherosclerose. De interacties tussen lipiden en het immuunsysteem zijn daarom samengevat in **hoofdstuk 2.** Omdat lipiden niet water oplosbaar zijn, worden lipiden (en andere niet water oplosbare stoffen) vervoerd in het bloed verpakt in lipoproteïnen. Lipoproteïnen vervoeren onder meer vetzuren en cholesterol, wat belangrijke bouwstenen zijn voor onder andere celmembranen, en ze vervullen daarmee een belangrijke fysiologische rol. Door een hoge concentratie lipoproteïnen in het bloed kunnen er echter op bepaalde plekken in het arterieel vaatstelsel ophopingen van lipoproteïnen in de vaatwand ontstaan. Dit is een cruciale stap in de ontwikkeling van atherosclerose omdat dit zorgt voor het aantrekken van macrofagen naar de plaats in de vaatwand, waar lipoproteïnen zich ophopen. De geoxideerde en geaggregeerde lipoproteïnen activeren macrofagen, wat samen met andere pro-inflammatoire factoren in de atherosclerotische omgeving ervoor zorgt dat macrofagen een pro-inflammatoir fenotype aannemen. Door afgifte van ontstekingsbevorderende cytokinen en chemokines trekken pro-inflammatoire macrofagen andere immuuncellen aan naar de atherosclerotische laesie, en houden op deze manier de ontsteking op gang.

Opname van grote hoeveelheden plaque materiaal door dendritische cellen en macrofagen en presentatie van deze plaque materialen, gecombineerd met de aanwezigheid van co-stimulerende moleculen op antigeen presenterende cellen, stimuleren de interactie met Th1 CD4 T helper cellen die specifiek reageren op plaque antigenen. Op deze manier wordt

gedurende de ontwikkeling van atherosclerose de van nature aanwezige immuun tolerantie voor lichaamseigen eiwitten in de plaque gebroken. De daaruit voortvloeiende auto-immuunachtige reactie tegen plaque eiwitten wordt atherosclerose stimulerend beschouwd. Onder andere de hoge IFN-γ en TNF-α niveaus in de atherosclerotische laesie, die karakteristiek zijn voor Th1 immuun reacties, zijn gecorreleerd met verergering van atherosclerose. Omdat immuunreacties tegen al dan niet geoxideerd LDL goed gedocumenteerd zijn in de context van atherosclerose, hebben we de immuunreactie tegen LDL op verschillende manieren gemoduleerd in **hoofdstuk 3-5** om atherosclerose te verminderen. In **hoofdstuk 6** probeerden we de pathogene immuunreactie als geheel te remmen door immunoproteasomale remming, wat verrassend genoeg ook verbeterde parameters van metabool syndroom en lipide homeostase induceerde.

Omdat atherosclerose wordt gekenmerkt door accumulatie van LDL in de vaatwand, wat leidt tot inductie van onder meer auto-reactieve (ox)LDL-specifieke CD4 T-cellen, zou het aantrekkelijk zijn om de natuurlijke tolerantie voor LDL herstellen, waardoor de pathogene ontsteking wordt verminderd en atherosclerose geremd. Omdat regulatoire CD4 T-cellen (Tregs) cruciaal zijn voor het behoud van immuuntolerantie en in staat om auto-immuun reacties te verminderen wilden we Tregs vermeerderen om atherosclerose te bestrijden in **hoofdstuk 3**. Voor de immunosuppressieve functie van Tregs is het niet perse nodig dat Tregs een antigeen specifiek herkennen, maar het immunosuppressieve effect van antigeen specifieke Tregs is wel groter dan dat van Tregs die een niet gerelateerd antigen herkennen. Een mogelijkheid voor inductie van antigeen specifieke Tregs is de orale toediening van antigenen. Via deze route wordt het toegediende antigen opgenomen en gepresenteerd door een tolerogene dendritische cel populatie in de darmen, waardoor inductie van onder meer antigeen specifieke induceerbare Tregs (iTregs) kan worden bevorderd. Via orale toediening van geoxideerd LDL werd eerder al atherosclerose verminderd door inductie van oxLDL-specifieke Tregs. Na de laatste behandeling met geoxideerd LDL nemen de Treg aantallen echter snel af tot controle niveaus. Het therapeutische effect van orale toediening van geoxideerd LDL zou daarom mogelijk verbeterd kunnen worden door de Treg aantallen kunstmatig hoog te houden na het induceren van geoxideerd LDL specifieke Tregs. Daarom hebben we muizen na orale toediening van geoxideerd LDL, behandeld met IL-2 gekoppeld aan een antilichaam (JES6-1A12). IL-2 is een belangrijke groeifactor voor regulatoire T cellen maar kan ook veel andere cellen aanzetten tot groei en celdeling. De meeste cellen hebben echter een andere receptor voor IL-2 dan Tregs. Door het koppelen van IL-2 aan JES6-1A12 blijft binding gewaarborgd aan de hoge affiniteit receptor voor IL-2 die aanwezig is op Tregs, maar wordt binding van IL-2 aan andere IL-2 receptoren verhinderd. Daarmee leidt toediening van IL-2 gekoppeld aan JES6-1A12 tot specifieke vermeerdering van Tregs.

Ondanks dat IL-2 behandeling inderdaad leidde tot verhoogde Tregs niveaus in onze studie, samenvallend met verlaagde aantallen circulerende immuuncellen wat een geslaagde immunosuppressie aangeeft, resulteerde alleen behandeling met geoxideerd LDL in een

significante reductie in atherosclerose. De reden voor het uitblijven van een additief effect van orale toediening van geoxideerd LDL en IL-2 is lastig aan te wijzen doordat antigeen specifieke Tregs moeilijk te onderscheiden zijn van andere Tregs, en omdat er geen sterke kenmerken zijn om verschillende Treg populaties, iTregs en Tregs geproduceerd in de thymus (nTregs), van elkaar te onderscheiden. Wel is aangetoond dat er een negatieve terugkoppeling bestaat tussen nTregs en iTregs door de transfusie van nTregs, wat leidde tot een reductie van iTregs. Het zou daarom kunnen dat IL-2 behandeling in plaats van het vergroten van de geoxideerd LDL specifieke Treg cellen, deze cellen juist heeft onderdrukt door het vergroten van de nTreg populatie. Na het specifiek stimuleren van geoxideerd LDL specifieke Tregs lijkt non-specifieke expansie van Tregs niet gunstig voor het behandelen van atherosclerose.

Door de heterogene en slecht gedefinieerde samenstelling van intact LDL is het niet geschikt voor gebruik in vaccins. Daarom zijn een groot aantal onderzoeken gewijd aan het vinden van immunogene epitopen in ApoB100, aanwezig in LDL, om te gebruiken voor modulatie van de immuunrespons tegen LDL. Een peptidebibliotheek die het volledige ApoB100-eiwit overspant, werd gescreend met humaan bloedplasma voor antilichaambinding om delen in ApoB100 te identificeren die worden herken door antilichamen. Eén van de peptiden die werd herkend door antilichamen uit humaan serum is p210, genoemd naar het peptidenummer in de peptidenbibliotheek. Verschillende vaccinatiestrategieën met p210 zijn succesvol gebleken in het verminderen van atherosclerose, maar de werkingsmechanismen van p210 die zijn gerapporteerd zijn uiteenlopend. In lijn met de identificatie van p210 is bescherming tegen atherosclerose gekoppeld aan het opwekken van p210 antilichamen, maar ook gekoppeld aan inductie van regulatoire B cellen, regulatoire CD4 T cellen, en CD8 T-cellen. Voor therapeutisch toepasbaarheid, en om de vaccinformulering en toediening te optimaliseren, is het belangrijk om het werkingsmechanisme van immuunmodulatie met p210 te identificeren.
Omdat p210 een humaan ApoB100 afgeleide sequentie is met 90% homologie met de overeenkomstige sequentie in de muis hebben we een experimenteel model met endogene expressie van humaan ApoB100 gebruikt. Dit kan de selectie van T cel klonen specifiek voor menselijk apoB100 en p210 in de thymus beïnvloeden, en maakt de in vivo presentatie van het volledige p210 mogelijk. Zoals eerder getoond en herhaald in **hoofdstuk 3**, zorgt het opwekken van tolerantie voor geoxideerd LDL voor verminderde atherosclerose. Daarom hebben we eerst p210 gekoppeld aan cholera toxine B (CTB), waarvan bekend is dat het opname via de slijmvliezen verbeterd en tolerantie bevordert, oraal toegediend. Zoals al eerder beschreven na nasale toediening van CTB-p210, leidde orale CTB-p210 administratie tot hogere aantallen regulatoire B cellen en p210 antilichamen, maar observeerden wij geen verhoging in Tregs wat wel werd gezien na nasale toediening van dit vaccin. Orale behandeling met p210-CTB resulteerde in dit experiment niet in verminderde atherosclerose. Om te bekijken of hogere p210 antilichaam niveaus wel atheroprotectief zouden werken, hebben we vervolgens muizen gevaccineerd met p210 gekoppeld aan een sterk CD4 T cel epitoop (pan-DR-epitoop, PADRE) en in combinatie met aluminiumhydroxide. Aluminiumhydroxide is een adjuvans dat zorgt voor een depot effect waardoor antigenen langer gepresenteerd worden en het versterkt ook de fagocytose van antigenen door antigen

presenterende cellen. De koppeling van een B cel epitoop aan een CD4 T cel epitoop induceert T cel hulp voor een versterkte antilichaam productie. Door eerst hoge antilichaam levels tegen p210 op te wekken met 3 vaccinaties met tussenposen van 3 weken, alvorens muizen op westers dieet te zetten, zorgden we ervoor dat gedurende het complete proces van atherogenese hoge antilichaam levels aanwezig waren. Echter ook in deze studie konden we geen effect vinden van vaccinatie met p210 op atherosclerose parameters. In experimenten in een ander atherosclerose model (ApoE deficiënte muizen), was toediening van p210 echter wel effectief in het reduceren van atherosclerose. Ook in de mens zijn p210 IgM en IgG niveaus gecorreleerd met verbeterde (kleinere) intima-media dikte van de carotiden, wat wijst op atheroprotectieve eigenschappen van p210-antilichamen in de mens.

Het ontbreken van door p210-antilichaam geïnduceerde atherobescherming in onze studies zou kunnen komen door de biologische functie van p210 in ApoB100. In gekweekte adipocyten werd namelijk LDLr gemedieerde LDL opname geremd door p210 antilichamen, wat verklaard kan worden doordat p210 onderdeel uit maakt van LDLr binding plaats A in ApoB100. Verder tonen verschillende studies dat p210 antilichamen de vorming van schuimcellen kan voorkomen, onder andere door de verhoogde expressie van genen die gelinkt zijn aan de verwerking en van cholesterol. In onze studies naar p210 vaccinatie hebben we in plaats van ApoE deficiënte muizen, LDLr deficiënte muizen gebruikt. Het ontbreken van de interactie tussen p210 en de LDLr door afwezigheid van de LDLr in het door ons gebruikte atherosclerose model, zou het ontbreken van een effect van p210 antilichamen kunnen verklaren.

Naast de inductie van beschermende antilichamen, wordt het beschermende effect van vaccinatie met p210-formuleringen verklaard door de inductie van atheroprotectieve T cel populaties. Wij konden echter geen inductie van CD4 T cel of CD8 T cel populatie detecteren. Dit zou te wijten kunnen zijn aan een gebrek aan sterke T cel epitopen in p210, wat bevestigd lijkt te worden door in silico predictie modellen voor MHC-I binding, die voorspellen dat p210 niet goed kan binden aan MHC-I en MHC-II, een voorwaarde voor een goede T cel activatie. De eerder beschreven T cel effecten na immuun modulatie met verschillende p210 formuleringen zou verklaard kunnen worden door een adjuvans functie van p210. Koppeling van p210 aan een fluorescent eiwit (FITC) verhoogde namelijk eerder de opname van FITC in dendritische cellen. Verder werden de bindingeigenschappen van de LDLr bindingsplaatsen in ApoB100, die binding met heparansulfaat proteoglycanen en de LDLr mogelijk maken, gebruikt om de CD8 T cel reactie tegen een peptide te versterken door het peptide te koppelen aan de LDLr bindingsplaatsen afkomstig van ApoB100. Ook zagen wij in celkweken van de milt dat incubatie met CTB-p210, maar niet met p210 of CTB leidde tot verhoogde cel dood. Aangezien CTB in hoge concentraties in de cel apoptose teweeg kan brengen, lijkt p210 ook hier een adjuvans functie vervuld te hebben. Veel van de vaccinatie studies met p210 die een reductie aan atherosclerose hebben geweten aan stimulatie van verschillende T cel populaties hebben ook cationisch runderserum albumine als dragereiwit gebruikt voor de vaccinaties met p210. Aangezien vaccinatie met cationisch runderserum albumine afzonderlijk al eerder leidde tot een reductie in atherosclerose, zou adjuvans functie van p210

mogelijk dit effect versterkt kunnen hebben in studies waarin cationisch runderserum albumine gebruikt werd als dragereiwit.

Het verminderen van atherosclerose door vaccinatie met van ApoB100 afgeleide CD4 T cel epitopen blijkt wel mogelijk, aangezien immunisatie met ApoB100 afgeleide peptiden die voorspeld zijn aan MHC-II te binden, leidde tot een reductie in atherosclerose. Vaccinatie met deze CD4 T cel epitopen leidde tot een verhoogde IL-10-expressie in aorta's van ApoB100-peptide-gevaccineerde muizen wat suggereert dat regulerende CD4-T-cellen werden geïnduceerd door vaccinatie, alhoewel de totale FoxP3-cel levels niet waren verhoogd. In deze studie werd vaccinatie echter aangevangen voordat extensieve atherosclerotische laesies waren gevormd. In een klinische setting zou vaccinatie in eerste instantie waarschijnlijk plaats vinden in risicogroepen en mensen die al zware plaque vorming hebben. Daarom zou het interessant zijn om te beoordelen of deze tolerogene en protectieve immuun reactie op ApoB100 afkomstige CD4 epitoop vaccinatie nog steeds kan worden bewerkstelligd als pas met vaccinatie wordt begonnen als atherosclerose al reeds aanwezig is. Gedurende de ontwikkeling van atherosclerose wordt namelijk de immuun tolerantie tegen plaque antigenen afgebroken, wat zou kunnen leiden tot een andere, misschien wel pathogene, immuunreactie na vaccinatie met CD4 epitopen van plaque antigenen in latere stadia atherosclerose ontwikkeling. Het aanbieden van deze CD4 T cel epitopen in een meer tolerogene setting, zoals een mucosale toediening in combinatie met tolerogene adjuvans, zou een aantrekkelijke strategie kunnen zijn om plaque antigen specifieke CD4 Tregs op te wekken om de atherosclerotische immuunrespons te remmen.

Er is veel minder bekend over de relevantie van CD8 T-cellen in de context van atherosclerose, hoewel CD8 T-cellen in grote hoeveelheden aanwezig zijn in de atherosclerotische plaque en daar een geactiveerd fenotype lijken te hebben. CD8 T-cellen zijn gespecialiseerd in het doden van specifieke cellen die herkend worden aan een specifiek peptide gepresenteerd op MHC-I via interactie met de T-cel receptor (TCR) op de CD8 T cel. Het algemene beeld is dat CD8 T cellen, door afgifte van inflammatoire cytokines vooral atherosclerose bevorderen. Bepaalde antigen specifieke CD8 T cel reacties zijn echter geïdentificeerd die atherosclerose remmen. Omdat ApoB100 wordt beschouwd als een van de belangrijkste plaque antigenen, wilden we de rol van ApoB100-specifieke CD8 T-cellen in atherosclerose beoordelen. Presentatie van plaque-bestanddelen, waaronder ApoB100 door antigen presenterende cellen in de plaque, zou antigen presenterende cellen vatbaar kunnen maken voor het dood maken door ApoB100-specifieke CD8 T-cellen. Omdat verminderde celdood van macrofagen resulteert in toegenomen atherosclerose, stelden we de hypothese dat het vermeerderen van ApoB100-specifieke CD8 T-cellen het doden van plaque-macrofagen door CD8 T-cellen zou kunnen bevorderen en daarmee therapeutisch relevant kan zijn.

Daarom hebben we deze hypothese getest in een gehumaniseerd muismodel met muizen met transgene expressie van humaan ApoB100 en MHC-I (HLA-A2), en hebben deze muizen geïmmuniseerd met van humaan ApoB100 afkomstige CD8 T cel epitopen die op humaan

MHC-I (HLA-S2) kunnen worden gepresenteerd in **hoofdstuk 5.** Aan de hand van modellen voor peptideverwerking en HLA-A2-binding, selecteerden we 6 van ApoB100 afkomstige peptiden die voorspeld waren gepresenteerd te worden op HLA-A2. HLA-A2-binding werd bevestigd voor alle 6 peptiden. Door middel van toediening van dendritische cellen beladen met de peptiden konden we in HLA-A2 transgene muizen zonder humaan ApoB100, ook voor 5 van de 6 peptiden een peptide specifieke CD8 T cel reactie opwekken. Daarna hebben we in atherosclerose studies met muizen die humaan ApoB100 en HLA-A2 tot expressie brengen atherosclerose studies gedaan. Opnieuw activeerden we initieel CD8 T-cellen met peptide beladen dendritische cellen. Na een week kregen de muizen een booster vaccinatie met poly(I: C) en een CD40 stimulerend antilichaam. Dit vaccinatieregime was eerder effectief in het induceren van specifieke CD8 T cellen tegen neo-epitopen uit tumoren. De CD8 T cellen geactiveerd door deze vaccinatie waren effectief in het penetreren en doden van tumoren, wat aangeeft dat deze vaccinatieaanpak migrerende en functionele CD8 T cellen oplevert.

Voor ApoB $_{406-414}$, ApoB $_{3070-3078}$ en ApoB $_{4531-4539}$, werden immuunspecifieke CD8 T cellen gedecteerd in de milt van ApoB100-peptide gevaccineerde muizen, 8-9 weken na boostervaccinatie bij opoffering, wat aangeeft dat de vaccinatie succesvol was. Interessant genoeg konden we in deze studie geen ApoB $_{406-417}$ en ApoB $_{2356-2364}$ specifieke CD8 T cellen detecteren in de met ApoB100 peptide behandelde muizen. Het zou kunnen dat endogene expressie van humaan ApoB100 in deze muizen heeft geleid tot negatieve selectie in de thymus of inductie van perifere tolerantie van ApoB $_{406-417}$ en ApoB $_{2356-2364}$ specifieke CD8 T cel klonen. In mediastinale lymfeknopen waren ook ApoB100 peptide specifieke CD8 T cellen aanwezig maar dit konden we niet detecteren in de aortabogen, een plek met veel atherosclerose formatie. Het verhoogde percentage effector CD8 T cellen in het bloed, en het verhoogde CD8 T cel percentage in de aorta na vaccinatie met de CD8 T cel epitopen, en eerder behaalde resultaten met dit immunisatieregime, suggereert dat het zeer aannemelijk is dat ApoB100 peptide specifieke CD8 T cellen circuleerden in het bloed en ook naar de plaque migreerden. Het zou kunnen dat de plaque omgeving ervoor zorgt dat CD8 T cellen minder effectief reageren op stimulatie van de T cel receptor door de ApoB100 peptiden. Dit zou een CD8 T cel intrinsiek effect kunnen zijn. Chronische antigeen blootstelling in de plaque zou kunnen leiden tot CD8 T cel uitputting. In overeenstemming met CD8 T cel uitputting produceren CD8 T cellen afkomstig uit de plaque minder cytokines en werd verhoogde expressie van het activatie remmende PD-1 waargenomen bij atherosclerosepatiënten. Aan de andere kant werd ook verhoogde expressie van het activatie remmende PD-L1 gevonden op macrofagen uit menselijke laesies, waarmee activatie van CD8 T cellen in de plaque geremd zou kunnen worden door andere cellen in de plaque omgeving.

In lijn met de afwezigheid van detecteerbare CD8 T cel activatie door de ApoB100 peptiden in de aortabogen, zagen we geen verschil in cellulaire inhoud van de plaques en de grootte en stabiliteit van de plaque in behandelde en onbehandelde muizen. Omdat vaccinatie met deze van ApoB100 afgeleide CD8 T cel epitopen wel duidelijke CD8 T cel reacties opwekte maar dus geen invloed had op atherosclerose, lijkt vaccinatie met van ApoB100 afgeleide CD8

T cel epitopen niet effectief om atherosclerose te behandelen. Bovendien zou het opwekken van een sterke CD8 T cel reactie tegen een endogeen eiwit kunnen leiden tot auto-immuniteit en weefselbeschadiging in organen waarin het eiwit endogeen tot expressie wordt gebracht. Daarom zal de inductie van specifieke tolerantie tegen plaque-antigenen of het verminderen van de algehele ontsteking waarschijnlijk effectiever zijn voor de behandeling van atherosclerose dan het induceren van antigeen-specifieke CD8 T cel reacties.

Alhoewel momenteel geen geneesmiddel gericht op het vermindering van ontsteking is goedgekeurd voor de behandeling van atherosclerose door de FDA en EMA, hebben de LODOCO en CANTOS klinische studies aangetoond dat een reductie in cardiovasculair risico kan worden bereikt door remming van het immuunsysteem. Omdat remming van het immuunsysteem via remming van het immunoproteasoom met specifieke remmer ONX-0914 het ziektebeeld in meerdere experimentele auto-immuun modellen verbeterde, hebben we effect van behandeling met ONX-0914 op atherosclerose bestudeerd in **hoofdstuk 6**. Het immunoproteasome is een proteasoom-variant die vooral aanwezig is in immuuncellen, maar ook tot expressie gebracht kan worden door inflammatoire signalen zoals interferon-γ. Proteasomen breken het overgrote deel van de eiwitten af die moeten worden afgebroken in de cel. Daartoe bevatten proteasomen 3 verschillende catalytische subunits die na verschillende aminozuren eiwitten kunnen knippen. Immunoproteasomen en normale proteasomen verschillen in deze katalytische subunits, waardoor de verschillende proteasomen door afbraak van eiwitten ook verschillende peptides creëren. Meer peptides gecreëerd door het immunoproteasome kunnen goed binden aan MHC-I. Daarmee is het immunoproteasome belangrijk voor het mogelijk maken van CD8 T cel reacties. Meer recent hebben gen-deletie studies en studies met specifieke immunoproteasoom remmers laten zien dat het immunoproteasoom betrokken is bij de regulatie van veel meer celprocessen in immuuncellen, zoals cytokine productie, proliferatie en differentiatie. Hoe immunoproteasoom remming precies leidt tot immunosuppressie is niet precies duidelijk. Er zijn echter aanwijzingen dat door de remming van het immunoproteasome, door ophoping van niet gevouwen-, slecht gevouwen-, geoxideerde-, en geubiquitineerde eiwitten de ongevouwen eiwit reactie wordt gestart. Voor sommige eiwitten waarvan de hoeveelheid omhoog gaat gedurende de ongevouwen eiwit reactie is een immunosuppressieve werking bekend. Het is mogelijk dat op deze manier activatie van immuun cellen wordt beperkt die stimuli uit de omgeving niet goed kunnen verwerken, waarmee mogelijk onnodige immuun gemedieerde weefselschade voorkomen wordt. In lijn met de eerder gerapporteerde immunosuppressieve werking van ONX-0914, werd de hoeveelheid en activatie van conventionele dendritische cellen verminderd door ONX-0914 behandeling. Verder waren in verschillende immuun compartimenten verminderde niveaus van geheugen CD4 en CD8 T cellen zichtbaar. Omdat de verhoogde activatie van DCs en verhoogde niveaus van circulerende geheugen T cellen gecorreleerd zijn met meer atherosclerose, zullen de waargenomen veranderingen in dendritische cellen en T cellen geïnduceerd door ONX-0914, waarschijnlijk bij hebben gedragen aan de verminderde atherosclerose gezien in met ONX-0914 behandelde groep .

Naast het ontwikkelen van atherosclerose ontwikkelen de LDLr deficiënte muizen die we gebruikt hebben in **hoofdstuk 6** op westers dieet, obesitas, metabool syndroom en insulineresistentie. Naast het verbeteren van atherosclerose, verminderde ONX-0914 het lichaamsgewicht in meerdere experimenten met WTD gevoede muizen. De massa van wit vetweefsel was aanzienlijk verminderd in alle onderzoeken, terwijl de vetvrije massa niet werd beïnvloed door de behandeling met ONX-0914. Samenvallend met de vermindering in wit vet, zagen we verbeteringen in metabole parameters zoals verlaagde insulineniveaus, verlaagde bloedglucose na vasten, verlaagde TG-spiegels, en verlaagde cholesterol gehaltes in het bloed. Voor zover bekend bij ons, is dit de eerste studie die aantoont dat remming van het immunoproteasoom met behulp van de LMP7- en LMP2- specifieke remmer ONX-0914 behalve zijn immuun modulerende effecten ook metabole effecten heeft. Dit is waarschijnlijk gekoppeld aan het gebruik van een westers dieet in onze studie, wat leidt tot obesitas en de ontwikkeling van metabool syndroom, in plaats van het gebruik van magere muizen op een normaal dieet in andere onderzoeken met ONX-0914. Bovendien is ONX-0914 veel getest in andere ziektemodellen waarin gewichtsverlies door het opgewekte ziektebeeld aanwezig is, zoals in arthritis of colitis modellen, waardoor het effect van ONX-0914 op gewichtsverlies in deze studies misschien gemaskeerd is geweest.

Gewichtsverlies bleek niet afhankelijk van verminderde voedselinname of verhoogd energieverbruik wat we bepaalden in metabole kooien. Verder zagen we in deze studie ook geen versnelde klaring van cholesterol en vetzuren uit in de bloedbaan toegediende op VLDL lijkende deeltjes. Bij orale toediening van olijfolie werd echter een verminderde verhoging van de triglyceride niveaus in het bloed vastgesteld in ONX-0914 behandelde muizen. Omdat de klaring van cholesterol en triglyceriden uit het bloed eerder niet was verhoogd, duiden deze resultaten op verlaagde lipidenopname in de darmen door ONX-0914 behandeling. Evenzo werd verminderde opname van lipiden in de darmen waargenomen in dieren zonder expressie van LMP7. In LMP7 deficiënte muizen werd verminderde lipide opname geweid aan een verlaagde expressie van lipases uit de pancreas. Dit observeerden wij echter niet. Wij observeerden dat ONX-0914 herhaaldelijk leidde tot accumulatie van neutrofielen en macrofagen in wit vetweefsel. Mature vetcellen geïsoleerd uit gonadaal wit vetweefsel bleken ook immunoproteasomale katalytische subunits tot expressie te brengen en verhoogden expressie van CCL2 na incubatie in kweek met ONX-0914. CCL2 is zeer potent in het aantrekken van immuuncellen. Daarom zou de verhoogde expressie van CCL2 in vetweefsel na behandeling met ONX-0914 zeer waarschijnlijk bij hebben gedragen aan de accumulatie van neutrofielen en macrofagen in wit vetweefsel. Interessant is dat patiënten met een verlies van functiemutatie in het PSMB8 gen, dat codeert voor het LMP7 eiwit, zichzelf presenteren met vergelijkbare symptomen zoals ontsteking van het witte vetweefsel en sterk verminderde opbouw van vetweefsel. Behandeling met ONX-0914, na het doden van macrofagen in de buikholte met clodronate liposomen, leidde niet tot verminderde triglyceride niveaus in het bloed zoals het geval was met ONX-0914 apart. Daarmee lijkt het effect dat wij zien op lipide opname in de darm afhankelijk te zijn van macrofagen. Er zijn meerdere factoren die door macrofagen geproduceerd kunnen worden

waarvan is beschreven dat ze of gewichtsverlies of verminderde opname van vetten in de darm kunnen bewerkstelligen, zoals GDF15, IL-15 en IL-1β. Aangezien we in muizen zonder expressie van GDF15 ook gewichtsverlies observeerden door behandeling met ONX-0914, lijkt GDF15 niet betrokken bij het gewichtsverlies tot stand gebracht door ONX-0914. Een veel voorkomende bijwerking van het (atheroprotectieve) IL-1β neutraliserende antilichaam canacinumab (Ilaris) is gewichtstoename. Het zou daarom interessant zijn om te onderzoeken of IL-1β verantwoordelijk was voor het door ONX-0914 veroorzaakte gewichtsverlies.

We hebben aangetoond dat de behandeling van muizen met ONX-0914 atherosclerose en de witte vet massa aanzienlijk vermindert, en parameters van metabool syndroom verbetert. Atherosclerose als wereldwijde doodsoorzaak nummer 1, en de wereldwijde obesitas epidemie die leidt metabool syndroom gerelateerde ziekten, vragen om effectieve therapieën. Ons onderzoek wekt de suggestie dat immunoproteasomale inhibitie een waardevol therapeutisch hulpmiddel zou kunnen zijn voor deze gezondheids dilemma's. Op dit moment zijn fase 1b-onderzoeken bij SLE-patiënten met de immunoproteasoom remmer KZR-616 bezig, met fase 2 klinische onderzoeken gericht op de behandeling van lupus nefritis, dermatomyositis, polymyositis, auto-immuun hemolytische anemie en immuuntrombocytopenie, op de planning. Het is interessant om in de gaten te houden of immunoproteasomale remming in deze patiënten groepen ook leidt tot gewichtsverlies en of er aanwijzingen zijn voor verminderde cardiovasculaire mortaliteit.

Toekomstperspectieven

Het recente succes van de CANTOS-studie, waarin zware cardiovasculaire gebeurtenissen vermindert werden door toediening van een neutraliserend monoklonaal antilichaam tegen het pro-inflammatoire cytokine IL-1β (Canakinumab), laat zien dat modulatie van het immuunsysteem ook in de mens een haalbare manier is om atherosclerose te behandelen en het cardiovasculaire risico te verminderen. In overeenstemming met het paradigma dat het systemisch verlagen van ontsteking atherosclerose kan verminderen en belangrijke cardiovasculaire gebeurtenissen kan voorkomen, zijn klinische onderzoeken met lage doses van de immunosuppressiva methotrexaat en colchicine bezig. Indien succesvol, zouden lage dosis methotrexaat en colchicine de eerste op immunomodulatie toegespitste goedgekeurde behandelingen voor atherosclerose kunnen worden. Afhankelijk van het succes van de klinische onderzoeken met immunoproteasomale remmers in de context van andere auto-immuunziekten, zouden klinische studies met betrekking tot immunoproteasomale remming voor behandeling van atherosclerose en obesitas kunnen worden overwogen. Nadelen van algemene immunsuppressiva zijn natuurlijk de verhoogde vatbaarheid voor infectieziekten, maar methotrexaat en colchicine zijn ook andere bijwerkingen gemeld, zoals gastro-intestinale problemen. Vervelende bijwerkingen van medicatie die voor langere tijd gebruikt moeten worden, leiden tot verminderde therapietrouw van de patiënt.

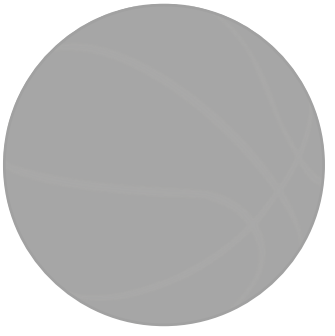
Aan vaccinatiebenaderingen om atherosclerose te verminderen die specifiek zijn gericht op het moduleren van de immuunreactie tegen plaque-antigenen, en met name het herstellen van tolerantie voor plaque-antigenen, zullen waarschijnlijk veel minder bijwerkingen vast zitten. In preklinische atherosclerose modellen is het induceren van tolerantie tegen plaque antigenen, waaronder (geoxideerd) LDL en ApoB100 afkomstige peptiden, effectief geweest in het reduceren van atherosclerose, maar heeft nog geen vervolg gevonden in klinische studies. Het opwekken van tolerantie voor myeline afkomstige peptiden leidde bij multiple sclerose (MS) patiënten in een afname van antigeen specifieke T cel reacties tegen de myeline peptiden in een fase 1 onderzoek. Dit onderzoek laat zien dat het haalbaar is om auto-immuniteit te doorbreken in de mens met gerichte tolerogene vaccinatie strategieën . Momenteel is onze kennis over de antigeen specifieke T-celreacties die plaatsvinden in atherosclerose echter zeer beperkt maar zijn er CD4 T cel reacties tegen onder andere LDL, collageen type V en HSP's in humane atherosclerose gerapporteerd. In **hoofdstuk 3** waren we in staat atherosclerose te verminderen door het opwekken van tolerantie voor geoxideerd LDL door orale toediening van geoxideerd LDL. We konden echter geen bescherming tegen atherosclerose bereiken door de orale toediening van het p210-peptide, afkomstig van het ApoB100-eiwit dat aanwezig is in LDL. Het is waarschijnlijk dat een atheroprotectief effect van orale p210 administratie uit bleef door het ontbreken van een CD4 T cel epitoop in p210. Dit is direct ook een moeilijkheid voor het ontwikkelen van een tolerogeen vaccin voor de behandeling van atherosclerose. De identificatie van geschikte CD4 T-cel epitopen voor tolerisatie is lastig en door de vele variaties in menselijke MHC-allelen zullen veel epitopen slechts bij een deel van de patiënten het MHC-II kunnen binden, en dus effectief kunnen zijn. Daarom zal voor tolerantie inductie voor de behandeling van atherosclerose moeten worden overwogen om met (delen van) plaque antigenen, of meerdere CD4 T cel epitopen te vaccineren. Het gebruik van verschillende plaque antigenen en meerdere epitopen voor vaccinatie zou ook meer T cel klonen kunnen moduleren, wat waarschijnlijk een sterker effect teweeg zal brengen dan het tolerogeen moduleren van een enkele T cel kloon. Betere karakterisatie van de adaptieve immuunrespons in humane atherosclerosis, zoals identificatie van antigenen en antigene epitopen zou daarmee zeer gunstig kunnen zijn voor de behandeling van atherosclerose. Een andere belangrijke hindernis voor het toepassen van tolerantie-inductie bij de mens is de ontwikkeling van de meest effectieve en veilige tolerantieregimes.

Naast het moduleren van de CD4 T cel respons, zijn er studies die laten zien dat vaccinatie met p210 en runderserum albumine als dragereiwit resulteerde in verminderde atherosclerose via activatie van CD8 T cellen. Wij konden echter geen beschermende effecten van vaccinatie met p210 onderscheiden, en zagen geen activatie van CD8 T cellen door vaccinatie met p210 in **hoofdstuk 4**. Verder zagen we ook geen reductie in atherosclerose in muizen gevaccineerd met CD8 T cel epitopen, wat wel leidde tot sterke CD8 T cel activatie, in **hoofdstuk 5**. Naast dat we geen atheroprotectief effect konden onderscheiden van vaccinatie met ApoB100 afkomstige CD8 T cel epitopen, is activatie van CD8 T cellen tegen plaque antigenen waarschijnlijk geen goed idee voor behandeling van atherosclerose. Door het opwekken van een CD8 T cel reactie tegen endogeen tot expressie komende eiwitten

bestaat de kans op weefselschade op plekken waar het eiwit endogeen wordt geproduceerd. Vaccinatiestrategieën met CD8 T cellen in een experimentele omgeving kunnen echter nog steeds zeer bruikbare inzichten verschaffen over de rol van CD8 T-cellen in atherosclerose.

Naast modulatie van T-cel responsen, is inductie van antilichamen (IgG) tegen verschillende plaque-antigenen in staat geweest om atherosclerose in preklinische studies te verminderen. Ook antilichamen tegen (epitopen) van (ox)LDL, waaronder p210, resulteerden in verlaagde atherosclerose in preklinisch studies. Wij konden de atheroprotectieve eigenschappen van p210-antilichamen in **hoofdstuk 4** niet onderschrijven. Mogelijk is dit een gevolg van het gebruik van LDLr deficiënte muizen in onze studie doordat p210 deel uitmaakt van de LDLr bindingsplaats in ApoB100 in plaats van ApoE deficiënte muizen gebruikt in andere studies. Van p210 antilichamen en andere antilichamen tegen LDL is beschreven dat het de klaring van LDL kan versnellen en daarmee het circulerende cholesterol kan verlagen, wat dan weer leidde tot verminderde atherosclerose. Als dit inderdaad het mechanisme vormt waarmee antilichamen gericht tegen LDL atherosclerose verminderen, is het de vraag of deze antilichamen nog een additioneel gunstig effect hebben in combinatie met huidige en toekomstige lipide verlagende therapieën. Van antilichamen tegen collageen type VI werd gevonden dat het activatie van macrofagen kan remmen en expressie van cholesterol efflux-genen kan bewerkstelligen door te binden aan FC-receptoren. Onze kennis van de effecten en de werkingsmechanismen van deze antilichamen op atherosclerose is nog zeer beperkt, maar er zijn indicaties dat antilichamen gericht tegen plaque antigenen atheroprotectief kunnen werken. Via mucosale toediening van (delen van) plaque antigenen zouden tegelijkertijd therapeutisch gunstige antilichamen en tolerogene regulatoire CD4 T cellen opgewekt kunnen worden, en activatie van pathogene CD4 T cel en CD8 T cel types voorkomen kunnen worden.

Er zijn verschillende uitvoerbare benaderingen waarmee modulatie van het immuunsysteem kan worden ingezet om atherosclerose te verminderen. Algemene immunosuppressie in de vorm van lage dosis colchicine wordt momenteel getest in klinische studies voor de behandeling van atherosclerose, en zal indien succesvol waarschijnlijk de eerste op immunomodulatie toegespitste behandelingen vormen die beschikbaar komen voor de behandeling van atherosclerose. Antigen-specifieke modulatie van de immuunrespons voor behandeling van atherosclerose is verder weg, maar preklinische studies hebben aangegeven dat dergelijke benaderingen mogelijk zijn voor de behandeling van atherosclerose. Een combinatie van lipide- en immuun management, en het bevorderen van een gezonde levensstijl zullen waarschijnlijk de preventieve maatregelen van de toekomst omvatten om atherosclerose en hart- en vaatziekten te voorkomen.



Curriculum Vitae

Scientific Publications

PhD Portfolio

Curriculum Vitae

Frank Schaftenaar werd op 24 februari 1986 geboren te Utrecht. In 2004 behaalde hij zijn gymnasium diploma aan het Blaucapel College in Utrecht. In het najaar van 2004 begon hij aan de studie Biomedische Wetenschappen in Utrecht. Tijdens zijn bachelor stage ambieerde hij nog een professionele basketbal carriere, wat leidde tot een lichte studievertraging, en uiteindelijk het behalen van de Bachelor of Science titel in begin 2010. Toen bleek dat een professionele basketbalcarriere er toch echt niet in zat, begon hij september 2010 aan de master "Biology of Disease" in Utrecht.

Zijn eerste masterstage liep hij bij de afdeling Pathobiology van de Faculteit Diergeneeskunde van de Universiteit Utrecht onder leiding van dr. W.J. Bakker en prof. dr. A. de Bruin. Tijdens deze stage in het veld van fundamenteel kanker-onderzoek onderzocht hij welke transcriptiefactor-bindingsplaatsen van invloed zijn op de activatie van de VEGFA promotor onder invloed van transcriptiefactor E2F7. Deze stage resultereerde in een co-auteurschap van een publicatie in *the EMBO journal*. In 2012 voerde hij een tweede wetenschappelijke stage uit bij het Centrum Infectieziektebestreiding van het Rijksinstituut voor Volksgezondheid en Milieu onder begeleiding van S.K. Rosendahl Huber en Dr. J. Van Beek. Daar zette hij een dendritische cell – CD8 T cell co-culture protocol op om de effectiviteit van gemodificeerde CD8 T cell epitopen afkomstig van het influenza virus te testen.

Zijn masterscritptie voerde hij eind 2012 wederom uit bij de afdeling Pathobiology van de Faculteit Diergeneeskunde van de Universiteit Utrecht onder begeleiding van dr. ir. M.J.M. Toussaint en prof. dr. A. de Bruin, met also onderwerp het de rol van ischemie en reperfusie in de ontwikkeling van leverkanker. Met het afronden van de master thesis behaalde hij eind 2012 de Master of Science titel.

Van februari 2014 tot oktober 2018 werkte hij als promovendus bij de afdeling BioTherapeutics van het Leiden Academic Centre for Drug research, begeleidt door dr. G.H.M van Puijvelde en prof. dr. J. Kuiper.

Vanaf 16 april 2019 is hij terug in dienst bij de afdeling BioTherapeutics van het Leiden Academic Centre for Drug research als onderzoeker, onder prof. dr. J. Kuiper.

Scientific Publications

F.H. Schaftenaar, V. Frodermann, J. Kuiper, E. Lutgens, Atherosclerosis: the interplay between lipids and immune cells., *Curr. Opin. Lipidol.* 27, 209–15 (2016). (Review)

F.H. Schaftenaar, J. Amersfoort, H. Douna, M.J. Kröner, G.H.M van Puijvelde, I.Bot, J. Kuiper. Protection from atherosclerosis induced by oxLDL tolerization is not reinforced by polyclonal Treg induction. Manuscript submitted.

F.H. Schaftenaar, J. Amersfoort, H. Douna, M.J. Kröner, P.J. van Santbrink, A.C. Foks, G.H.M van Puijvelde, l. Bot, Johan Kuiper. Vaccination with ApoB100 derived peptide p210 does not provide atheroprotection in LDLr deficient, hApoB100/100 transgenic mice. Scientific Reports, accepted.

F.H. Schaftenaar, J. Amersfoort, H. Douna, M.J. Kröner, A.C. Foks, I. Bot, B.A. Slütter, G.H.M. van Puijvelde, J.W. Drijfhout, J. Kuiper. Induction of HLA-A2 restricted CD8 T cell responses against ApoB100 peptides does not affect atherosclerosis in a humanized mouse model. Manuscript submitted.

F.H. Schaftenaar, A.D. van Dam, G. de Bruin, J. Amersfoort, H. Douna, M.J. Kröner, P.J. van Santbrink, A.C. Foks, G.H.M van Puijvelde, I. Bot, B.I. Florea, H.S. Overkleeft, P.C.N. Rensen, J. Kuiper. Immunoproteasomal inhibition with ONX-0914 attenuates atherosclerosis and reduces white adipose tissue mass and metabolic syndrome. Manuscript submitted.

J. Amersfoort, **F. H. Schaftenaar**, H. Douna, P. J. van Santbrink, M. J. Kröner, G. H. M. van Puijvelde, P. H. A. Quax, J. Kuiper, I. Bot, Lipocalin-2 contributes to experimental atherosclerosis in a stage-dependent manner., *Atherosclerosis* 275, 214–224 (2018).

H. Douna, J. Amersfoort, **F. H. Schaftenaar**, S. Kroon, G. H. M. van Puijvelde, J. Kuiper, A. C. Foks, Bidirectional effects of IL-10+ regulatory B cells in Ldlr-/- mice., *Atherosclerosis* 280, 118–125 (2019).

J. Amersfoort, H. Douna, **F. H. Schaftenaar**, A. C. Foks, M. J. Kröner, P. J. van Santbrink, G. H. M. van Puijvelde, I. Bot, J. Kuiper, Defective Autophagy in T Cells Impairs the Development of Diet-Induced Hepatic Steatosis and Atherosclerosis., *Front. Immunol.* 9, 2937 (2018).

H. Douna, J. Amersfoort, **F. H. Schaftenaar**, M. J. Kröner, M. G. Kiss, B. Slütter, M. A. C. Depuydt, M. N. A. Bernabé Kleijn, A. Wezel, H. J. Smeets, H. Yagita, C. J. Binder, I. Bot, G. H. M. van Puijvelde, J. Kuiper, A. C. Foks, B- and T-lymphocyte attenuator stimulation protects against atherosclerosis by regulating follicular B cells, *Cardiovasc. Res.* (2019), doi:10.1093/cvr/cvz129.

E. Kritikou, J. van Duijn, J. E. Nahon, T. van der Heijden, M. Bouwman, C. Groeneveldt, **F. H. Schaftenaar**, M. J. Kröner, J. Kuiper, G. H. M. van Puijvelde, I. Bot, Disruption of a CD1d-mediated interaction between mast cells and NKT cells aggravates atherosclerosis., *Atherosclerosis* 280, 132–139 (2019).

B. G. M. W. Weijts, W. J. Bakker, P. W. A. Cornelissen, K. H. Liang, **F. H. Schaftenaar**, B. Westendorp, C. A. C. M. T. De Wolf, M. Paciejewska, C. L. G. J. Scheele, L. Kent, G. Leone, S. Schulte-Merker, A. De Bruin, E2F7 and E2F8 promote angiogenesis through transcriptional activation of VEGFA in cooperation with HIF1, *EMBO J.* 31, 3871–3884 (2012).

E. Kritikou, G. H. M. van Puijvelde, T. van der Heijden, P. J. van Santbrink, M. Swart, **F. H. Schaftenaar**, M. J. Kröner, J. Kuiper, I. Bot, Inhibition of lysophosphatidic acid receptors 1 and 3 attenuates atherosclerosis development in LDL-receptor deficient mice., *Sci. Rep.* 6, 37585 (2016).

A. B. Ouweneel, M. Hoekstra, E. J. van der Wel, **F. H. Schaftenaar**, O. S. C. Snip, J. Hassan, S. J. A. Korporaal, M. Van Eck, Hypercholesterolemia impairs megakaryopoiesis and platelet production in scavenger receptor BI knockout mice., *Atherosclerosis* 282, 176–182 (2019).

J. van Duijn, E. Kritikou, N. Benne, T. van der Heijden, G. H. van Puijvelde, M. J. Kröner, **F. H. Schaftenaar**, A. C. Foks, A. Wezel, H. Smeets, H. Yagita, I. Bot, W. Jiskoot, J. Kuiper, B. Slütter, CD8+ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4+ T-cell responses., *Cardiovasc. Res.* 115, 729–738 (2019).

Phd Portfolio

Courses

2015	Data Management Course for PhD students
2014	LACDR Introductory Course on Drug Research & PhD Education Program
2014	Introduction to teaching and supervision for LACDR PhD students
2014	Time management, self -management
2014	Communication in Science
2014	Proefdierkunde

Presentations

2019	Genius consortium meeting, Amsterdam, Nederland
2018	International atherosclerosis society (IAS), Toronto, Canada
2018	Scandinavian Atherosclerosis Society (SSAR), Humlebæk, Denemarken
2018	LACDR Spring Symposium, Leiden, Nederland
2017	Rembrandt Symposium, Noordwijkerhout, Nederland
2017	Scandinavian Atherosclerosis Society (SSAR), Humlebæk, Denemarken
2017	LACDR Spring Symposium, Leiden, Nederland
2016	LACDR Spring Symposium, Leiden, Nederland
2016	Vaccination in Atherosclerosis consortium meeting, Trolleholm, Zweden
2015	Rembrandt Symposium, Noordwijkerhout, Nederland
2015	LACDR Spring Symposium, Leiden, Nederland
2014	Vaccination in Atherosclerosis consortium meeting, Leiden, Nederland
2014	LACDR Spring Symposium, Leiden, Nederland