CD8+ T-cells in Atherosclerosis: mechanistic studies revealing a protective role in the plaque microenvironment

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"Not even knowledge takes all strangeness from the world."

Sarah Perry, *The Essex Serpent*

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General Introduction

1

1. Cardiovascular disease

The collective term cardiovascular disease (CVD) entails all diseases that involve the heart and blood vessels. CVD is a major killer worldwide, accounting for 31% of all deaths [1]. The main manifestations of CVD are coronary heart disease and cerebrovascular disease, in which the blood vessels of the heart or brain become obstructed and reduce regional blood flow. This induces a deficiency of oxygen and nutrients in the myocardial or brain tissue, resulting in myocardial infarction or stroke [2, 3]. Major risk factors for developing CVD include high levels of serum low-density lipoprotein (LDL)-cholesterol and triglycerides, hypertension, smoking, diabetes, a sedentary lifestyle and obesity [4]. Furthermore, familial hypercholesteremia and underlying autoimmune diseases, such as rheumatoid arthritis, Graves' disease, and systemic lupus erythematosus, also increase the risk of CVD [5, 6]. Recent advances in the treatment of CVD have resulted in a decrease in stroke incidence in western countries [7, 8]. These improved strategies include improvement in visualization techniques (which enables doctors to identify high-risk patients that require treatment more effectively), promoting healthy lifestyle choices and new medical treatments. Nevertheless, the consequences of CVD remain a major economic burden in both Europe and the United States [9, 10]. Moreover, CVD is becoming a growing problem in developing countries, where there also is a higher incidence of case-fatality rates compared to the Western world [11].

2. Atherosclerosis

The main underlying pathological process that drives CVD is atherosclerosis, a chronic inflammatory disease affecting medium- and large-sized arteries. Atherosclerotic pathology is characterized by a progressive, but slow, build-up of lesions within the vessels that already starts during childhood [12]. These plaques were described by Fallopius in 1575 as degeneration of arteries into bone. The first detailed microscopic studies were performed by Virchow in 1858, who observed that the plaques formed in, rather than on, the intima at locations of high pressure from the bloodstream by a process of inflammatory proliferation as well as cholesterol accumulation [13]. Great advances in techniques and methodology since these times have led to the generally accepted model that atherosclerosis is the result of endothelial dysfunction which leads to accumulation of cholesterol in the vessel wall, which in turn results in the recruitment of a plethora of immune cells driving ongoing inflammation and plaque development.

Atherosclerotic lesions can remain asymptomatic throughout long periods of time but can become symptomatic upon perfusion defects and the resulting ischemia that can damage tissues. Reduced blood flow to the heart muscle can lead to angina pectoris (chest pain) [14]. Moreover, atherosclerosis can give rise to serious events such as myocardial infarction, which is the result of plaque rupture or erosion, which causes a thrombus to occlude a coronary artery and subsequently leads to the death of myocardial tissue [15]. Furthermore, thrombus formation as a result of atherosclerosis can also cause the dissociation of the thrombus, after which it can circulate and occlude blood

vessels elsewhere, such as in the brain, causing a stroke [16].

2.1. Atherosclerosis initiation

Atherosclerotic lesion formation is initiated at sites where there is a local disturbance in blood flow. The innermost layer of the vessel wall (the intima) consists of vascular endothelial cells (ECs), which are especially sensitive to shear stress, the frictional force generated by blood flow. At sites of low shear stress (such as the aortic arch or bifurcation), mechanoreceptors on the ECs are triggered, which in turn alters the gene expression, triggers inflammatory responses, and results in the upregulation of adhesion receptors upon the ECs such as vascular cell adhesion molecule 1 (VCAM-1) [17]. These changes allow for the adhesion and transmigration of leukocytes into the subintimal space, as well as enhance the permeability of the EC layer. This enables lipoproteins to cross the EC barrier and accumulate in the subendothelial space as well.

Lipoproteins are the main carriers for cholesterol present in the bloodstream and are categorized based on their densities: chylomicrons, very-low-density lipoprotein (VLDL), LDL, intermediate-density lipoprotein (IDL) and high-density lipoprotein (HDL). The cholesterol-rich VLDL and LDL particles deliver cholesterol to the tissues and are considered the most atherogenic lipoproteins. HDL, on the other hand, mediates the reverse transport of cholesterol from the tissues back to the liver for excretion and is therefore considered anti-atherogenic [18]. Infiltration of especially LDL particles into the subendothelial space is an important step in the initiation of atherosclerosis. These particles consist of a high molecular weight protein named apolipoprotein B-100 (ApoB100), neutral and polar lipids, and lipophilic antioxidants. Upon entrapment in the vessel wall, LDL particles can be subjected to oxidative modification both via enzymatic- and non-enzymatic reactions [19]. The resulting oxidized LDL (oxLDL) further stimulates the expression of adhesion molecules on the ECs, as wells as the production of chemoattractant proteins, such as monocyte chemoattractant proteins (MCP-1), leading to the recruitment of monocytes to the lesions [20].

Interactions between monocytes and VCAM-1 as well as intracellular adhesion molecule-1 (ICAM-1) result in the capture of these cells. Subsequently, trans-endothelial diapedesis is facilitated by junctional adhesion molecule-A and platelet/endothelial cell adhesion molecule 1 [21]. Local stimuli within the subendothelial layer induce the differentiation of monocytes into macrophages [22], a cell-type that is specialized in the uptake of cell debris and is able to engulf oxLDL as well. After engulfment, the lipoproteins are converted into cholesterol esters and stored as lipids within the macrophages. In the case of atherosclerosis, there is an overload with cholesterol esters, resulting in the transformation into lipid-rich foam cells [23]. These cells are a hallmark of early atherosclerotic lesions and have received this nomenclature because of their “foamy” appearance upon viewing the many lipid droplets within these cells via microscopy. Foam cells are known to secrete inflammatory factors such as cytokines and chemokines [24, 25]. This ongoing inflammation results in further recruitment of inflammatory cells towards the lesion, among which

are the cells of the adaptive immune system, T- and B-cells [26]. The continued cholesterol accumulation within the foam cells eventually surpasses the toxic threshold, inducing cell death via apoptosis (a regulated, non-inflammatory process) or necrosis (a disruptive, pro-inflammatory process). Apoptotic bodies can be cleared by phagocytic cells in the environment, whereas necrosis leads to the release of a myriad of danger-associated molecular patterns (DAMPs) that contribute to atherogenesis [27]. The remnants of the dying foam cells contribute to the formation of a necrotic core in the lesion.

The aforementioned recruited monocytes can differentiate not only into macrophages but also into dendritic cells (DCs). These are antigen-presenting cells (APCs) specialized in activating the adaptive immune system. DCs express receptors, such as the Toll-like receptors, that recognize pathogen-associated molecular patterns (PAMPs) in the case of infection but are also able to recognize DAMPs. Ligation of these receptors by dying-cell fragments or oxLDL will activate the DCs, which are able to take up antigens and travel to the secondary lymphoid organs, where they can activate T-cells [28].

During atherosclerosis initiation, the inflammatory process within the vessel wall is not resolved but continues in a vicious cycle. An increasing number of cells are recruited that subsequently cannot clear all the cholesterol present in the vessel wall, thereby furthering the inflammatory response, resulting in the formation of an early atherosclerotic lesion that is classified as a “fatty streak” [29] (Fig. 1). This stage of lesion development is asymptomatic and is able to disappear upon normalization of serum cholesterol levels [30]. However, sustained high levels of serum cholesterol combined with a progressive inflammatory cycle can give rise to more progressed atherosclerotic lesions.

2.2. Atherosclerosis progression

The progression from fatty streak lesions towards more advanced atherosclerotic lesions involves an increase in extracellular lipids as well as necrotic area [31, 32]. Furthermore, these plaques show a migration of smooth muscle cells (SMCs) from the arterial media to the intima. Here, the SMCs can proliferate and produce extracellular matrix molecules, resulting in the formation of a fibrous cap [33] (Fig. 1). Expansion of the lesion and its fibrous cap into the lumen leads to partial occlusion of the blood vessel, disturbing normal blood flow [32]. Conditions of hypoxia within the lesion can locally induce neovascularization, facilitating an increased influx of immune cells into the lesion [34]. Moreover, these new blood vessels are subject to a high risk of microvessel leakage, which may lead to intraplaque hemorrhage [34, 35]. Furthermore, the presence of apoptotic bodies, cholesterol crystals and osteogenic factors in the plaque microenvironment can drive arterial calcification, resulting in intraplaque calcium deposits [36].

Thinning of the fibrous cap can occur upon release of extracellular-degrading enzymes, such as matrix metalloproteinases, by macrophages or neutrophils [37]. Gradual thinning of the cap decreases the stability, and may eventually lead to plaque rupture, an event that usually occurs at the “shoulder” regions of the lesion where there is a high

influx of inflammatory cells [38, 39]. Rupture of the plaque exposes pro-thrombotic signals, such as tissue factor and platelet-adhesive molecules, to the bloodstream [40]. This leads to the initiation of the coagulation cascade, resulting in the formation of a thrombus within the blood vessel (Fig. 1). The thrombus can either block the blood flow locally upon the site of lesion rupture, or travel with the blood and block smaller arteries, such as the coronary arteries in the heart, resulting in infarction, or in the brain, resulting in stroke [41]. Alternatively to direct rupture, thrombi can also arise from a gradual erosion of the cap of the lesion, which exposes thrombogenic extracellular matrix material to the blood flow, activating the coagulation cascade and leading to similar clinical events [42].

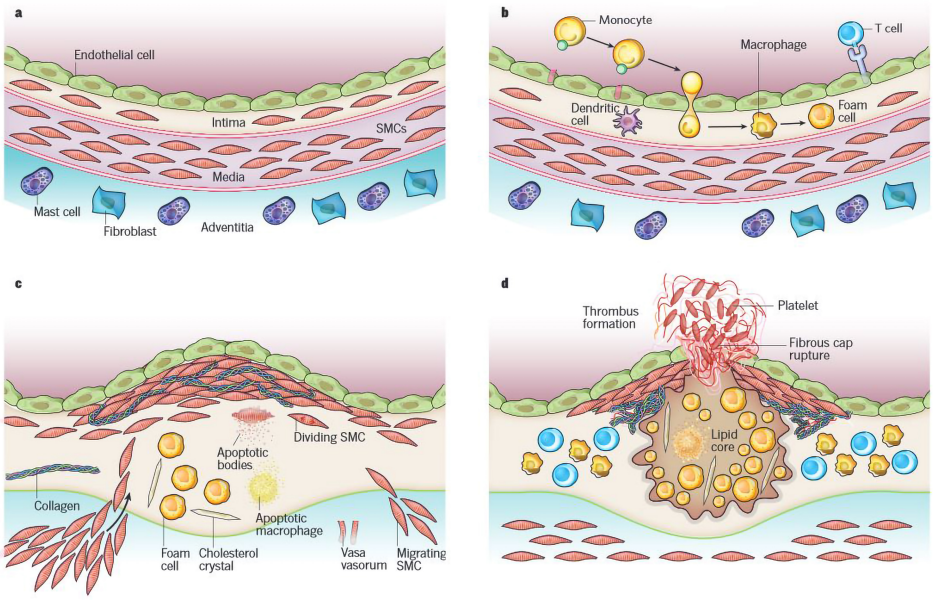

Figure 1: Stages in the development of atherosclerotic lesions. The healthy artery and the changes that occur during disease progression to thrombosis are shown. (A) The normal artery is lined by ECs that are in contact with blood. (B) Atherosclerosis is initiated by adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes into macrophages, and their uptake of lipid, yielding foam cells that form a fatty streak lesion. (C) Lesion progression involves the migration of SMCs to form the cap of the plaque, as well as the heightened synthesis of extracellular matrix macromolecules. Extracellular lipids derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. (D) Vulnerable lesions are prone to rupture, which enables blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow. *Adapted from Libby et al. Nature 2011;473:317*

2.3. Mouse models of atherosclerosis

The use of preclinical models of atherosclerosis is important in order to gain a better understanding of this complex disease. *In vitro* experimentation on cell lines, primary cultures or a mixture of different cell types may be used to investigate impor-

tant aspects of atherogenesis [43, 44]. However, these models cannot (as of yet) mimic the complexity of the atherosclerotic lesion. Alternatively, *ex vivo* culturing of human atherosclerotic lesions offers a more physiologically relevant model to study atherosclerosis, but still shows limited viability over time and cannot reflect the early stages of the disease [45]. Therefore, atherosclerosis is also investigated in different animal models, including non-human primates [46–48], swines [49, 50], rabbits [51], rats [52], and mice [53, 54]. The mouse is the preferred research animal, as they are relatively easy to modify genetically, relatively cheap to purchase and keep, easy to house and breed, and require only a short period of time to develop atherosclerotic lesions compared to the other animals mentioned above [53].

Several different mouse strains are used to study atherosclerosis development and progression. The wild-type (WT) laboratory mouse C57BL/6 is relatively resistant to atherosclerosis and will only develop small lesions when fed an atherogenic diet for 14 weeks [55]. Therefore, genetically modified mouse strains that develop advanced atherosclerotic lesions are more widely used for investigating the development of atherosclerosis as well as for testing therapeutic interventions. LDL receptor (LDLr) deficient mice on a C57BL/6 background have increased levels of circulating VLDL and LDL due to the lack of uptake of these particles by the liver, and are therefore commonly used in atherosclerosis research. On a normal chow diet, these cholesterol levels in the blood are only modestly (2-fold) increased compared to wild-type mice, and atherosclerotic lesions develop slowly [56]. However, feeding these mice a high-fat, high-cholesterol diet results in up to 10-fold increases in plasma cholesterol levels and rapid formation of atherosclerotic lesions [57]. Another widely used atherosclerotic murine model is the apolipoprotein E (apoE) deficient mouse on a C57BL/6 background. ApoE functions as a ligand for receptors that bind VLDL particles and remove them from the circulation. Therefore, apoE$^{-/-}$ mice show a 5-fold increase in plasma cholesterol levels upon feeding a normal chow diet. Feeding these mice a western-type diet (WTD) can further quadruple the plasma cholesterol levels and speed up atherosclerosis development [58]. Lesions of WTD-fed apoE$^{-/-}$ mice are enriched in foam cells, whereas chow-fed apoE$^{-/-}$ mice display more complex and cellular atherosclerotic lesions [53].

In order to investigate the role of the immune system in atherosclerosis development, the generation of new murine models via the transfer of bone marrow into atherogenic mice has proven to be very useful. This involves irradiation of the recipients in order to remove their endogenous bone marrow, followed by reconstitution of the bone marrow via intravenous injection of bone marrow derived from wild-type or genetically modified donor animals. The donor bone marrow will give rise to new blood cells, allowing for the discernment of the genetic defect in the circulating cells from that of genetically normal ECs and SMCs in atherosclerotic lesion development. Importantly, transfer of donor bone marrow expressing apoE into an apoE$^{-/-}$ recipient reduces atherosclerosis, by significantly reducing plasma lipid levels, whereas the transfer of LDLr-expressing bone marrow in WTD-fed LDLr$^{-/-}$ mice does not have a large impact on plasma lipid levels and thus on atherosclerosis development [53, 59, 60]. Therefore, bone marrow transfer experiments are preferably conducted in LDLr$^{-/-}$ mice.

2.4. Treatment of atherosclerosis

Upon a clinical event caused by thrombus formation within the vessel wall, acute treatment of the patient is necessary. Balloon angioplasty can be performed in which a balloon is inflated within the atherosclerotic vessel in order to widen the lumen and restore the blood flow, after which stents can be placed that are left behind once the balloon is removed [61]. Alternatively, coronary bypass surgery can be performed in which the blocked blood flow in a coronary artery is rerouted by transplantation of a healthy vessel onto the coronary artery [62]. Finally, endarterectomy surgery is another option to treat an acute event, in which the affected vessel is opened up and the plaque is surgically removed [63]. The disadvantages of these treatments are that they are invasive and can cause complications, such as restenosis [61, 64, 65]. In the non-acute stage of atherosclerosis, lifestyle changes are advised and pharmacological intervention is possible. The main therapy currently available focuses on lowering plasma cholesterol levels by statins. These drugs inhibit the enzyme HMG-CoA reductase in the cholesterol synthesis pathway, thereby reducing the amount of endogenously produced cholesterol. This induces an upregulation of the LDLr, resulting in increased uptake of circulating LDL and consequently lowers the LDL-cholesterol levels in patients [66]. As stated above, high cholesterol levels result in an elevated risk of a cardiac event. Unfortunately, statin treatment reduces cardiovascular events by only 30% [67]. Recently, protein convertase subtilisin/kexin type 9 (PCSK9) inhibitors have been added to the repertoire of lipid-lowering drugs [68]. However, there are currently no drugs on the market that target the immune component of atherosclerosis. A large clinical trial studying the effect of canakinumab, a monoclonal antibody that blocks the function of the pro-inflammatory cytokine IL-1β, was shown to reduce the incidence of cardiovascular events compared to placebo treatment on top of state-of-the-art lipid treatment [69]. This trial illustrates the potential for the development of new therapeutics that target the inflammatory aspects of atherosclerosis in order to prevent cardiovascular complications.

3. The immune system in atherosclerosis

As mentioned above, dyslipidemia is an important driving force of atherosclerosis development. However, it is the ongoing immune response that drives the disease towards its advanced stages [70]. An immune response is generated toward pathogens in the case of infection, or toward self-molecules that are erroneously identified as non-self in the case of autoimmune disease.

In the first phase of an immune response, innate immune cells are recruited towards the site of infection or damage. This innate response relies on humoral components, such as acute phase proteins, as well as cellular components. The main innate immune cells are macrophages, neutrophils and natural killer cells, which employ non-selective responses in order to clear the infection. Neutrophils are the first responders to tissue damage and release pro-inflammatory mediators as well as neutrophil extracellular traps in order to capture the pathogen and induce a protective inflammatory re-

sponse [71]. Macrophages are phagocytic cells that can take up and destroy pathogens, as well as produce soluble inflammatory mediators [72]. Natural killer cells express receptors that can sense (stress-related) changes in cells and upon activation by these signals, trigger a cytotoxic response that kills the target cells in order to contain an infection.

If despite these mechanisms the inflammatory response persists, adaptive immune cells are recruited that generate a specific response towards an antigen and induce immunological memory. This part of the immune response requires the activation of T- and B-lymphocytes by antigen-presenting cells [71]. Most immune cell types have been shown to play a role in atherosclerosis development, illustrating the importance of the inflammatory component in this disease.

3.1. Macrophages

As previously mentioned, macrophages are derived from circulating monocytes in the blood. Monocytes circulate in the blood, and populate the spleen and bone marrow under homeostatic conditions. They are a short-lived, non-proliferative cell population that is involved in scavenging dead cells and toxic molecules. Monocytes can renew tissue-resident macrophages and DCs. Blood monocytes comprise a heterogeneous population of cells. The most important distinction is that between the classical, inflammatory monocytes (defined as $Ly6C^{high}CCR2^{+}$ in mice and $CD14^{high}CD16^{low}$ in humans) and the non-classical, patrolling monocytes (defined as $Ly6C^{low}CCR2^{-}$ in mice and $CD14^{low}CD16^{high}$ in humans) [73]. The $Ly6C^{high}$ monocytes give rise to the inflammatory macrophage type referred to as M1 macrophages [74], whereas their $Ly6C^{low}$ counterparts are more likely to differentiate into anti-inflammatory (M2) macrophages [75]. The depletion of all monocytes from the circulation is protective against plaque formation in rabbits, suggesting a pro-atherogenic role for (the majority of) these cells [76].

Hypercholesterolemia induces increased monocyte production in the bone marrow [77], resulting in increased levels of monocytes within the circulation of atherosclerosis-prone individuals. Monocytes travel toward atherosclerotic lesions in response to the production of MCP-1, which is produced in the lesion in response to oxLDL. MCP-1 binds the CCR2 receptor on the monocytes, leading to chemotaxis towards the lesions [78]. Upon infiltration into the subintimal space, monocytes can differentiate into macrophages under the influence of local growth factors, such as macrophage colony-stimulating factor (M-CSF) [79]. Macrophages in the lesions can express scavenger receptors, such as CD36 and scavenger receptor B-1, through which they are able to take up oxLDL [80, 81]. This triggers a release of inflammatory cytokines, such as TNFα, interleukin (IL)-1α, IL-1β, and IL-6, which further the inflammatory response [82]. Moreover, macrophages are known to secrete matrix metalloproteinases, which degrade the extracellular matrix and reduce plaque stability, as described above [37].

Different subsets of macrophages in atherosclerotic lesions are described. The classi-

cal model of macrophage activation describes two different types of macrophages: the pro-inflammatory M1 and alternative M2 subsets. Recently, two new subsets have been added to the macrophage repertoire: the Mox and M4 subsets. Cell markers specific for inflammatory M1 macrophages are preferentially detected in rupture-susceptible shoulder regions of atherosclerotic lesions [83]. In contrast, M2 macrophages are found in more stable plaque regions outside the lipid core and show more resistance to foam cell formation [84]. Uptake of oxidized phospholipids by macrophages induces the pro-atherogenic Mox macrophage phenotype in an nrf-2 dependent manner. This subset of macrophages produces high levels of the pro-inflammatory cytokine IL-1β, as well as secreting high levels of reactive oxygen species (ROS), which induces oxidative stress [85]. Finally, the prevalence of M4 macrophages in atherosclerotic lesions is associated with plaque instability, suggesting a pro-inflammatory role for this subset [86].

3.2. Dendritic cells

As mentioned above, monocytes can differentiate into both macrophages and DCs. DCs are the most potent APCs and can activate the adaptive immune system. In the atherosclerotic context, DCs recognize oxLDL as a "non-self" molecule and danger signal and consequently take up oxLDL particles via phagocytosis. This stimulates migration of the DCs towards the afferent lymphatic vessels and draining lymph nodes, where there is a vast repertoire of T-cells able to recognize various epitopes [87, 88]. Moreover, the uptake of oxLDL induces maturation of the dendritic cells, which involves the downregulation of endocytic activity and upregulation of costimulatory molecules and the antigen-presenting molecules major histocompatibility complex (MHC) class I and II and CD1 molecules [89–92]. Mature DCs are able to present the antigens they have taken up via the MHC molecules to naïve and memory T-cells [88, 93]. The binding of the costimulatory molecules expressed on DCs, such as CD80/86 and CD40, to their corresponding receptors on T-cells, CD28 and CD40L, respectively, provides the second signal needed to induce T-cell activation [94]. Finally, cytokines secreted by the DCs provide the final signal needed to induce T-cell activation [95]. Importantly, the types of cytokines secreted as well as the type of costimulatory molecules expressed on the DCs are able to skew the T-cell towards differentiating into pro- or anti-inflammatory subsets [96, 97] (Fig. 2).

The presence of different subsets of DCs in atherosclerotic lesions is established in both murine models and human patients. Conventional DCs are shown to be present in murine atherosclerotic lesions [98], and overexpression of these cells aggravates atherosclerosis [99]. A more recent study describes two subsets of DCs in mice: Flt3-dependent classical DCs and M-CSF dependent monocyte-derived DCs. Both of these cell types are found to be present in diseased mouse aortas. However, deficiency of Flt3 in LDLr$^{-/-}$ mice, leading to lower numbers of classical DCs, results in more severe atherosclerosis. This indicates that classical DCs, in contrast to monocytes and macrophages, have an atheroprotective function [100]. On the other hand, interferon (IFN) α-producing plasmacytoid DCs are observed in the shoulder regions of human atherosclerotic lesions and are associated strongly with plaque instability [101].

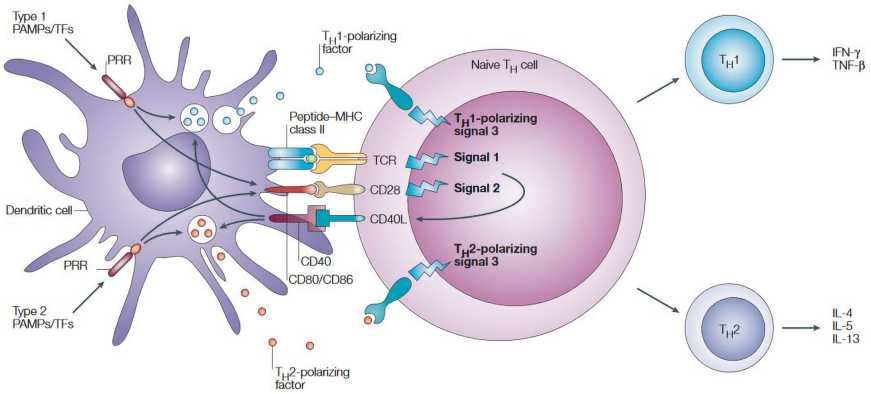

Figure 2: T-cell stimulation and polarization require three dendritic cell-derived signals. Signal 1 is the antigen-specific signal that is mediated through T-cell receptor (TCR) triggering by MHC-associated peptides processed from pathogens or self-molecules after internalization through Pattern Recognition Receptors (PRRs). Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by DCs after ligation of PRRs, that sense infection through recognition of PAMPs or DAMPs. Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors that promote the development of different T-cell subsets, such as Th1 and Th2 cells. The nature of signal 3 depends on the activation of particular PRRs on the DCs. *Adapted from Kapsenberg, Nature Reviews Immunology 2003;3:984.*

Of note, DCs can also be involved in maintaining peripheral tolerance. DCs are able to obtain a tolerogenic phenotype based on which immune-stimulatory or -modulatory signals are available in the tissue environment, as well as the strength of signaling [102]. Tolerogenic DCs generate T-cells with regulatory properties that dampen the immune response. Alternatively, tolerogenic DCs can induce apoptosis or anergy in T-cells, which also limits inflammation [103, 104]. In an atherosclerotic-specific context, tolerogenic DCs can dampen inflammatory T-cell responses [105]. This immune-modulatory potential makes tolerogenic DCs interesting for the treatment or prevention of atherosclerosis.

As of yet, the immunogenic peptides presented upon DCs that drive the activation of the adaptive immune response in atherosclerosis are still unknown. The most likely candidates include peptides derived from oxLDL [106]. DCs are able to take up oxLDL via scavenger receptors, which triggers their activation and differentiation [92]. It is therefore likely that antigens derived from ApoB100, the main protein component of oxLDL, are presented by the DCs to T-cells. For MHC-II molecules, it was indeed shown via peptide elution from bone marrow-derived DCs incubated with serum from $LDLr^{-/-}$ mice that they do present ApoB100-derived peptides. Moreover, vaccination against one of these peptides was protective against atherosclerosis development [107]. Many antibodies in human plasma have been identified that recognize modified peptide sequences within the ApoB-100 protein. Moreover, immunization against these apoB-100 peptide sequences was shown to confer protection against atherosclerosis in $apoE^{-/-}$ mice [108]. Furthermore, T-cells derived from human atherosclerotic lesions can mount an autoimmune response upon stimulation with oxLDL [109]. Together,

these studies indicate that peptide sequences derived from the ApoB100 protein likely are presented as autoantigens on DCs in this disease. Alternatively, other work has suggested that the immune response is directed against heat shock proteins that are produced in response to the oxidative stress within the lesion [110]. Tolerization against heat shock proteins was also shown to protect against atherosclerosis development [111], suggesting that antigen-specific responses towards these proteins may play a role in atherogenesis as well.

3.3. B-cells

B-cells play an important role in the pathophysiology of atherosclerosis through both the production of antibodies and cytokines. However, opposing effects are described for different B-cell subsets. B-cells can be broadly divided into two lineages: B1 and B2 cells [112]. B1 cells develop from fetal tissues and are characterized by their production of neutralizing IgM antibodies that recognize self- and foreign antigens [113, 114]. B2 cells comprise the large majority of B-cells in the adult and include both conventional follicular B-cells and marginal zone B-cells. Conventional B-cells are activated by T-cell dependent antigens and subsequently become plasma cells that secrete large amounts of antibodies against their cognate antigen. Alternatively, they can become memory B-cells with the ability to produce specific antibodies upon antigen re-exposure [115]. Marginal zone B-cells are considered a part of the innate immune system, as the main function of these cells is to respond immediately to antigens in the blood that are filtering through the spleen [116]. Finally, regulatory B-cells (Bregs) are defined by their immunomodulatory activities, mainly through the production of the anti-inflammatory cytokine IL-10 [117]. However, several B-cell subsets are able to produce this cytokine and as of yet no unifying marker or transcription factor has been described to more precisely identify regulatory B-cells [118].

B1 cells are established to be atheroprotective through their production of neutralizing IgM antibodies against oxidation-specific epitopes [119, 120]. Indeed, plasma levels of IgM against modified LDL associate inversely with the risk of coronary artery disease in a human population [121]. In contrast, an atherogenic role for B2 cells is established [122], although the mechanisms that mediate this effect remain unclear. Possible mechanisms include an increase in IL-17-mediated pro-inflammatory signaling [123], stimulation of T-cell proliferation [124], or the production of pathogenic antibodies [112, 122, 125]. Finally, regulatory B-cells are shown to protect against atherosclerosis development through inhibition of inflammation via IL-10 production [126, 127].

3.4. T-cells

As explained above, naïve T-cells can be activated upon exposure to their cognate antigens presented upon DCs. Subsequent to antigen recognition, the T-cells undergo clonal expansion, resulting in a large pool of T-cells able to recognize the same antigen and differentiate into effector or memory T-cells. Activated T-cells are able to migrate

towards sites of inflammation, where they can be activated in a secondary manner by APCs present at the site. Both CD4-expressing helper T-cells and CD8-expressing cytotoxic T-cells are found in human atherosclerotic lesions [128]. Most of these T-cells are in an activated state [129] and the presence of clonally expanded T-cells in both murine and human atherosclerosis indicates that antigen-specific responses are important in the pathogenesis of atherosclerosis [130, 131].

3.4.1. Helper T-cells

$CD4^+$ T-cells play a crucial role in atherosclerosis, as a deficiency in this cell type significantly reduces atherosclerotic lesion size by 70% [132], whereas adoptive transfer of $CD4^+$ T-cells into immunodeficient $apoE^{-/-}$ mice results in a 164% increase in lesion size [133]. In contrast, deficiency of MHC-II in $apoE^{-/-}$ mice is reported to increase atherosclerotic lesion size. Of note, MHC-II deficiency in this study not only decreases in the inflammatory $CD4^+$ T-cell subsets, but is also associated with a decrease in regulatory subsets and a compensatory increase in $CD8^+$ T-cells [134]. This emphasizes the importance of studying the contribution of different T-cell subsets, instead of studying the $CD4^+$ T-cell compartment as a whole. As discussed above, different T-cell subsets can be induced based on the cytokines that are produced by the DCs upon activation of the T-cells. The most well-known of these subsets are T-helper 1 (Th1), Th2, Th17 and regulatory T-cells (Tregs), which are discussed in more detail below. Of note, the more recently discovered Th9 and Th22 cells might also play a role in atherosclerosis development and progression [135, 136].

3.4.1.1 Th1 $CD4^+$ T-cells

When dendritic cells secrete IL-12 during the activation of $CD4^+$ T-cells, this results in differentiation towards the Th1 phenotype [137]. IL-12 induces the expression of the transcription factor T-bet [138], which is the hallmark transcription factor of Th1 cells [139]. Th1 cells are a pro-inflammatory cell type and produce a plethora of inflammatory cytokines, such as IFN-γ, TNF-α, and IL-2 [140]. IFN-γ is known to promote atherosclerosis development, as IFN-$\gamma^{-/-}$$apoE^{-/-}$ mice show marked reductions in lesion development compared to IFN-γ-competent controls [141]. Moreover, administration of exogenous IFN-γ to $apoE^{-/-}$ mice increases plaque size by a factor of two [142], again demonstrating a pro-atherogenic role of this cytokine. Atherosclerosis is significantly attenuated in T-$bet^{-/-}$$LDLr^{-/-}$ mice, which lack Th1 cells [143]. Together, these studies show a strong pro-atherogenic function for Th1 cells.

3.4.1.2 Th2 $CD4^+$ T-cells

The presence of IL-4 during the activation of naïve $CD4^+$ T-cells skews them towards a Th2 phenotype. Th2 cells secrete large amounts of IL-4 and IL-5, but little IL-2 or IFN-γ. Thereby, these cells provide help for antibody production by B-cells [144]. This subset of helper T-cells is characterized by the expression of the transcription factor GATA-3, which inhibits Th1-specific factors and induces Th2-related cytokine production [145].

Interestingly, in advanced human atherosclerosis, expression of IL-4 and IL-5 is rarely observed [146], suggesting a limited presence of Th2 cells in the plaques.

There is conflicting evidence of the contribution of these cells to atherosclerosis development. IL-4 is shown to limit atherosclerosis development by suppressing Th1-mediated inflammation [147] and IL-5 stimulates B1 B-cells to produce more naturalizing IgM antibodies, thereby reducing plaque development [148]. Treatment of apoE$^{-/-}$ mice with IL-33, a cytokine that induces the Th2 phenotype via the production of IL-4, -5, and -13, reduces atherosclerosis development [149]. Notably, higher numbers of Th2 cells in the blood of human patients are associated with a reduced risk of myocardial infarction [150]. On the other hand, IL-4 deficiency in apoE$^{-/-}$ mice results in a 27% reduction in lesion size compared to controls, suggesting that this Th2 cytokine might drive atherogenesis [151]. Similarly, IL-4 deficiency in LDLr$^{-/-}$ mice leads to a reduced lesion size in the thoracic aorta and aortic arch, suggesting a pro-atherogenic role for this cytokine [152]. Another study showed that blocking the costimulatory molecule OX40 ligand reduces Th2 responses, as indicated by decreased expression levels of GATA-3 and IL-4. This induces a significant regression of atherosclerotic lesions, again showing a pro-atherogenic role for this subset [153].

The different effects of the cytokines associated with the Th2 phenotype complicate the process of elucidating the role of Th2 cells in atherosclerosis lesion development. Moreover, these cytokines are not solely produced by Th2 cells, which poses even greater difficulties. On balance, the general consensus is that the function of these cells in atherosclerosis is dependent on their cytokine profile, but they are likely less atherogenic than their Th1 counterparts [87, 154].

3.4.1.3 Th17 CD4$^+$ T-cells

The third helper T-cell subset to be identified was the Th17 subset. A combination of transforming growth factor β (TGF-β) and IL-6 drive the expression of the characteristic transcription factor RORγt in these cells. Furthermore, IL-21 and IL-23 are needed for the proliferation and stabilization of this subset, respectively. Th17 cells mainly produce the IL-17A cytokine, after which the subset was named [155]. IL-17A is present in both human and murine atherosclerotic lesions [156, 157]. Interestingly, oxLDL is able to induce Th17 differentiation and activation [158], suggesting these cells could play an important role in atherogenesis. Indeed, IL-17A is known to activate endothelial cells and stimulate cytokine and chemokine production by macrophages. Consequently, treatment of apoE$^{-/-}$ mice with an inhibiting IL-17A antibody results in a reduction in atherosclerotic lesion area, reduced T-cell and macrophage content, as well as a reduced vulnerability of the plaques [157]. In agreement with this, blockade of IL-17A in apoE$^{-/-}$ mice using adenoviral-produced IL-17 receptor A also reduces plaque burden via decreasing circulating IL-6 and granulocyte colony-stimulating factor levels, limiting chemotaxis and reducing macrophage content within the aorta [159]. Transplantation of LDLr$^{-/-}$ recipient mice with IL-17 receptor-deficient bone marrow also reduces lesion size compared to controls, which, interestingly, is associated with an increase in plaque macrophages that could be indicative of a more initial plaque phenotype [160]. Controversially, T-cell specific deletion of suppressor of cytokine signaling (SOCS) 3 in

LDLr$^{-/-}$ mice, increasing both IL-17 and IL-10 production, reduces atherosclerotic lesion size. This is associated with an anti-inflammatory macrophage phenotype, and a reduction in vascular inflammation [161]. Moreover, IL-17A has also been established to contribute to plaque stabilization by stimulating collagen production [162]. This conflicting evidence concerning the role of IL-17A and Th17 cells in atherosclerosis underlines the need for more studies in which the effect of IL-17A produced specifically by T-cells can be studied.

3.4.1.4 Regulatory CD4$^+$ T-cells

Tregs are involved in the regulation of the immune response by suppressing both proliferative and inflammatory responses of other immune cell subsets. Tregs are important for maintaining self-tolerance and for mitigating tissue damage during inflammation. Two different types of Tregs can be distinguished: the naturally occurring CD25-expressing Tregs that are derived from the thymus where they were trained to maintain tolerance to self-antigens [163]; and the induced Tregs that are differentiated from naïve T-cells in the periphery by stimulation of tolerogenic DCs, exposure to TGF-β and/or weak T-cell receptor (TCR) stimulation [164]. Tregs are characterized by their expression of the transcription factor forkhead box P3 (FoxP3), which induces the expression of suppressive genes [165]. The immunosuppressive function of Tregs is mediated via secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β, as well as through direct inhibitory contact with other cell types. TGF-β is known to suppress T-cell proliferation, whereas IL-10 plays an important role in the suppression of inflammation. Direct inhibitory contact is mediated via the expression of coinhibitory molecules on the Tregs, such as CTLA-4 and LAG-3 [166].

It is thought that in many chronic inflammatory diseases, atherosclerosis included, there is a dysfunction in the inhibitory capacity of the Treg population, resulting in overt inflammatory response directed against self-antigens [167]. Indeed, there is an association between reduced numbers of circulating Tregs in humans and the occurrence of myocardial infarction [168]. In mice, stimulation of T-cells derived from apoE$^{-/-}$ mice with oxLDL attenuates the suppressive properties of the Tregs within this population [169]. Moreover, apoE$^{-/-}$ mice have a significantly lower number of Tregs in the spleen compared to WT mice [170]. LDLr$^{-/-}$ mice deficient in Tregs show increased lesion size compared to controls [171]. Furthermore, adoptive transfer of Tregs is known to reduce atherosclerosis development [169, 172], whereas depletion of these cells aggravates the lesion formation [173]. Together, these studies demonstrate a protective role for Tregs against the development of atherosclerosis.

Restoring the suppressive function of Tregs has great therapeutic potential for the treatment of atherosclerosis. Increasing the number of antigen-specific Tregs via the induction of oral tolerance towards oxLDL reduces atherosclerosis [174]. Moreover, adoptive transfer of Tregs or induction of Tregs and tolerogenic DCs in vivo are promising therapeutic options, as reviewed elsewhere [175].

3.4.2. Cytotoxic T-cells

Although the presence of $CD8^+$ T-cells in atherosclerotic lesions has been established a long time ago [176], research into their phenotype and function in atherosclerosis has lagged behind that of $CD4^+$ T-cells. Of note, plaque-derived $CD8^+$ T-cells from human atherosclerotic lesions show a more highly activated phenotype compared to $CD4^+$ T-cells, as well as their $CD8^+$ counterparts in the circulation [177]. As $CD8^+$ T-cells are normally involved in the defense of the host against intracellular pathogens, they can exert an array of different effector functions. Firstly, they can produce pro-inflammatory cytokines such as TNF-α, which induces apoptosis, and IFN-γ, which promotes inflammation. Furthermore, they can induce cell death via the ligation of Fas ligand (FasL), expressed on the surface of the $CD8^+$ T-cells, to the Fas receptor on target cells, which leads to signaling within the target cell that initiates the apoptotic process. Alternatively, $CD8^+$ T-cells can release granzymes and perforin, cytotoxic molecules that result in lysis of the target cells [178]. These inflammatory functions would suggest a pro-atherogenic role for these cells in atherosclerosis, but it appears that the role of $CD8^+$ T-cells in atherosclerosis is complex and subset dependent [179, 180].

WT mice that are deficient in MHC-I, and therefore have no activated $CD8^+$ T-cells, show a large increase in plaque area upon WTD-feeding compared to controls, suggesting a protective role for $CD8^+$ T-cells against plaque formation [181]. However, $CD8^+$ T-cell deficiency in $apoE^{-/-}$ mice is reported not to affect atherosclerotic lesion development [182, 183]. More mechanistic work reveals that $CD8^+$ T-cells in $apoE^{-/-}$ mice produce increased amounts of IFN-γ upon feeding a high-fat diet compared to a control diet, suggesting that they exert a pro-inflammatory role under hypercholesterolemic conditions. However, a population of $CD8^+$ T-cells producing the anti-inflammatory cytokine IL-10 is also found in the spleens of these mice, suggesting different roles for different subsets of $CD8^+$ T-cells [184]. The depletion of $CD8^+$ T-cells in $LDLr^{-/-}$ mice ameliorates atherosclerosis due to a decreased influx of monocytes [185]. In contrast, adoptive transfer of a regulatory subset of $CD8^+CD25^+$ T-cells reduces atherosclerosis development [186]. Thus, it appears $CD8^+$ T-cells may exert either pro- or anti-atherogenic effects depending on what phenotype is induced by the local stimuli and the stage of lesion progression.

4. Thesis outline

In recent years, it has become apparent that the immune system plays a crucial role in the development of atherosclerotic lesions. Due to the limited effect of lipid-lowering treatments in reducing cardiovascular events, it may be interesting to identify new therapeutic strategies targeting the immune component of atherosclerosis. Cells involved in the adaptive immune response would be interesting to target, as these cells are able to mount an antigen-specific immune response. Thus, targeting the adaptive immune system minimizes the risk of non-specific adverse effects. Whereas B-cells and $CD4^+$ T-cells have been extensively studied in the atherosclerotic context, there is a knowledge gap concerning the role of $CD8^+$ T-cells in this disease. The aim of this thesis is to

elucidate the role of $CD8^+$ T-cells in advanced atherosclerosis. Furthermore, we set out to harness our newfound knowledge of $CD8^+$ T-cell function in atherosclerosis by developing a novel therapeutic strategy designed to modulate the function of these cells in the disease process.

In **Chapter 2** we provide an overview of the current knowledge on the role of $CD8^+$ T-cells in atherosclerosis and provide a therapeutic outlook on treatment strategies targeting these cells.

In **Chapter 3** we report a protective role for $CD8^+$ T-cells in both human and murine atherosclerosis. $CD8^+$ T-cells limit the content of macrophages and inflammatory $CD4^+$ T-cells in the plaques. This effect is at least in part mediated via Fas-FasL interactions. Moreover, these protective effects of $CD8^+$ T-cells were found to be limited to the lesional microenvironment.

In **Chapter 4** we further investigated how the lesion-localized protective effects of $CD8^+$ T-cells described in chapter 3 are mediated. We report that TCR signaling induces increased expression of the extracellular enzyme CD39 specifically in the lesion microenvironment. This results in an immunomodulatory environment that reduces inflammatory cytokine production by lesional $CD8^+$ T-cells compared to their circulating counterparts in both humans and murine models.

Chapter 5 describes the study of different cytokine-producing $CD8^+$ T-cell subsets in atherosclerosis. We report a lesion-localized increase in IL-17-producing Tc17 cells in an atherosclerotic mouse model. Adoptive transfer of these cells into atherosclerotic mice that lack endogenous $CD8^+$ T-cells reduced lesion size compared to the transfer of undifferentiated Tc0 cells, suggesting a protective role for Tc17 cells in atherosclerosis.

In **Chapter 6** we review the current knowledge on how particulate carrier systems can be designed in order to obtain the desired immunogenicity upon vaccination with these particles. In particular, we focus on the role of the parameters of size, shape, and rigidity of liposomal particles in enhancing and skewing T-cell responses.

In **Chapter 7** we employ a liposomal vaccination in order to boost protective $CD8^+$ T-cell responses in a mouse model of atherosclerosis. We successfully formulated a vaccine of DOPC:DOTAP liposomes encapsulating an ApoB100-derived peptide. Unexpectedly, vaccination with this formulation did not induce any antigen-specific immune responses and affected neither plaque burden nor stability.

Finally, in **Chapter 8**, we summarize the data reported in this thesis and discuss the latest therapeutic potential of these findings.

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2

The many faces of CD8$^+$ T-cells in atherosclerosis

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ABSTRACT

Purpose of review Atherosclerosis and the clinical consequence of cardiovascular disease remain the leading cause of death worldwide. Both an increase in cholesterol levels, as well as immune responses drive the pathogenesis of this disease. Although much is known about the role of many immune cell subsets in atherogenesis, research into the role of CD8$^+$ T-cells is limited.

Recent findings Both atheroprotective and atherogenic functions of CD8$^+$ T-cells have been reported. On the one hand, the inflammatory cytokines produced by CD8$^+$ T-cells exacerbate inflammatory responses, and the cytotoxic activity of these cells towards lesion-stabilizing cells such as endothelial cells drives the progression and instability of atherosclerotic lesions. On the other hand, cytotoxic activity towards antigen presenting cells and the presence of regulatory CD8$^+$ T-cell subsets dampen immunity and can limit atherosclerosis.

Summary Here we review the different roles of CD8$^+$ T-cells in atherosclerosis and discuss possible treatment strategies targeting these cells to reduce atherosclerotic lesion burden.

Keywords Atherosclerosis, Immunity, CD8$^+$ T-cells, Vaccination

1. Introduction

Atherosclerosis remains a major public health concern, as it is the leading cause of mortality worldwide. The burden of atherosclerotic cardiovascular disease is not only high in Western countries but increases fast in developing countries as well, stressing the need for adequate treatment strategies [1].

Atherosclerosis is initiated by high levels of serum cholesterol, particularly when present in low-density lipoproteins (LDL), driving infiltration of lipoproteins into the vascular intima. This, in turn, triggers an inflammatory response, resulting in the recruitment and accumulation of monocyte-derived macrophages within the vessel wall. Local activation of the macrophages results in increased cytokine production, as well as the release of enzymes and reactive oxygen species that can modify LDL to its oxidized form. The ongoing inflammation further results in the recruitment of T-cells to the lesion. Monocyte-derived dendritic cells have the capacity to take up a variety of lesion-derived antigens and travel to the lymph nodes, where they can activate T-cells. Moreover, plaque-resident antigen presenting cells can present antigen locally to the T-cells [2]. Extensive research into the role of $CD4^+$ T-cells has elucidated the role of $CD4^+$ T-cell subsets in this disease, such as the pro-inflammatory function of Th1 cells and the protective role of Tregs [3–5]. However, research into the function of $CD8^+$ T-cells in atherosclerosis lags behind, although they can play an important role in atherosclerosis initiation and progression, as they comprise a large portion of lymphocytes in both early and advanced human lesions [6, 7]. In this review, we provide a brief overview of the role of $CD8^+$ T-cells in this disease and discuss how these cells could be targeted therapeutically to reduce atherosclerotic disease burden.

2. $CD8^+$ T-cells in atherosclerosis

$CD8^+$ T-cells, also known as cytotoxic T lymphocytes, play an important role in protection against intracellular pathogens. They continuously monitor every cell in the body and are activated upon binding of their T-cell receptor (TCR) to a peptide-loaded MHC (or HLA) class I molecule. Upon recognition of a target cell, $CD8^+$ T-cells exert three main effector functions. Firstly, they secrete pro-inflammatory cytokines such as TNF-α, which can induce apoptosis, and IFN-γ, which induces upregulation of MHC-I and further promotes the inflammatory response. Secondly, Fas ligand expressed on the $CD8^+$ T-cells can bind the Fas receptor on target cells, which leads to downstream activation of the caspase cascade and subsequent apoptosis of the target cell. Finally, $CD8^+$ T-cells can release granzymes and perforin into the cleft between their own membrane and the target cell membrane, resulting in lysis of the target cell [8]. In the past few years, a number of studies have addressed the role of the different functions of $CD8^+$ T-cells in atherosclerosis (Fig. 1).

Early experimental studies using full body knockouts provided limited evidence towards a role for $CD8^+$ T-cells in atherogenesis. The introduction of $CD8^+$ T-cell deficiency in apolipoprotein E $(apoE)^{-/-}$ mice by disrupting the Antigen Peptide Trans-

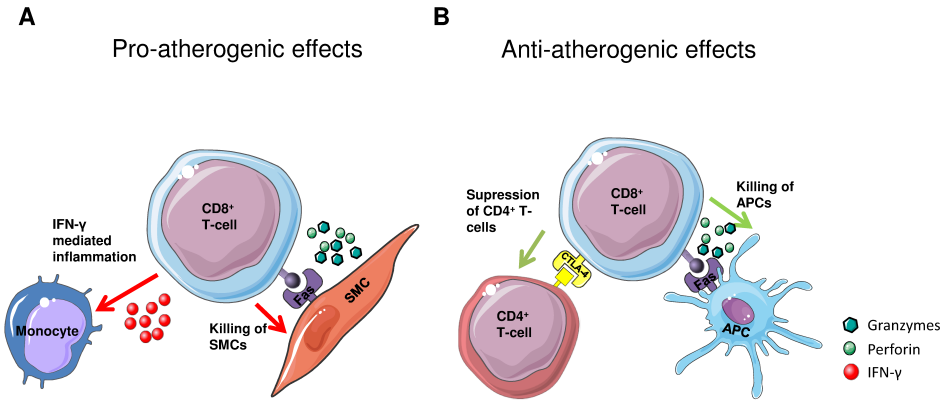

Figure 1: The pro- and anti-atherogenic effects of CD8$^+$ T-cells in atherosclerosis. (A) CD8$^+$ T-cells are reported to promote atherosclerosis via the release of inflammatory cytokines, which affects monocyte recruitment, as well as via cytolytic killing of smooth muscle cells and endothelial cells. (B) The protective functions of CD8$^+$ T-cells are mediated via suppression of CD4$^+$ T-cells by stimulating inhibitory receptors and cytolytic killing of antigen-presenting cells.

porter 1 (TAP-1)-dependent MHC class I antigen presentation, shows no difference in lesion development compared to apoE$^{-/-}$ controls [9]. Moreover, deficiency of the CD8a gene in apoE$^{-/-}$ mice does not affect the development of atherosclerosis at either 18 weeks and 1 year of age [10]. Feeding a high-fat diet to MHC-I deficient C57Bl/6 mice, which display greatly reduced numbers of CD8$^+$ T-cells as they are unable to positively select them in the thymus, does result in a large increase in plaque area compared to wild-type controls, suggesting a protective role for CD8$^+$ T-cells in atherosclerosis [11].

However, more recent studies provide a more detailed and a better mechanistic insight into CD8$^+$ T-cell function. There is a collection of work that suggests a pro-atherogenic role for CD8$^+$ T-cells in atherosclerosis. Firstly, high-fat diet fed apoE$^{-/-}$ mice show an increase in IFN-γ production by activated (CD28$^+$) CD8$^+$ T-cells in the mediastinal lymph nodes (which drain the major arteries around the heart and thus drain major lesion sites), compared to chow-fed controls, suggesting that CD8$^+$ T-cells mount a pro-inflammatory response to hypercholesterolemia [12]. However, in the splenic compartment, two distinct CD8$^+$ T-cell phenotypes were identified: a proliferative population that produces IFN-γ and a quiescent population producing IL-10, indicating that different subsets of CD8$^+$ T-cells may exert different functions. Cochain *et al.* report the presence of pro-inflammatory cytokine-producing CD8$^+$ T-cells in plaques as well as in splenic tissue [13]. Furthermore, upon depletion of these cells in initial atherosclerosis in LDL receptor (LDLr)$^{-/-}$ mice, they observe a decrease in plaque macrophage content. Interestingly, CD8$^+$ T-cells did not appear to directly affect trafficking of monocytes into the lesion. Rather, the authors suggest that the systemic absence of IFN-γ produced by CD8$^+$ T-cells contributed to reduced monopoiesis and lower levels of circulating inflammatory monocytes, resulting in the observed decrease in plaque macrophages. In contrast with this, Kyaw *et al.* do not observe a reduc-

tion in atherosclerosis development after the introduction of IFN-γ deficient $CD8^+$ T-cells compared to wild-type controls, suggesting that the role of $CD8^+$ T-cell-derived IFN-γ in the pathogenesis of atherosclerosis is limited [14]. However, they do report that the cytotoxic functions of $CD8^+$ T-cells contribute to their pro-atherogenic role. Perforin- and granzyme-B release by $CD8^+$ T-cells in early stages of lesion development in $apoE^{-/-}$ mice results in apoptosis of smooth muscle cells, macrophages, and endothelial cells, thereby destabilizing the lesion and increasing necrotic core formation.

On the other hand, a body of work has shown a protective role for $CD8^+$ T-cells in atherosclerosis. Immunization of $apoE^{-/-}$ mice with p210, an ApoB100-derived peptide, activates and expands $CD8^+$ T-cells and reduces atherosclerosis compared to controls [15]. The atheroprotective effects of the $CD8^+$ T-cells in this study are mediated by their cytolytic activity towards dendritic cells, which present ApoB100-derived peptide fragments via MHC-I molecules. More recently, these authors described a population of self-reactive $CD8^+$ T-cells to p210-derived peptides using fluorescently labeled H2Kb pentamers in $apoE^{-/-}$ mice [16]. These pentamers consist of five peptide-MHC-I complexes, which only bind to antigen-specific $CD8^+$ T-cells via recognition of the peptide presented on the pentamer by the TCR of the T-cell. Moreover, pentamer-mediated blocking of the $CD8^+$ T-cells significantly reduces the cytolytic activity of these cells, although its effect on atherosclerosis remains to be determined. $CD8^+$ T-cells are also able to confer protection against neointima formation after arterial injury by mounting a cytotoxic response against smooth muscle cells [17]. In support of that, there is a marked absence of $CD8^+$ T-cells in $apoE^{-/-}$ mice with completely ligated common carotid artery lesions (a model for neointima formation) upon Western-type diet feeding, whereas they are present in chow-fed mice [18]. This suggests a protective role of $CD8^+$ T-cells in atherosclerosis but requires further research into the mechanisms by which the Western-type diet affects the $CD8^+$ T-cells.

The contradictory role of $CD8^+$ T-cells may be explained by their heterogeneity and the various subsets that have been identified. Zhou *et al.* report the presence of a $CD8^+CD25^+$ regulatory T-cell subset in the lesions of 25-week old $apoE^{-/-}$ mice fed an atherogenic diet. These cells have a high expression of regulatory markers such as TGF-β, FoxP3, and CTLA-4 [19]. Thereby, they are able to inhibit the proliferation of $CD4^+CD25^-$ T-cells and the cytolytic activity of $CD8^+CD25^-$ T-cells. As anticipated, adoptive transfer of $CD8^+CD25^+$ T-cells reduces atherosclerosis development. Clement *et al.* report a protective role for Qa1-restricted $CD8^+CD44^{high}CD122^+$ regulatory T-cells in the development of atherosclerosis in $apoE^{-/-}$ mice [20]. These $CD8^+$ Tregs interact via their TCR with the Qa1 antigen-presenting molecules (the murine variant of HLA-E) on T follicular helper cells and inhibit their function. However, as the lesions advance, the numbers of these regulatory cells decrease, associated with an increase in T follicular helper cells. These cells, in turn, stimulate the expansion of germinal center B cells, tertiary lymphoid organ formation and increase immunoglobulin production, resulting in an increase in atherosclerosis. More recently, a study in $LDLr^{-/-}Stat4^{-/-}$ mice reported a similar phenotype, characterized by a reduction in T follicular helper cells and plasma B cells associated with an increase in $CD8^+CXCR5^+CD275^+CD122^+$ T reg-

ulatory cells. Besides the effects on CD8$^+$ Treg generation, STAT4 deficiency also affects macrophages by altering the cytokine profile of these cells, further promoting CD8$^+$ Treg development [21].

In human atherosclerosis, CD8$^+$ T-cells also make up a large proportion of the lymphocytes present in the lesion and are mainly found in the shoulder regions and fibrous caps [6, 7, 22]. Moreover, the CD8$^+$ T-cell numbers in blood correlate with the incidence of coronary events [23]. Naïve CD8$^+$ (CCR7$^+$CD45RA$^+$) T-cells are present in higher numbers in patients without significant coronary disease, whereas they are decreased in patients with more advanced atherosclerosis, suggesting activated CD8$^+$ T-cells have a pro-atherogenic effect [24]. In support of this, there is a negative correlation between these naïve CD8$^+$ T-cells and pulse wave velocity, a well-documented risk factor of coronary artery disease, thus implying that higher numbers of differentiated CD8$^+$ T-cells may augment atherosclerosis. In particular, expression of the IL-6 receptor α chain (IL-6Rα) correlates with pathogenicity of the CD8$^+$ T-cells [25]. Expression of the IL-6Rα on CD8$^+$ T-cells in the circulation is decreased in patients suffering from coronary artery disease, which is associated with an increase in CD8$^+$ effector T-cells that express granzyme B and the pro-inflammatory transcription factor T-bet, indicating a pathogenic role for CD8$^+$ T-cells in coronary artery disease. However, similar to murine models, different CD8$^+$ T-cell subsets appear to exert different functions in humans. Whereas a high percentage of CD8$^+$CD25$^+$ T-cells shows a correlation with a high degree of carotid stenosis, CD8$^+$CD56$^-$IFN-γ+ T-cells are associated with less carotid stenosis [23]. Qiu *et al.* describe a subset of CD8$^+$ T-cells expressing both PD-1 and Tim-3, which is increased in human atherosclerotic patients compared to controls [26]. This subset is associated with decreased production of pro-atherogenic cytokines and an associated increase in anti-atherogenic cytokines.

3. Therapeutic outlook on treatment strategies targeting CD8$^+$ T-cells

Overall, these studies show that CD8$^+$ T-cells play an intricate role in atherosclerosis, but targeting the right subsets may provide new avenues for treatment. An interesting therapeutic approach that may prove useful to induce protective CD8$^+$ T-cells, is vaccination. Several studies have already demonstrated the atheroprotective potential of vaccination using LDL-derived peptides via induction of CD4$^+$ regulatory T-cells [27–29]. As mentioned above, vaccination with p210 can induce a CD8$^+$ T-cell population that can lyse dendritic cells and reduce lesion formation [16, 17]. Moreover, p210 vaccination is also reported to be protective against aortic aneurysm formation by boosting CD8$^+$ T-cell responses that have cytolytic activity against macrophages and via reducing the polarization of CD4$^+$ T-cells into the Th17 phenotype [30]. Furthermore, vaccination using BSA conjugated to the ApoB100-derived p2 and p45 peptides, but not the p210 peptide, induces a significant increase in CD8$^+$CD25$^+$FoxP3$^+$ and CD8$^+$CD25$^-$FoxP3$^+$ regulatory T-cells when compared to controls [29]. Inducing CD8$^+$ T-cell responses against vascular endothelial growth factor receptor 2 and CD99 (a membrane protein involved in transmigration of monocytes through endothelial

cells) using DNA vaccinations are also proven to be effective in reducing atherosclerosis development [31, 32]. Taken together, these studies suggest that boosting $CD8^+$ T-cell responses in atherosclerosis holds promise as a treatment against atherosclerosis. However, some challenges remain to be met before an optimal vaccine can be designed. Although promising results have been obtained with vaccination against LDL-derived antigens, the epitope against which $CD8^+$ T-cell responses are directed in atherosclerosis remains to be determined [33]. Furthermore, it is of vital importance to identify the optimal adjuvant, as several studies have reported that adjuvants alone can affect atherosclerosis development [34–36]. Finally, one must be careful to induce only protective $CD8^+$ T-cell responses and avoid activation of the pro-atherogenic $CD8^+$ T-cell subsets discussed above.

Other potential treatment strategies in that could boost protective $CD8^+$ T-cell responses include TCR stimulation and administration of IL-2 complexes. Stimulation of the TCR using modified anti-CD3 antibodies induces regulatory $CD8^+$ T-cells which can inhibit antigen-specific $CD4^+$ T-cell responses in several *in vitro* models [37, 38]. Although oral of administration of anti-CD3 in a murine model was shown to reduce atherosclerosis development by the induction of $CD4^+$ regulatory T-cells, no effects on $CD8^+$ T-cells were reported [39]. Similarly, intravenous administration of anti-CD3 in $LDLr^{-/-}$ mice reduces atherosclerosis via increased $CD4^+$ regulatory T-cell responses, and a significant decrease in aortic CD8 mRNA levels was observed, although no $CD8^+$ T-cell subsets were investigated in this work [40]. Immune complexes of IL-2 with anti-IL-2 monoclonal antibodies can also activate and expand $CD8^+$ T-cell populations [41, 42]. Different IL-2 complexes can be used to stimulate $CD4^+$ regulatory T-cells or $CD8^+$ T-cells, based on their binding affinity to CD25 [43]. Thus far, several studies have shown the protective effect of inducing $CD4^+$ Tregs in atherosclerosis using IL-2 complexes [44–46], but to our knowledge, the effect of IL-2 complexes on $CD8^+$ T-cells has not been studied in this disease. Therefore, there is a need for research that sheds more light on the effects of TCR stimulation and IL-2 complex therapy on $CD8^+$ T-cells in atherosclerosis to investigate the clinical potential of this treatment.

On the other hand, disabling atherogenic $CD8^+$ T-cell subsets may prove to be effective as well. MHC-I peptide multimers are commonly used to detect antigen-specific $CD8^+$ T-cells [47, 48]. Coupling peptide-MHC complexes to nanoparticles can be used to target antigen-specific $CD8^+$ T-cells [49–51], which opens up avenues for specific killing of autoreactive $CD8^+$ T-cells in atherosclerosis. DNA vaccination in a type 1 diabetes model against proinsulin, towards which the $CD8^+$ T-cell response is directed in this disease, results in a deletion of $CD8^+$ T-cells that are reactive to proinsulin [52]. This is likely mediated via increased apoptosis of $CD8^+$ T-cells due to a reduction in costimulatory signals, or via active suppression by regulatory T-cells. Once antigens against which $CD8^+$ T-cells are directed in atherosclerosis have been identified, a similar vaccination strategy may prove useful to delete pro-atherogenic $CD8^+$ T-cell subsets.

Finally, current treatment of atherosclerosis focuses mainly on lipid-lowering strategies by lifestyle changes and the use of statins. However, statin treatment results in a 25% reduction in cardiovascular disease events [53], stressing the need for new therapies that target the underlying immunologic pathogenic mechanisms as well. Of note, choles-

terol lowering in human cells, as well as murine models, has been reported to reduce CD8$^+$ T-cell proliferation, due to reduced lipid raft formation and TCR signaling [54]. Indeed, inhibiting cholesterol esterification in T-cells enhances proliferation and effector function of CD8$^+$ T-cells, but not CD4$^+$ T-cells, in a murine cancer model [55]. In this study, cholesterol esterification is inhibited via knockout of Acyl-CoA cholesterol acyltransferase-1, as well as pharmacological inhibition of this enzyme using avasimibe, which was in clinical trials as a drug against atherosclerosis development. The commercially available statins simvastatin and pitavastatin are reported to reduce the proliferative response and expression of IFN-γ in human T-cells [56]. Furthermore, atorvastatin and pravastatin treatment in virologically suppressed HIV-infected persons also reduces CD8$^+$ T-cell proliferation and activation, but also down-regulates markers of CD8$^+$ T-cell exhaustion [57]. Finally, statin treatment reduces the number of CD8$^+$ T-cells in the blood of patients with Hashimoto's thyroiditis, and *in vitro* experiments show that Pravastatin, Mevastatin, Cerivastatin, and Simvastatin are able to induce CD8$^+$ T-cell apoptosis [58]. Thus, statin treatment may impair CD8$^+$ T-cell function in cardiovascular disease patients, which may affect the pathogenesis of the disease depending on which subsets are most affected. This stresses the need for a better understanding of the effects of current and future therapeutics on the CD8$^+$ T-cell response in atherosclerosis.

4. Conclusion

Studies in both humans and in murine models have identified an important role for CD8$^+$ T-cells in atherosclerosis development and progression. Different CD8$^+$ T-cell subsets are present in atherosclerotic lesions and can either augment or limit lesion development. The discovery of the protective functions of CD8$^+$ T-cells is highly relevant for the development of new treatments for atherosclerosis, as methods to induce protective CD8$^+$ T-cells, such as vaccination, can be considered as possible therapies. However, future studies will need to show that no pro-atherogenic functions of CD8$^+$ T-cells are boosted as well before these therapies can be considered as treatments in human patients.

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3

CD8$^+$ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4$^+$ T-cell responses

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ABSTRACT

Aims T lymphocytes play an important role in atherosclerosis development, but the role of the $CD8^+$ T-cells remains debated, especially in the clinically relevant advanced stages of atherosclerosis development. Here, we set out to determine the role of $CD8^+$ T-cells in advanced atherosclerosis.

Methods and Results Human endarterectomy samples analyzed by flow cytometry showed a negative correlation between the percentage of $CD8^+$ T-cells and macrophages, suggesting a possible protective role for these cells in lesion development. To further test this hypothesis, $LDLr^{-/-}$ mice were fed a Western-type diet (WTD) for 10 weeks to induce atherosclerosis, after which they received CD8α-depleting or isotype control antibody for six weeks. Depletion of $CD8^+$ T-cells in advanced atherosclerosis resulted in less stable lesions, with significantly reduced collagen content in the trivalve area, increased macrophage content and increased necrotic core area compared to controls. Mechanistically, we observed that $CD8^+$ depletion specifically increased the fraction of Th1 $CD4^+$ T-cells in the lesions. Treatment of WTD-fed $LDLr^{-/-}$ mice with a FasL-neutralizing antibody resulted in similar changes in macrophages and $CD4^+$ T-cell skewing as $CD8^+$ T-cell depletion.

Conclusion These findings demonstrate for the first time a local, protective role for $CD8^+$ T-cells in advanced atherosclerosis, through limiting accumulation of Th1 cells and macrophages, identifying a novel regulatory mechanism for these cells in atherosclerosis.

1. Introduction

Atherosclerosis is a chronic disorder characterized by inflammation and accumulation of lipids in the vessel wall. Inflammation plays a key role throughout all stages of atherosclerosis development, involving a complex interplay between different inflammatory cell types. Among these are CD8$^+$ T-cells, which could play an important role in all stages of atherogenesis, as they represent 29% of all leukocytes in early human lesions, increasing to 50% in advanced plaques [1]. In accordance, CD8$^+$ T-cell numbers are shown to increase significantly as human lesions become more progressed and vulnerable to rupture but show a decrease in healed plaque ruptures and fibrotic calcified plaques [2]. Also in the circulation, CD8$^+$ T-cells have been linked to atherosclerosis development as the percentage of low-differentiated CD8$^+$CCR7$^+$CD45RA$^+$ T-cells is reduced in patients with advanced coronary atherosclerosis compared to those without significant coronary disease [3] Furthermore, a significant correlation between the total number of CD8$^+$ T-cells in the circulation and the occurrence of myocardial infarction was established [4], suggesting that CD8$^+$ T-cells contribute to lesion growth and instability. Interestingly, the same study demonstrates an inverse correlation between the number of IFN-γ^+CD8$^+$ T-cells and carotid stenosis, suggesting that certain CD8$^+$ T-cell subpopulations slow down lesion progression. The PD-1$^+$TIM-3$^+$CD8$^+$ T-cell subset has been identified in the circulation of atherosclerotic patients, which exhibit an increased production of anti-atherogenic cytokines and decreased pro-atherogenic cytokines, suggesting a regulatory function for these cells in atherogenesis [5].

Several studies in experimental models of atherosclerosis have been conducted to provide insight into the role of CD8$^+$ T-cells in atherogenesis, but the results are thus far conflicting [6–12]. Whereas CD8$^+$ T-cells can induce lesion growth and instability, through lysis of endothelial cells and vascular smooth muscle cells [9], CD8$^+$ T-cell mediated killing of dendritic cells and follicular helper T-cells can reduce plaque formation [11, 12]. Importantly, studies in murine atherosclerotic models focus mainly on initial lesion development, whereas it is clinically more relevant to study advanced and/or unstable lesions, as patients usually experience symptoms related to severe stenosis when lesions are advanced. Therefore, from a drug development perspective, it is most valuable to understand the role of CD8$^+$ T-cells in this stage of disease progression, as this is the stage when pharmacological intervention is possible.

In the present study, we aimed to assess how CD8$^+$ T-cells affect plaque composition and stability of advanced lesions. We show a negative correlation between the percentage of CD8$^+$ T-cells and macrophages in human endarterectomy samples, suggesting a possible protective role for CD8$^+$ T-cells in the more advanced stages of atherogenesis. We next set out to investigate the role of CD8$^+$ T-cells in advanced plaques by CD8$^+$ T-cell depletion in the low-density lipoprotein receptor (LDLr) knockout mouse model. We show here for the first time that CD8$^+$ T-cells contribute to increased plaque stability, as well as to a microenvironment-specific skewing of CD4$^+$ T-cells within the lesions.

2. Materials and methods

2.1. Human studies

7 plaques from the carotid artery and 12 plaques from the femoral artery were obtained during endarterectomy from anonymous individuals, from whom we did not receive any patient details. The patients underwent endarterectomy surgery between July and December 2016 at the Haaglanden Medical Center, Westeinde, The Hague, NL. The handling of all human samples complied with the "Code for Proper Secondary Use of Human Tissue" and conforms with the principles outlined in the Declaration of Helsinki. Single-cell suspensions were obtained from human plaques by cutting the tissue into small pieces, followed by a 2-hour digestion at 37 °C with an enzymatic mix consisting of collagenase IV (Gibco) and DNase (Sigma) as previously described [13]. Cells were stained for flow cytometric analysis as described below.

2.2. Animals

LDLr$^{-/-}$ and apolipoprotein E (apoE)$^{-/-}$ mice were purchased from Jackson Laboratory (Sacramento, CA, USA) and bred in-house. Animals were kept under standard laboratory conditions; food and water were provided *ad libitum*. All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

2.3. Murine studies

For the pilot study to determine the dosing regimen of the CD8α-depleting antibody, male LDLr$^{-/-}$ mice (n = 3) were injected with 50 µg anti-CD8α antibody (clone 2.43, BioXcell, NH, USA). 100 µL of blood was drawn for a baseline measurement and at 1, 4 and 7 days post injection from the tail vein in EDTA containing tubes (Sarstedt) and subsequently analyzed by flow cytometry. For the CD8 depletion study, male LDLr$^{-/-}$ mice (n = 24) were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK) for 10 weeks, and subsequently randomized into two groups based on age, weight, and plasma cholesterol levels. WTD feeding was continued for another 6 weeks combined with twice weekly i.p. injections of 50 µg rat IgG2b isotype control (clone LTF-2) or anti-CD8α antibody. During the 6-week injection period, depletion efficiency was monitored by drawing 100 µL of blood from the tail vein in EDTA containing tubes (Sarstedt) from n = 6 mice per group every two weeks the day after injection, alternating each week between the mice. At the end of the experiment, mice were sacrificed via subcutaneous injection with a mix of ketamine (100 mg/ml), sedazine (25 mg/ml) and atropine (0.5 mg/ml) and tissues were harvested after *in situ* perfusion using PBS. Total cholesterol levels were assessed using an enzymatic colorimetric assay (Roche Diagnostics).

To investigate FasL expression on CD8^{+} T-cells of atherosclerotic mice, 3 male apoE$^{-/-}$

mice of 65 weeks old were used, which display advanced atherosclerotic lesions at this age. Mice were sacrificed and spleens and aortas were harvested after *in situ* perfusion with PBS.

For the FasL blocking study, male LDLr$^{-/-}$ mice (n = 20) were fed a WTD for 12 weeks, and subsequently randomized into two groups based on age, weight, and plasma cholesterol levels. WTD feeding was continued for 2 weeks combined with i.p. injections on alternate days of 500 µg FasL-neutralizing antibody (clone MFL4 [14]) or Armenian hamster isotype control (Innovative Research, MI, USA). After a total of 8 injections, mice were sacrificed, and tissues were harvested after *in situ* perfusion with PBS.

2.4. Cell preparation and flow cytometry

Mice were sacrificed and blood, spleens, and aortas were harvested. WBCs were obtained by lysing the blood twice for 2 minutes with lysis buffer (0.15 M NH_4Cl,1 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.3). Single-cell suspensions of spleens were obtained by using a 70 µm cell strainer (Greiner Bio-One). Splenocytes were lysed for 1 minute with lysis buffer to obtain WBCs. Aortas were cleaned of perivascular fat and cut up into small pieces and digested by incubation with digestion mix (collagenase I 450 U mL^{-1}, collagenase XI 250 U ml^{-1}, DNAse 120 U ml^{-1} and hyaluronidase 120 U ml^{-1}; all Sigma-Aldrich) for 30 min at 37 °C while shaking and subsequently strained over a 70 µm strainer. A maximum of 200 000 cells was stained with the appropriate antibodies (Table S1). To stain apoptotic cells, Annexin V Apoptosis Detection Kit (eBioscience) was used according to manufacturer's protocol. For intracellular staining, cells were fixed and permeabilized by using an intracellular staining kit (eBioscience) according to manufacturer's protocol. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S or BD Biosciences Canto II and FlowJo software (Treestar).

2.5. Histological analysis

All hearts were embedded in O.C.T. compound (Sakura) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, 10 µm sections were collected. Analysis of lesion size was performed on cryosections of the aortic root lesion stained with Oil-red O and haematoxylin (Sigma-Aldrich). Corresponding sections were stained with Sirius Red (Sigma-Aldrich) to determine collagen content and with Masson's Trichrome staining (Sigma-Aldrich) to determine the necrotic area. Plaque macrophages were stained immunohistochemically by using a monocyte/macrophage (MOMA)-2 antibody (1:1000 rat IgG2b, Serotec Ltd.) as a primary antibody, goat anti-rat IgG alkaline phosphatase conjugate (1:100; Sigma-Aldrich) as a secondary antibody, and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. Furthermore, sections were stained with an antibody against CD4 (1:90 clone RM4-5, BD Biosciences), biotinylated rabbit anti-rat IgG (1:200, Vector) as a secondary antibody, and 3-amino-9-ethyl carbazole (Dako) for visualization. For VCAM-1 staining,

sections were incubated with CD106 antibody (1:100, BD Biosciences), followed by incubation with biotinylated rabbit anti-rat IgG (1:200, Vector) as a secondary antibody, and stained with 3-amino-9-ethyl carbazole (Dako). TUNEL staining was performed using the In Situ Cell Death Detection Kit, POD (Sigma-Aldrich). The average plaque size (in μm^2) was calculated from 5 sequential sections. For all other stainings, three subsequent sections displaying the highest plaque content per mouse were analyzed. All microscopic analyses were performed on a Leica DM-RE microscope using Leica QWin software and were blinded for independent analysis. The percentages of collagen, VCAM-1^+ cells, and macrophages in the atherosclerotic lesions were determined by dividing the area in μm^2 stained positive for collagen, VCAM-1 or MOMA-2 by the total lesion surface area, and calculated as a percentage. The percentage of necrosis was determined by dividing the acellular area by the total lesion surface area and again calculated as a percentage. The total number of $CD4^+$ T-cells or $TUNEL^+$ cells in each stained section were counted, and the average was divided by the total lesion surface area in order to obtain the number of $TUNEL^+$ or $CD4^+$ T-cells per mm^2 of lesion area.

2.6. Cell culture

$CD4^+$ and $CD8^+$ T-cells were isolated from splenocytes of a male $LDLr^{-/-}$ mouse of 18 weeks old using the $CD4^+$ and $CD8^+$ T-cell isolation kits (Miltenyi Biotec). Isolated cells were mixed in a 1:1 ratio and 0.5×106 total cells were plated out in 12-well plates (Greiner Bio-One) and cultured overnight at 37 °C and 5% CO_2 in RPMI 1640 medium containing 25 mM HEPES (Lonza) supplemented with 10% fetal calf serum (FCS), 60 µM β-mercaptoethanol (Sigma), 100 U mL^{-1} mix of penicillin/streptomycin (PAA), 1% non-essential amino acids (NEAA; Gibco), 1% sodium pyruvate (Sigma) and 2% L-glutamine (Lonza). The medium was supplemented with 1 µg mL^{-1} of anti-CD3 and anti-CD28 (ThermoScientific) and 10 µg mL^{-1} of either FasL-neutralizing antibody (clone MFL414) or Armenian hamster isotype control (Innovative Research, MI, USA).

2.7. Statistical analysis

Data are presented as individual dot plots with bars denoting the mean, the number of animals in each group is stated in the text. Data were tested for normal distribution and analyzed by using a two-way ANOVA, two-tailed Student's T-test or Mann-Whitney test, as appropriate. Statistical analysis was performed by using Prism (GraphPad). Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. CD8$^+$ T-cell content negatively correlates with macrophage content in human atherosclerosis

With the progression of atherosclerosis, the total number of CD8$^+$ T-cells in the plaque increases [2]. However, other leukocyte populations may also increase in number. We sought to investigate whether there is a correlation between the number of these CD8$^+$ T-cells and other atherogenic cell types in human atherosclerosis. Nineteen endarterectomy samples were obtained from atherosclerosis patients and analyzed by flow cytometry for CD8$^+$ T-cells, CD4$^+$ T-cells and macrophages (Fig. 1A-C, for gating strategy, see Fig. S1). Interestingly, there was a significant inverse correlation between the percentage of CD8$^+$ T-cells and macrophages (p = 0.03, Fig. 1D), which could indicate that CD8$^+$ T-cells limit macrophage content in human atherosclerosis. Importantly, this correlation was not observed between the percentage of CD4$^+$ T-cells and macrophages (Fig. 1E), suggesting that a change in macrophage content does not lead to an increase in every T-cell subset and the correlation between CD8$^+$ T-cells and macrophages may have functional relevance.

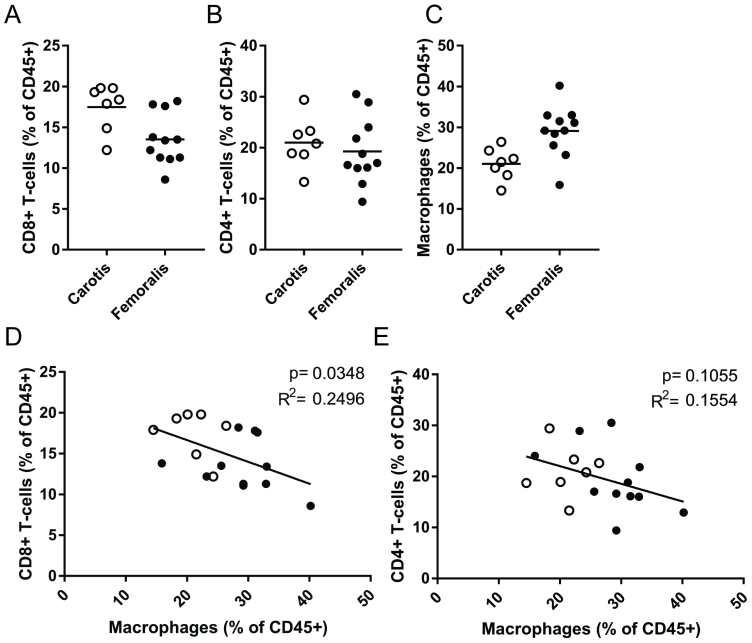

Figure 1: Inverse correlation between the percentages of CD8$^+$ T-cells and macrophages in human atherosclerotic lesions. Flow cytometry analysis of (A) CD8$^+$ T-cells (B) CD4$^+$ T-cells and (C) macrophages in endarterectomy samples from the arteria carotis (n = 7, open circles) or arteria femoralis (n = 12, closed circles) expressed as a percentage of live CD45$^+$ cells. Cells were gated as shown in Fig. S1 (D) Correlation between the percentage of CD8$^+$ T-cells and macrophages in all endarterectomy samples. (E) Correlation between the percentage of CD4$^+$ T-cells and macrophages in all endarterectomy samples. Significance was determined using linear regression analysis.

3.2. Lesion size is not affected by CD8$^+$ T-cell depletion in advanced stages of murine atherosclerosis

To further elucidate the role of CD8$^+$ T-cells in advanced atherosclerosis, we fed LDLr$^{-/-}$ mice a WTD for 10 weeks to establish lesions, followed by another 6 weeks of WTD combined with twice weekly administration of a CD8α-depleting antibody or an isotype control antibody (see Fig. S2A for the experimental setup). The dosing regimen was determined by a pilot experiment, in which 3 LDLr$^{-/-}$ mice were injected with 50 µg of CD8α-depleting antibody. CD8$^+$ T-cells were fully depleted for at least 4 days (Fig. S2B). As we saw a slight increase in the number of CD8$^+$ T-cells 7 days after injection of the anti-CD8 monoclonal antibody, we decided to inject the mice in the atherosclerosis experiment twice weekly. Administration of the CD8 depleting antibody in the atherosclerosis study resulted in successful depletion of CD8$^+$ T-cells in blood throughout the course of the experiment (Fig. S2C). At sacrifice, successful depletion was also observed in the spleen and aorta, whereas the CD8α^+dendritic cell population in the spleen was only slightly affected (Fig. S2D,E). We observed no differences in the percentages of NK cells, neutrophils or monocytes in the blood at sacrifice (Fig. S2F-H). Upon treatment with the CD8-depleting antibody, we did observe a percentual increase in CD4$^+$ T-cells within the T-cell population, as is to be expected, as well as a percentual increase of B-cells in the spleen. However, the absolute numbers of these cells were not different upon treatment (Fig. S2I-L). Depletion of CD8$^+$ T-cells in advanced atherosclerosis did not affect the aortic root lesion size as determined by Oil-red O staining (Fig. 2). We found no difference in body weight at any time during the treatment, nor did we find any differences in serum cholesterol levels (Fig. S2M,N).

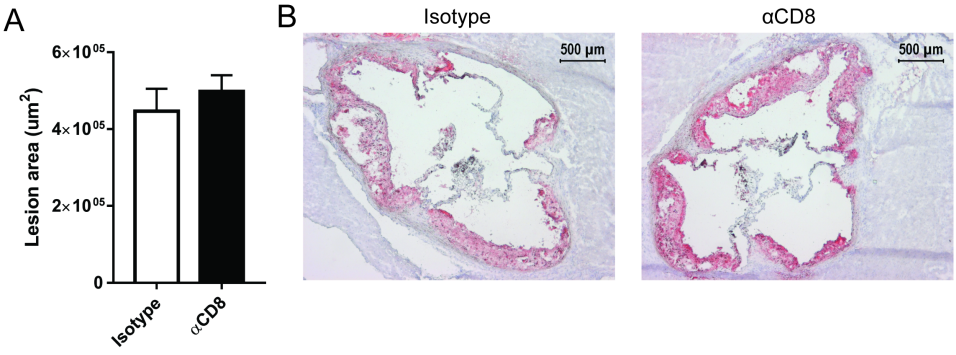

Figure 2: CD8$^+$ T-cell depletion does not affect lesion size in advanced atherosclerosis. (A) Quantification of lesion size in the aortic roots of LDLr$^{-/-}$ mice treated with CD8-depleting or isotype antibody by Oil-red O staining, n = 12 mice per group. Significance was determined using an unpaired T-test. (B) Representative images of ORO staining.

3.3. Advanced atherosclerotic lesions show decreased plaque stability upon CD8$^+$ T-cell depletion and increased inflammatory CD4$^+$ T-cells responses

Although lesion size was not significantly affected by CD8$^+$ T-cell depletion in advanced atherosclerosis, we investigated whether plaque stability and composition were altered in the aortic root lesions of these mice. The collagen content, assessed by Sirius Red staining, showed a significant decrease of 18% upon depletion of CD8$^+$ T-cells ($p = 0.02$, Fig. 3A,B). In addition, we found a 42% increase in necrotic core formation in the CD8$^+$ T-cell depleted group ($p = 0.04$, Fig. 3C,D). The content of total apoptotic cells in the lesions was not significantly different between both groups, as measured by TUNEL staining ($p = 0.48$, Fig. S3A). Regarding the monocyte/macrophage positive area, we observed a 27% increase upon CD8$^+$ T-cell depletion in the total area stained positive for MOMA-2 ($p = 0.045$, Fig. 3E,F). The most likely reasons for the increasing number of macrophages and decreased stability of the lesions after CD8$^+$ T-cell depletion would be an enhanced influx of monocytes or reduced cell death of macrophages in the lesion. Regarding monocyte influx, VCAM-1 is known to play an important role in monocyte adhesion upon the endothelium of the atherosclerotic lesion [15]. However, the expression of VCAM-1 in the aortic root endothelium was not found to be significantly different between both groups ($p = 0.14$, Fig. S3B). Furthermore, the percentage of monocytes in the blood did not show any differences between the two groups ($p = 0.86$, Fig. S3C). Based on these results, we cannot exclude effects of CD8$^+$ T-cell depletion on macrophage recruitment or adhesion. However, it is likely that other, lesion localized, mechanisms may be involved in the observed increase in lesional macrophage content.

Besides macrophages and CD8$^+$ T-cells, CD4$^+$ T-cells represent a third major leukocyte population in atherosclerotic lesions. Especially IFN-γ-producing Th1 cells have been associated with macrophage activation and plaque instability [16]. To determine whether CD4$^+$ T-cells play a role in destabilizing the plaques upon depletion of CD8$^+$ T-cells, we analyzed the presence and phenotype of CD4$^+$ T-cells in the lesions. We observed no difference in the number of CD4$^+$ T-cells in the aortic root lesions of these mice by immunohistochemistry ($p = 0.44$, Fig. 4A). However, we observed a skewing towards a more inflammatory Th1 phenotype in the aortic plaques of the CD8-depleted mice compared to the controls. The T-bet expression in CD4$^+$ T-cells in this group was significantly increased by 25% resulting in an over two-fold increase in the T-bet/GATA3 ratio ($p = 0.01$, Fig. 4B, Fig. S3D), indicating a shift from the Th2 towards the Th1 phenotype. Interestingly, this skewing of CD4$^+$ T-cell responses was not observed in the circulation (data not shown), whereas opposite trends were observed for T-bet expression ($p = 0.02$) and the T-bet/GATA3 ratio in the splenic compartment ($p = 0.09$, Fig. 4C, Fig. S3D). Total CD4$^+$ T-cell numbers in the spleen were not significantly different between both treatment groups ($p = 0.15$, Fig. 4D). Taken together, these results suggest a local, anti-inflammatory and lesion-stabilizing role of CD8$^+$ T-cells in advanced atherosclerosis.

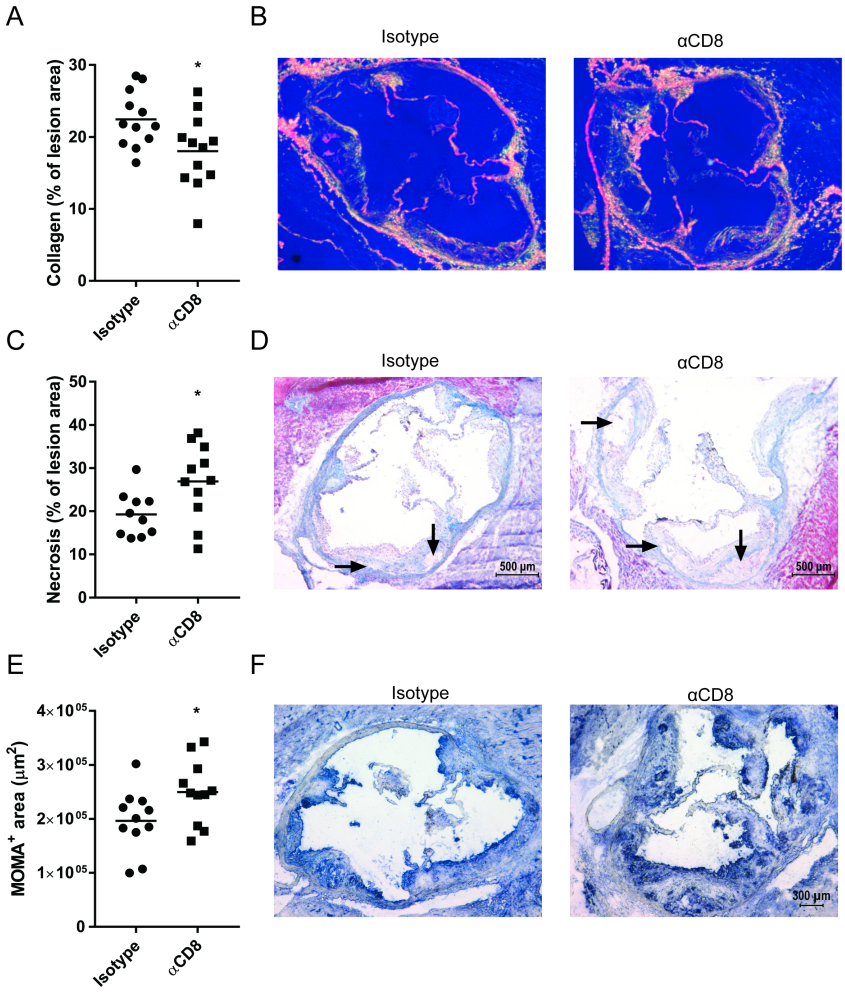

Figure 3: CD8$^+$ T-cell depletion in advanced lesions reduces plaque stability and increases necrosis and macrophage content. (A) Quantification of collagen content by Sirius Red staining in the aortic roots of LDLr$^{-/-}$ mice treated with CD8-depleting or isotype antibody. Significance was determined using a Mann-Whitney test. (B) Representative images of Sirius Red staining. (C) Quantification of necrosis in the aortic roots of the LDLr$^{-/-}$ mice. Significance was determined using an unpaired T-test (D) Representative images of the Masson's Trichrome staining, necrotic areas are indicated by arrows. (E) Macrophage quantification in the aortic roots of the LDLr$^{-/-}$ mice. (F) Representative images of MOMA-2 staining. n = 12 mice per group. Significance was determined using an unpaired T-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

3.4. FasL blockade increases inflammation in advanced atherosclerotic lesions

Although CD8$^+$ T-cells are foremost known as a pro-inflammatory cell type, various reports also support an immune regulatory role for these cells [11, 12, 17]. For instance, CD8$^+$ T-cells can kill dendritic cells in an antigen-specific, perforin-dependent man-

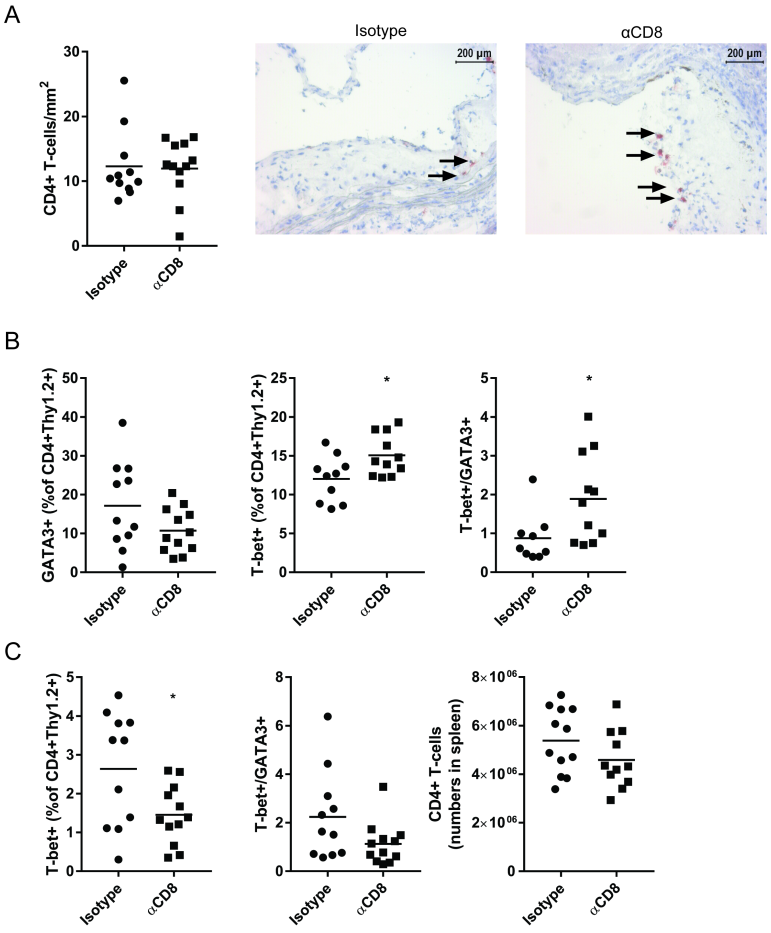

Figure 4: CD8$^+$ T-cell depletion in advanced lesions skews CD4$^+$ T-cell responses towards a more inflammatory phenotype specifically in the aortic microenvironment. (A) Quantification of CD4$^+$ T-cell staining per mm^2 of lesion in the aortic roots of LDLr$^{-/-}$ mice treated with CD8-depleting or isotype antibody, and representative images of the CD4 staining, arrows indicate CD4$^+$ cells. (B) Flow cytometry analysis of percentages of aortic CD4$^+$ T-cells expressing GATA3 and T-bet, as well as the T-bet$^+$/GATA3$^+$ ratio. (C) Flow cytometry analysis of the percentages of splenic CD4$^+$ T-cells expressing T-bet, as well as the T-bet$^+$/GATA3$^+$ ratio. (D) The total amount of CD4$^+$ T-cells in the spleens at the time of sacrifice. Cells were pregated on Live Thy1.2$^+$CD4$^+$ cells. n = 12 mice per group. All significance was determined using unpaired T-tests. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

ner [18] and can regulate T-cell homeostasis by killing activated T-cells via a Fas-FasL-mediated mechanism [19]. Interestingly, a deficiency in Fas on hematopoietic cells in LDLr$^{-/-}$ mice results in an enhanced inflammatory state [20] and a decrease in lesion stability [21], suggesting Fas-FasL interaction is an important immune regulatory pathway in the context of hypercholesteremia. As Th1 cells are more susceptible to FasL-induced apoptosis than Th2 cells [22], we hypothesized that the Th1 skewing effect we observed specifically in the lesions of CD8$^+$ T-cell depleted mice, was in part mediated

through the lack of FasL-induced apoptosis. Therefore, we first set out to determine whether $CD8^+$ T-cells in the lesions of advanced atherosclerotic mice express increased levels of FasL compared to their counterparts in the spleen. To this end, we isolated the spleens and aortas of mice with advanced atherosclerosis. Flow cytometry analysis confirmed that the mean fluorescence intensity (MFI) for FasL was 1.7-fold higher in the aortas compared to the spleens ($p = 0.008$, Fig. 5A, Fig. S4A). To assess whether FasL-mediated killing by $CD8^+$ T-cells specifically affects the Th1 $CD4^+$ T-cells in our model, $CD8^+$ and $CD4^+$ T-cells from $LDLr^{-/-}$ mice were isolated and stimulated *in vitro* in the presence of a blocking anti-FasL antibody or isotype control antibody and stained for apoptosis using Annexin V. Whereas apoptosis decreased only slightly and not significantly in Th2 cells upon anti-FasL treatment (1.5 fold, $p = 0.07$, Fig. 5B), there is a striking 4.1 fold decrease in apoptotic Th1 cells ($p = 0.0001$, Fig. 5C, Fig. S4B). This confirms previous work [22] and shows that FasL expressed on $CD8^+$ T-cells preferentially targets Th1 cells for apoptosis. Finally, we proceeded to block FasL activation in $LDLr^{-/-}$ mice in an advanced stage of lesion formation in order to assess whether this affected the $CD4^+$ T-cell responses and lesion development in a similar fashion as $CD8^+$ T-cell depletion. In agreement with the $CD8^+$ T-cell depletion study, treatment with anti-FasL antibody resulted in a significant increase in T-bet-expressing $CD4^+$ T-cells in the aortic lesion ($p = 0.04$, Fig. 5D) and not in the spleen (Fig. S4C), although GATA3 expressing $CD4^+$ T-cells were not changed ($p = 0.98$, Fig. 5E, Fig. S4D). We did observe a small, non-significant 1.3 fold increase in the T-bet/GATA3 ratio in the aortas of the treated mice ($p = 0.29$, Fig. 5F). Moreover, in agreement with the $CD8^+$ T-cell depletion study, we observed no changes in lesion size ($p = 0.09$, Fig. 5G), but immunohistochemical analysis of the aortic root lesions revealed a 69% increase in the monocyte/macrophage positive area upon treatment with the anti-FasL antibody ($p = 0.04$, Fig. 5H). Together, these data suggest that FasL-mediated interactions play an important immune regulatory role in atherosclerosis by decreasing Th1 $CD4^+$ T-cells and macrophages within the lesion.

4. Discussion

In this study, we show that $CD8^+$ T-cells may be protective in advanced stages of atherosclerotic lesion development. The negative correlation we observed between the percentages of $CD8^+$ T-cells and macrophages in human atherosclerosis indicates that $CD8^+$ T-cells may play a protective role by reducing plaque macrophage content. Additionally, in a murine model, we show that $CD8^+$ T-cells contribute to increased plaque stability in advanced atherosclerotic lesions, by restricting the accumulation of macrophages and pro-inflammatory Th1 cells. Importantly, we show that the effect of $CD8^+$ T-cells on Th1 cells is specific to the microenvironment of the lesion, as such effects are not observed in splenic tissue. Our results are in agreement with previously published studies, demonstrating that antigen-specific $CD8^+$ cells are protective against atherosclerosis by mounting a cytolytic response against antigen-presenting dendritic cells [11, 23]. Previous studies have shown that $apoE^{-/-}CD8^{-/-}$ mice show no difference in early or late atherosclerotic lesion development compared to $apoE^{-/-}$ mice [6]. Additionally, full body knockout of the antigen peptide transporter TAP1

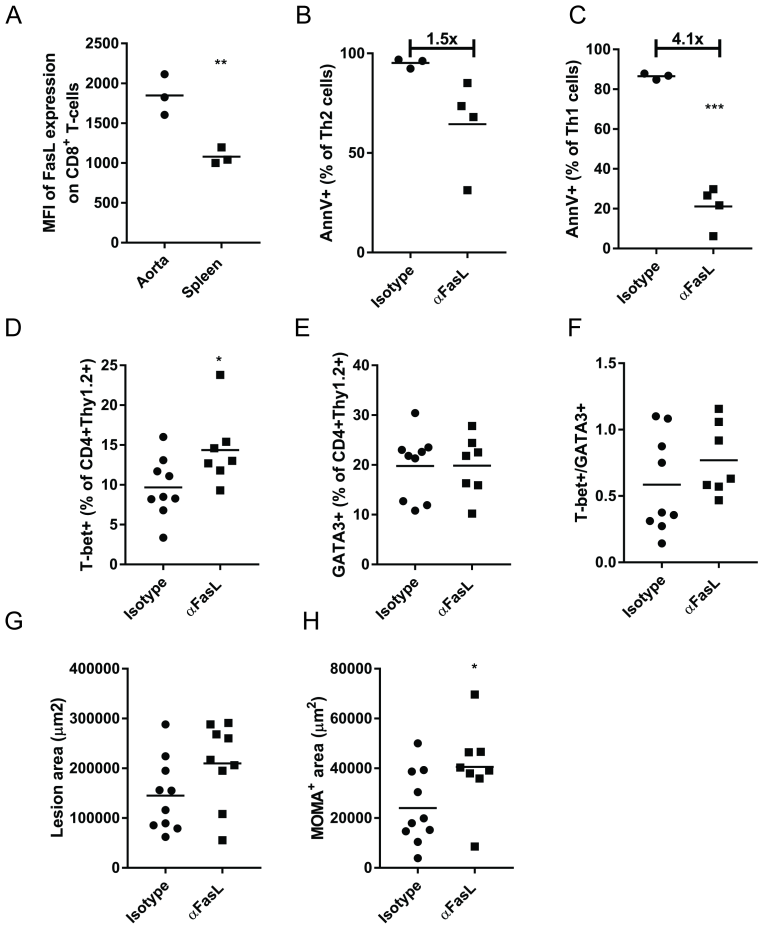

Figure 5: FasL blockade preferentially inhibits apoptosis of Th1 compared to Th2 CD4$^+$ T-cells and increases inflammatory CD4$^+$ T-cell responses in advanced atherosclerosis. (A) Flow cytometry analysis of FasL MFI on CD8$^+$ T-cells in single-cell suspensions obtained from the indicated organs of 3 male apoE$^{-/-}$ mice of 65 weeks old. (B-C) LDLr$^{-/-}$ derived CD4$^+$ and CD8$^+$ T-cells were cultured in a 1:1 ratio for 24h and stimulated with anti-CD3 and anti-CD28 antibodies in the presence of either anti-FasL or isotype antibody. Flow cytometry analysis of Annexin V staining on Thy1.2$^+$CD4$^+$GATA3$^+$ (B) and Thy1.2$^+$CD4$^+$T-bet$^+$ (C) cells, n = 4. (D-F) Flow cytometry analysis of aortic cells derived from LDLr$^{-/-}$ mice treated with anti-FasL or isotype antibody. Percentages of aortic CD4$^+$ T-cells expressing T-bet (D) and GATA3 (E), as well as the T-bet$^+$/GATA3$^+$ (F) ratio. Cells were pregated on Live Thy1.2$^+$CD4$^+$ cells. (G) Quantification of lesion size in the aortic roots of the LDLr$^{-/-}$ mice treated with anti-FasL or isotype antibody by Oil-red O staining. (H) Macrophage quantification in the aortic roots of the LDLr$^{-/-}$ mice. n = 9 in αFASL group, n = 8 in isotype group. All significance was determined using unpaired T-tests $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

in apoE$^{-/-}$ mice, resulting in deficient MHC-I antigen presentation, did not affect lesion development at either early or late stages of atherosclerosis [7]. However, these mutations are able to affect other cell types besides CD8$^+$ T-cells, and therefore do not provide conclusive evidence about CD8$^+$ T-cell function in atherogenesis. Other work has shown pro-atherogenic roles for CD8$^+$ T-cells in atherosclerosis, based on reduced

monopoiesis in the absence of $CD8^+$ T-cells [8]. In contrast to the data presented here, however, the aforementioned study focused on early stages of atherosclerotic lesion development, which suggests the role of $CD8^+$ T-cells may depend on the stage of atherogenesis. Indeed, another study investigating CD8α- and CD8β-depletion on initial lesion development in $apoE^{-/-}$ mice also showed reductions in lesion area, macrophage accumulation and necrotic core formation [9]. Together, this indicates a pro-atherogenic role for $CD8^+$ T-cells in initial atherosclerosis, whereas our data suggest a protective role for these cells in advanced atherosclerosis. In agreement with our findings, C57BL/6J mice that are deficient for MHC class I demonstrated a threefold increase in atherosclerotic lesion area compared to WT mice after 15 weeks on an atherogenic diet [10], suggesting a protective role for $CD8^+$ T-cells. Several regulatory $CD8^+$ T-cell subsets have been described that could exert protective effects on lesion development [12, 17], but as we used a CD8α-depleting antibody, we depleted all $CD8^+$ T-cell subsets and were therefore unable to identify which $CD8^+$ T-cell subset is responsible for the protective effects observed here.

The lesion size in advanced stages of atherosclerosis was not affected by $CD8^+$ T-cell depletion in our study. As we only started depleting the $CD8^+$ T-cells after 10 weeks of WTD feeding, upon which lesions are already established, we expected the treatment to mainly affect lesion composition. Indeed, we did observe a decreased plaque stability upon depletion in this stage. Specifically, $CD8^+$ T-cell depletion decreased collagen content, whereas it increased necrotic core formation and plaque macrophages. We observed a microenvironment-specific skewing of $CD4^+$ T-cells towards the Th1 phenotype, which may explain the observed effects on plaque stability. Th1 cells are known to produce high levels of IFN-γ, which has been shown to inhibit collagen synthesis by vascular smooth muscle cells [24] and may explain the reduced collagen content observed here. Indeed, vaccination against IL-12, a cytokine known to favor the development of Th1 cells, was previously shown to inhibit atherosclerosis development and promote lesion stability via a Th1/Th2 switch and the associated reduction in IFN-γ levels [25]. A Th1 shift and an increase IFN-γ may explain why we observed an increase in plaque resident macrophages upon CD8 depletion. Monocyte transmigration into atherosclerotic lesions is mediated by upregulation of IFN-γ inducible VCAM-1 and ICAM-1 [26]. Even though we found no increases in VCAM-1 expression at the moment of sacrifice, we cannot exclude regulation of these adhesion molecules at earlier time points during the experiment. Furthermore, we have not measured ICAM-1 expression, which may have contributed to the increased MOMA-2 staining observed in this study. Alternatively, the increased macrophage accumulation may be caused by decreased apoptosis of macrophages, however, we did not observe any changes in the number apoptotic ($TUNEL^+$) cells in lesions of $CD8^+$ T-cell depleted mice. Importantly, we provide data suggesting a direct role of $CD8^+$ T-cells in the regulation of Th1 $CD4^+$ T-cells and macrophages through Fas-FasL-mediated apoptosis. Fas-FasL interaction is a major contributor to apoptosis of activated $CD4^+$ T-cells, a process referred to as activation-induced cell death [27]. We propose that $FasL^+CD8^+$ T-cells may be able to regulate $CD4^+$ T-cell responses via Fas-FasL-induced apoptosis of Th1 cells. Fas expression on bone marrow-derived cells has been shown to play a protective role in atherosclerosis development as bone marrow transplantation of cells derived

from Fas-mutant lpr mice into LDLr$^{-/-}$ mice resulted in less fibrous lesions compared to transplantation with WT bone marrow, suggesting Fas expression contributes to a more stable lesion phenotype [21]. Here, we show that CD8$^+$ T-cells within atherosclerotic lesions express higher levels of FasL than their counterparts in the spleen, suggesting that the atherosclerotic microenvironment may affect CD8$^+$ T-cell phenotype and function. Th1 cells have an increased susceptibility to FasL-induced cell death, as they express lower levels of Fas-associated phosphatase 1, which plays an important role in inhibiting FasL-induced cell death by attenuating Fas export to the cell surface [22, 28]. Indeed, we observed that upon *in vitro* treatment of activated LDLr$^{-/-}$ derived CD4$^+$ and CD8$^+$ T-cells with a FasL-neutralizing antibody, the decrease in apoptotic cells was much larger in the Th1 subset compared to the Th2 subset. Upon *in vivo* treatment of atherosclerotic mice with a neutralizing FasL antibody, we observed a similar skewing of CD4$^+$ T-cell subsets in favor of Th1 CD4$^+$ T-cells as we did in CD8-depleted mice. Although this *in vivo* experiment cannot rule out contributions of other FasL$^+$ cell types; in light of the upregulation of FasL on CD8$^+$ T-cells and the increased susceptibility of Th1 cells to FasL-induced cell death these data suggest that FasL is an important effector molecule for CD8$^+$ T-cells to limit Th1 accumulation in the plaque.

Alternatively to direct CD4$^+$ T-cell inhibition, indirect effects on CD4$^+$ T-cell skewing can be exerted via cytolytic killing of macrophages by CD8$^+$ T-cells. Inflammatory macrophages are known to secrete cytokines that can recruit CD4$^+$ T-cells toward the lesion site and skew them towards the Th1 phenotype [29, 30]. CD8$^+$ T-cells have long been established to have the capacity to kill virus- or bacteria-infected APCs [31]. Inflammatory stimuli such as IFN-γ are able to increase Fas expression on cultured macrophages, which increases their susceptibility to Fas-mediated apoptosis [32]. Additionally, free cholesterol loading in WT macrophages was previously shown to result in caspase-induced apoptosis, which is much less pronounced in either gld or lpr macrophages [33]. This suggests that macrophage foam cells could be killed by CD8$^+$ T-cells in a Fas-FasL mediated fashion. As CD8$^+$ T-cell depletion in our study resulted in an increased lesional macrophage content, CD8$^+$ T-cells could potentially regulate lipid-loaded macrophage numbers in advanced atherosclerosis directly. Interestingly, upon treatment with an anti-FasL antibody, we observed an increase in lesion macrophage content as well, suggesting that FasL-induced apoptosis of macrophages by CD8$^+$ T-cells may contribute to their protective effect against atherosclerosis. Notably, we observed a link between CD8$^+$ T-cell percentages and the percentage of macrophages in human lesions, suggesting that the regulatory role of CD8$^+$ T-cells we describe here for a murine model of atherosclerosis, may hold true in humans as well.

Finally, our results indicate a microenvironment-specific role for CD8$^+$ T-cells in controlling Th1 responses in atherosclerotic lesions, as we observed this effect only in the aorta and not in the blood or spleen. This finding illustrates the importance of investigating local immune responses, in addition to systemic immune responses. We hypothesize that the atherosclerotic microenvironment contains many lipid-derived and inflammatory stimuli that alter the CD8$^+$ T-cell phenotype specifically at this site. A recent clinical trial with IL-1β-neutralizing antibodies (canakinumab) showed that systemic anti-inflammatory responses significantly reduce cardiovascular events [34], al-

though administration of this drug could also cause neutropenia and was associated with fatal infection. As the anti-inflammatory effect of $CD8^+$ T-cells appears to act locally, expanding these T-cells may provide an interesting strategy to lower inflammation associated with atherosclerosis without unwanted systemic immune suppression.

5. Conclusion

In conclusion, these *in vivo* experiments demonstrate a protective effect of $CD8^+$ T-cells in advanced atherosclerotic lesions via a reduction of macrophages and Th1 cells and show an immune modulatory role for FasL. The protective effect of $CD8^+$ T-cells may be exploited by stimulating $CD8^+$ T-cell responses in advanced stages of atherogenesis, which could translate into the suppression of atherosclerosis in humans.

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Supplementary information

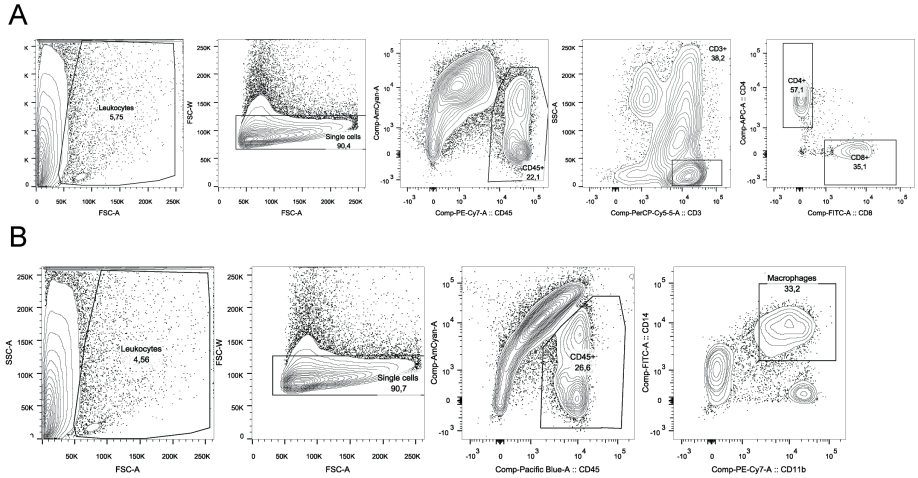

Figure S1: Flow cytometry gating strategy for human atherosclerotic lesions. (A) Flow cytometry gating strategy for determining the percentage of $CD4^+$ and $CD8^+$ T-cells in endarterectomy samples, cells were gated on leukocytes, $CD45^+$, $CD3^+$ and on $CD4^+$ or $CD8^+$. (B) Flow cytometry gating strategy for determining the percentage of macrophages cells in endarterectomy samples, cells were gated on leukocytes, $CD45^+$, and $CD14^+CD11b^+$. $CD45^+$ cells were gated against an empty channel in order to exclude autofluorescent cells.

Table S1: Antibodies used for flow cytometric analysis.

Antibody	Fluorochrome	Clone	Supplier
Anti-Mouse CD3	PerCP	145-2C11	BD Biosciences
Anti-Mouse CD4	Efluor450	GK1.5	eBioscience
Anti-Mouse CD4	PEcy5	GK1.5	eBioscience
Anti-Mouse CD45.2	APC	104	eBioscience
Anti-Mouse CD8	FITC	53-6.7	eBioscience
Anti-Mouse CD8	PerCP	53-6.7	BD Biosciences
Anti-Mouse CD8	PE-TR	5H10	ThermoScientific
Anti-Mouse CD11b	Efluor450	M1/70	eBioscience
Anti-Mouse FasL	Fitc	MFL3	eBioscience
Anti-Mouse GATA3	PE	TWAJ	eBioscience
Anti-Mouse Ly-6C	PerCP/cy5.5	HK1.4	eBioscience
Anti-Mouse Ly-6G	PE	1A8	BD Biosciences
Anti-Mouse NK1.1	FITC	PK136	BD Biosciences
Anti-Mouse T-bet	PEcy7	4B10	eBioscience
Anti-Mouse T-bet	Efluor660	4B10	eBioscience
Anti-Mouse Thy1.2	PerCP/cy5.5	53-2.1	Biolegend
Anti-Mouse Thy1.2	Pecy7	53-2.1	eBioscience
Anti-Human CD3	PerCP/cy5.5	OKT3	eBioscience
Anti-Human CD45	PEcy7	2D1	eBioscience
Anti-Human CD45	Efluor450	2D1	eBioscience
Anti-Human CD8	FITC	SK1	eBioscience
Anti-Human CD4	APC	RPA-T4	Biolegend
Anti-Human CD14	FITC	61D3	eBioscience
Anti-Human CD11b	PEcy7	ICRF44	eBioscience
Fixable Viability Dye	Amcyan	-	eBioscience
Fixable Viability	Efluor780	-	eBioscience

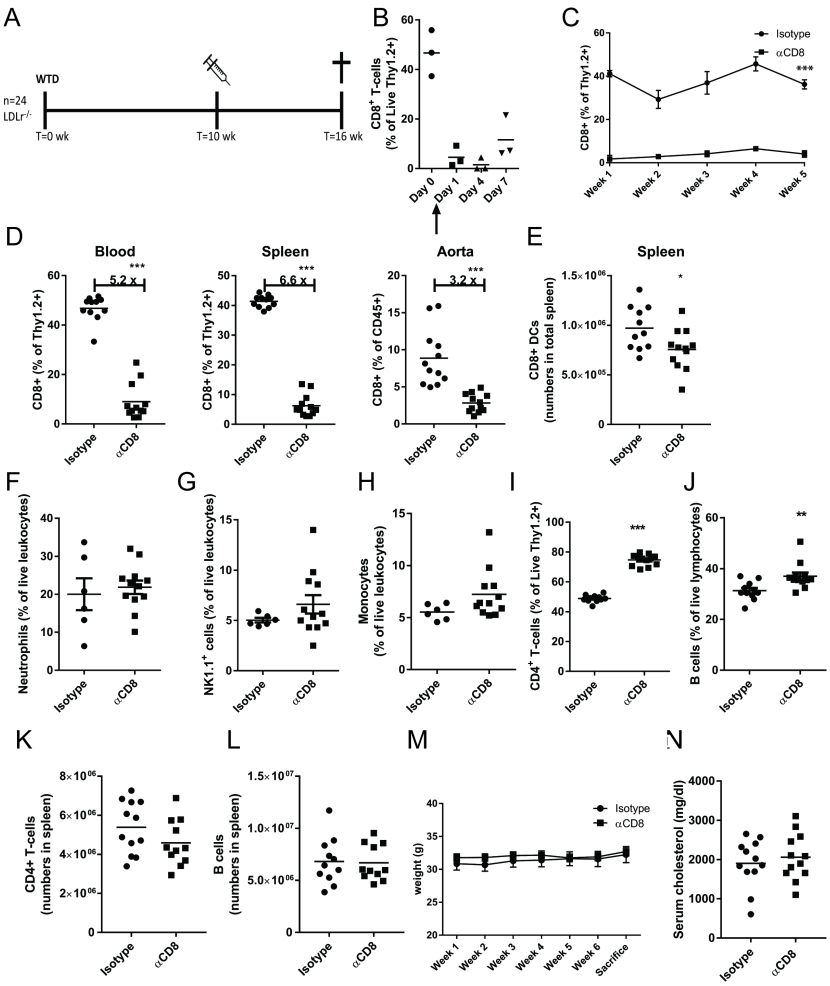

Figure S2: Treatment with CD8α-depleting antibody results in a significant reduction in the number of CD8$^+$ T-cells in various tissues (A) Experimental setup of the CD8 depletion study. 24 male LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks to establish lesions, followed by 6 weeks of diet combined with twice weekly i.p. injections of 50 µg isotype (n = 12) or anti-CD8 (n = 12) antibody. (B) Percentage of CD8$^+$ T-cells in the blood of 3 LDLr$^{-/-}$ mice injected with 50 µg of CD8-depleting antibody as measured in the blood over 7 days, arrow indicates injection time point. (C) Analysis of percentages of CD8$^+$ T-cells in blood lysates obtained each week during the injection period. n = 6 mice per group were sampled at each time point. Cells were pregated on Live Thy1.2$^+$ cells. Significance was determined using a two-way ANOVA. (D) Analysis of percentages of CD8$^+$ T-cells in single-cell suspensions obtained from the indicated organs at the time of sacrifice, and fold decrease between the groups, pregated on Live Thy1.2$^+$ cells. Significance was determined using unpaired T-tests. (E) Total number of CD8$^+$ dendritic cells in the spleen at the time of sacrifice. Significance was determined using an unpaired T-test. (F-G) frequencies of NK1.1$^+$ cells (F), neutrophils (G, gated as NK1.1$^-$Ly6G$^+$) and monocytes (H, gated as NK1.1$^-$Ly6G$^-$CD11b$^+$) in the blood of the LDLr$^{-/-}$ mice at the time of sacrifice. Significance was determined using unpaired T-tests. (I-L) Percentages and numbers of CD4$^+$ T-cells (I,K gated as % of Thy1.2$^+$ live lymphocytes) and B-cells (J,L gated as percentage of live lymphocytes) in the spleens of the LDLr$^{-/-}$ mice at the time of sacrifice. Significance was determined using unpaired T-tests. (M) Body weights over time during the period of injection with CD8-depleting antibody or isotype control. Significance was determined using a two-way ANOVA. (N) Serum cholesterol levels at the time of sacrifice. Significance was determined using an unpaired T-test. Data are presented as mean ± SEM or as individual dot plots with bars denoting the mean. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

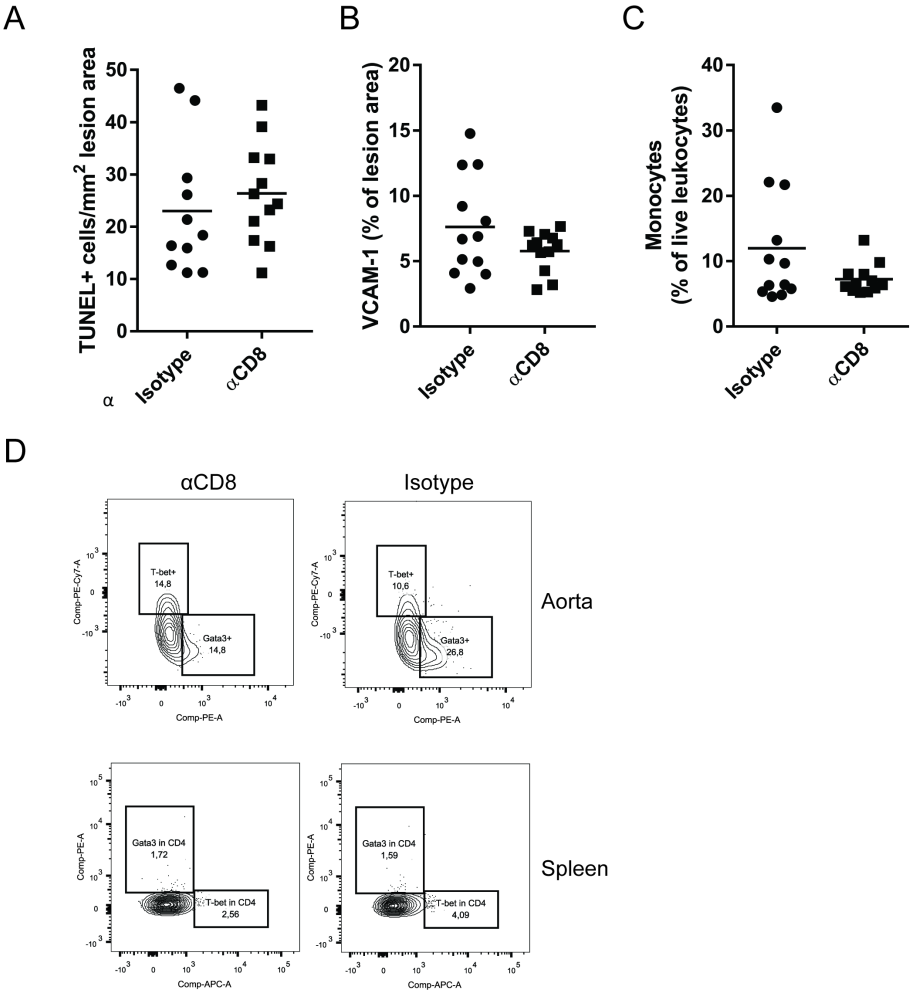

Figure S3: CD8 depletion in advanced lesions does not affect TUNEL staining, VCAM-1 expression or monocytes in the blood. (A) Quantification of TUNEL positive cells per mm^2 of lesion in the aortic roots at the time of sacrifice. (B) Quantification of VCAM-1 positive area in the endothelium of the aortic roots of $LDLr^{-/-}$ mice at the time of sacrifice. (C) Percentage of monocytes in the blood at the time of sacrifice. Monocytes were gated as Viable, $NK1.1^{-}Ly6G^{-}CD11b^{+}$ cells. Data are presented as individual dot plots with bars denoting the mean. n = 12 mice per group. All significance was determined using unpaired T-tests. (D) Representative flow cytometry plots of GATA3 and T-bet staining on aortic and splenic samples of anti-CD8 or isotype antibody-treated $LDLr^{-/-}$ mice. Cells were pregated on Live $Thy1.2^{+}CD4^{+}$ cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

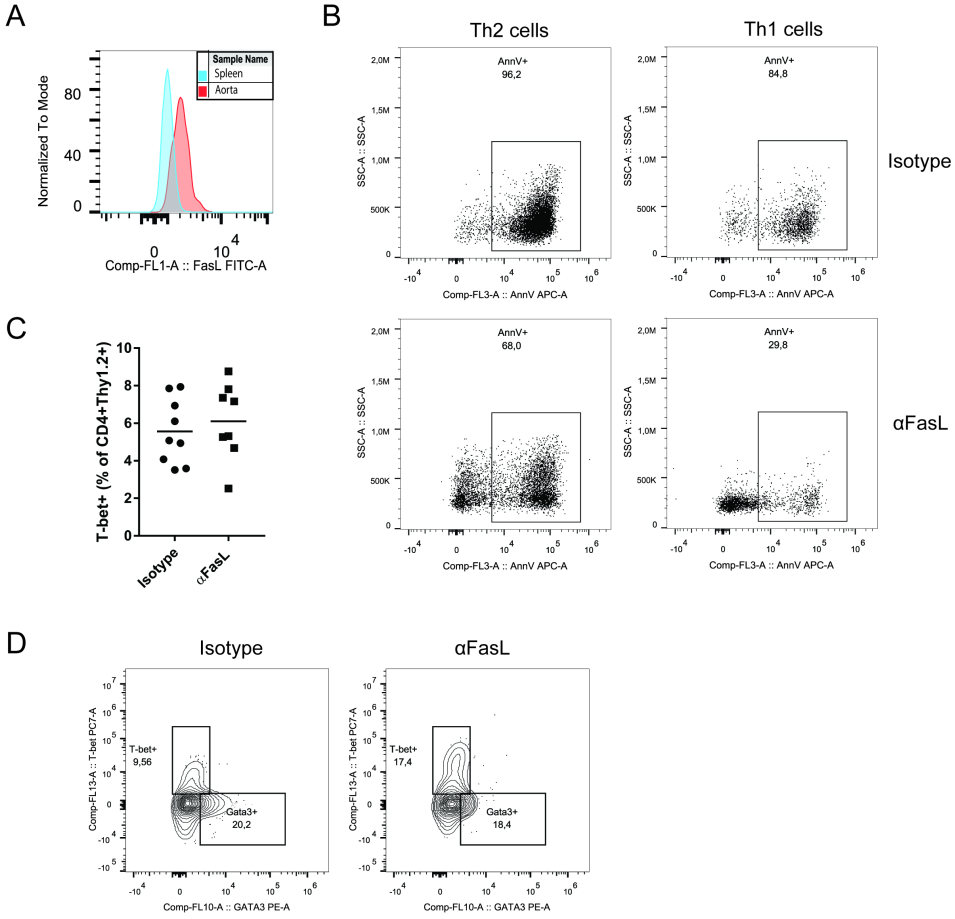

Figure S4: Representative flow cytometric images of FasL expression in aged apoE$^{-/-}$ mice, of annexin V staining on Th1 and Th2 cells and of T-bet and GATA3 staining in the anti-FasL experiment. (A) Representative histogram plots of FasL MFI on CD8^{+} T-cells in single-cell suspensions obtained from the indicated organs of 3 male apoE$^{-/-}$ mice of 65 weeks old. Cells were pregated on Live CD3^{+}CD8^{+} cells (B) LDLr$^{-/-}$ derived CD4^{+} and CD8^{+} T-cells were cultured in a 1:1 ratio for 24h and stimulated with anti-CD3 and anti-CD28 antibodies in the presence of either anti-FasL or isotype antibody. Representative flow cytometry plots of Annexin V expression on Th2 and Th1 cells, pregated on Live Thy1.2^{+}CD4^{+}GATA3^{+} or Live Thy1.2^{+}CD4^{+}T-bet^{+} cells, respectively. (C) Flow cytometry analysis of percentages of splenic CD4^{+} T-cells expressing T-bet upon treatment with anti-FasL antibody or isotype control antibody, cells were pregated on Live Thy1.2^{+}CD4^{+} cells. Significance was determined using an unpaired T-test. (D) Representative flow cytometry plots of GATA3 and T-bet staining on aortic samples of anti-FasL or isotype antibody-treated LDLr$^{-/-}$ mice. Cells were pregated on Live Thy1.2^{+}CD4^{+} cells, n = 9 in αFASL group, n = 8 in isotype group.

4

CD39 identifies a microenvironment-specific anti-inflammatory CD8$^+$ T-cell population in atherosclerotic lesions

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ABSTRACT

Background and aims CD8$^+$ T-cells have been attributed both atherogenic and atheroprotective properties, but analysis of CD8$^+$ T-cells has mostly been restricted to the circulation and secondary lymphoid organs. The atherosclerotic lesion however, is a complex microenvironment containing a plethora of inflammatory signals, which may affect CD8$^+$ T-cell activation. Here, we address how this environment affects the functionality of CD8$^+$ T-cells.

Methods and Results We compared the cytokine production of CD8$^+$ T-cells derived from spleens and enzymatically digested aortas of apoE$^{-/-}$ mice with advanced atherosclerosis by flow cytometry. Aortic CD8$^+$ T-cells produced decreased amounts of IFN-γ and TNF-α compared to their systemic counterparts. The observed dysfunctional phenotype of the lesion-derived CD8$^+$ T-cells was not associated with classical exhaustion markers, but with increased expression of the ectonucleotidase CD39. Indeed, pharmacological inhibition of CD39 in apoE$^{-/-}$ mice partly restored cytokine production by CD8$^+$ T-cells. Using a bone-marrow transplantation approach, we show that TCR signaling is required to induce CD39 expression on CD8$^+$ T-cells in atherosclerotic lesions. Importantly, analysis of human endarterectomy samples showed a strong microenvironment-specific upregulation of CD39 on CD8$^+$ T-cells in the plaques of human patients compared to matched blood samples.

Conclusion Our results suggest that the continuous TCR signaling in the atherosclerotic environment in the vessel wall induces an immune regulatory CD8$^+$ T-cell phenotype that is associated with decreased cytokine production through increased CD39 expression in both a murine atherosclerotic model and in atherosclerosis patients. This provides a new understanding of immune regulation by CD8$^+$ T-cells in atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease, characterized by the buildup of arterial plaques that contain both lipids and inflammatory cells. Among these cells are the $CD8^+$ T-cells, whose numbers have been shown to increase as the lesions progress towards more advanced stages [1]. However, the exact function of $CD8^+$ T-cells in atherosclerosis remains debated [2]. On the one hand, $CD8^+$ T-cells can contribute to monocyte recruitment [3] and increased plaque vulnerability [4], suggesting a pro-atherogenic role for these cells. On the other hand, $CD8^+$ T-cells can mount a cytolytic response against pro-atherogenic dendritic cells [5] and follicular helper T-cells [6], thereby limiting atherosclerosis development. Recently, we have shown a protective role for $CD8^+$ T-cells in the clinically relevant advanced stages of atherosclerosis [7]. Interestingly, we observed a microenvironment-specific skewing of $CD4^+$ T-cells upon CD8 depletion, suggesting that there is an interaction between $CD8^+$ T-cells and the local environment in the plaque.

The atherosclerotic lesion comprises a complex immunological environment. Cholesterol accumulates in the arterial intima in the form of low-density lipoproteins (LDLs). The excess of cholesterol cannot be cleared and becomes oxidized by various enzymes, forming oxidized LDL (oxLDL). This, in turn, is able to activate endothelial cells and drive the recruitment of inflammatory cells into the lesion. Many chemokines have been associated with atherosclerosis, among which are CCL2, CX3CL1, and CCL5 [8, 9]. The immune cells thus recruited to the plaque by these chemokines release an array of cytokines such as IFN-γ, TNF-α, and interleukin (IL)-12, which drives atherogenesis, as well as atheroprotective cytokines such as IL-10, IL-13 and transforming growth factor β [10]. Furthermore, antigen presenting cells (APCs) are able to process the intraplaque oxLDL and present oxLDL-derived antigens to induce adaptive immunity. Under the hyperlipidemic conditions in the lesion, APC migration to peripheral tissue is reduced, resulting in systemic as well as local T-cell activation [11]. Furthermore, cell death within the lesion due to the ongoing inflammation results in the release of damage-associated molecular patterns, which further drive the inflammatory response [12].

In this study, we aimed to determine how the aforementioned complex microenvironment in the atherosclerotic lesion affects local $CD8^+$ T-cells. We used the apolipoprotein E (apoE) knockout mouse model, which spontaneously develops atherosclerosis on a chow diet in response to increased plasma cholesterol levels. In these mice, we demonstrate that $CD8^+$ T-cells derived from aortic lesions show a dysfunctional phenotype, characterized by impaired cytokine production when compared to their counterparts in the spleen. This $CD8^+$ T-cell dysfunction was associated with an increased expression of the ectonucleotidase CD39. We further show that CD39 expression was induced by TCR signaling, and that pharmacological inhibition of CD39 could partially reverse the observed phenotypical changes of $CD8^+$ T-cells in the atherosclerotic microenvironment. Finally, we observed microenvironment-specific CD39 expression on $CD8^+$ T-cells derived from human atherosclerosis patients, indicating that the murine results may be translated to a clinical setting.

2. Materials and methods

2.1. Human studies

Plaques from the carotid artery were obtained after endarterectomy from 22 anonymous individuals. Matched blood samples were obtained before surgery from 12 of these patients. The patients underwent endarterectomy surgery between December 2017 and April 2018 at the Haaglanden Medical Center, Westeinde, The Hague, NL. The handling of all human samples complied with the "Code for Proper Secondary Use of Human Tissue" and are in accordance with the declaration of Helsinki regarding ethical principles for medical research involving human subjects (METC registration number 17-046). Single-cell suspensions were obtained from human plaques as described previously [13] (see supplementary data for a full description).

2.2. Animals

C57Bl/6, LDL receptor (LDLr)$^{-/-}$ and apoE$^{-/-}$ mice were purchased from Jackson Laboratory (Sacramento, CA, USA) and bred in-house. Animals were kept under standard laboratory conditions; food and water were provided *ad libitum*. In order to develop advanced atherosclerotic lesions in apoE$^{-/-}$ mice, animals were kept on a chow diet for 30-49 weeks before analysis of T-cell content in the lesion. Upon sacrifice, mice were subcutaneously anesthetized with an injection mix of ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL). All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

2.3. Bone marrow transplantation experiment

Bone marrow transplantation was performed as detailed in the supplementary data.

2.4. *In vitro* CD39 blockade

Splenocytes were derived from a 31 week-old male apoE$^{-/-}$ mouse. To obtain WBCs, splenocytes were lysed for 1 minute at room temperature in lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA; pH 7.3). CD8$^+$ T-cells were isolated using the CD8$^+$ T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Cells were cultured for 24 hours in the presence of 0, 25, 50 or 100 µM of the CD39 inhibitor sodium metatungstate (POM-1, Tocris) in RPMI supplemented with fetal calf serum (10%, PAA), L-glutamine (2%, Lonza), penicillin/streptomycin (1%, PAA), sodium pyruvate (1%, Sigma-Aldrich) and β-mercaptoethanol (60 µM, Sigma-Aldrich) at 37 °C and 5% CO_2.

2.5. *In vivo* CD39 blockade

To study the effect of CD39 blockade *in vivo*, apoE$^{-/-}$ mice with advanced atherosclerotic lesions (38-47 weeks old) were injected i.p. with 10mg/kg of POM-1 (Tocris) or with sterile PBS as a control (n = 5/group). After 24 hours, mice were sacrificed as described above.

2.6. Flow cytometry

Flow cytometry was performed as detailed in the supplementary data.

2.7. Statistical analysis

Data are presented as mean ± SEM, the number of animals in each group is stated in the text. Data were tested for normal distribution and analyzed by using a two-tailed Student's T-test, Mann-Whitney test, one-way ANOVA or two-way ANOVA, as appropriate. Statistical analysis was performed by using Prism (GraphPad). Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. Decreased cytokine production by CD8$^+$ T-cells in atherosclerotic lesions is associated with an upregulation of CD39 expression

Although the presence of CD8$^+$ T-cells in atherosclerosis has been established, the exact phenotype of these cells within the lesional microenvironment is as of yet unknown. Here, we examined the difference in phenotype between CD8$^+$ T-cells derived from aortic lesions and their counterparts in the spleen in advanced atherosclerosis using the murine apoE$^{-/-}$ model. Upon 2 hour stimulation of these cells with PMA and ionomycin, we observed a striking 46% decrease in the percentage of IFN-γ producing CD8$^+$ T-cells (Fig. 1A,C) and a 37% decrease in the percentage of TNF-α producing CD8$^+$ T-cells (Fig. 1B,D) in the lesion compared to the spleen. This decreased ability to produce cytokines could indicate that the CD8$^+$ T-cells derived from the lesion are exhausted. Nonetheless, we found no difference in the expression of the classical exhaustion markers PD-1 (Fig. 1E) and CTLA4 (Fig. 1F). However, using flow cytometry, we did observe a 9-fold increased expression of the ectonucleotidase CD39 on lesion-derived compared to splenic CD8$^+$ T-cells (Fig. 1G, 1H), which could not be explained by a different ratio of effector and central memory populations in aorta and spleen (Fig. S1A,B). This enzyme hydrolyzes extracellular ATP into ADP, which can further be converted into adenosine by the ectonucleotidase CD73. Interestingly, aortic CD8$^+$ T-cells show a slightly lower expression of CD73 compared to splenic CD8$^+$ T-cells (Fig. S1C), which may reflect their activation status [14]. Interestingly, CD39 has previously been reported to characterize exhausted CD8$^+$ T-cells during chronic infection [15]. We next set out to investigate whether CD39-expressing CD8$^+$ T-cells produce fewer cytokines. Unexpect-

edly, we observed that CD8$^+$CD39$^+$ T-cells are able to produce more IFN-γ compared to CD8$^+$CD39$^-$ T-cells in both aorta (13.3% vs. 1.7%, respectively) and spleen (35.6% vs. 9.5%, respectively, Fig. 1I), suggesting CD39 expression does not indicate exhaustion, but rather reflects the most activated CD8$^+$ T-cells in the plaque. Notably, although CD39$^+$CD8$^+$ T-cells produce more IFN-γ than CD39$^-$CD8$^+$ T-cells in the plaque, the production of IFN-γ by both of these subsets in the aorta is markedly lower compared to their counterparts in the spleen (Fig. 1I).

3.2. CD39 inhibition can increase cytokine production by CD8$^+$ T-cells in the lesions

Although CD39 does not appear to mark exhausted CD8$^+$ T-cells in the atherosclerotic plaque, we did observe decreased cytokine production in the aortic compared to the splenic CD8$^+$ T-cells. We, therefore, hypothesized that the role of CD39 in the stepwise conversion of ATP into adenosine may result in high adenosine levels specifically in the lesion, which has been reported to reduce cytokine production [16]. This may allow CD39$^+$CD8$^+$ T-cells to affect cytokine production of CD39$^-$ T-cells in a paracrine manner as well as CD39$^+$CD8$^+$ T-cells in an autocrine manner. To test this hypothesis, CD39 was inhibited by using the small molecule inhibitor sodium metatungstate (POM-1). *Ex vivo* stimulation of apoE$^{-/-}$ derived splenic CD8$^+$ T-cells with increasing concentrations of POM-1 resulted in a dose-dependent increase in IFN-γ production by CD8$^+$ T-cells (Fig. 2A). The expression levels of CD39 were also slightly increased upon treatment with POM-1 (Fig. 2B). Upon stratification of IFN-γ production by each subset, it appeared that CD39$^-$CD8$^+$ T-cells (which made up over 80% of all CD8$^+$ T-cells in this experiment) were for the most part responsible for this marked increase in IFN-γ production, as they showed a 3.9-fold increase in cytokine production upon treatment with the highest concentration of POM-1 (Fig. 2C). Within the CD39$^+$CD8$^+$ T-cell subset we only observed a modest 1.7-fold increase in IFN-γ production. This suggests indeed that CD39 expression may regulate the cytokine responses of both CD39$^-$ and CD39$^+$ cells by affecting paracrine and autocrine adenosine signaling.

To examine the effects of this inhibitor *in vivo*, apoE$^{-/-}$ mice were injected i.p. with 10 mg/kg POM-1 or PBS as a control and sacrificed 24 hours after treatment. Analysis of the aortic phenotype by flow cytometry revealed a trend towards an increased IFN-γ production by CD8$^+$ T-cells after POM-1 treatment in percentages (3.9% vs. 5.9%, p = 0.079 Fig. 2D,E) and a significant increase in mean fluorescent intensity (470 vs. 617 MFI, Fig. 2F). However, the levels are not returned to those observed in the spleen. Concomitantly, we observed an increased percentage of inflammatory mononuclear cells (CD11b$^+$/Ly6C^{hi}/Ly6G$^-$) in the aorta (Fig. S2), suggesting the increased IFN-γ production affected mononuclear cell recruitment and/or activation. Interestingly, POM-1 treatment increased the production of IFN-γ by both CD39$^+$ and CD39$^-$ CD8$^+$ T-cells. The pro-inflammatory cytokine production increased by 21% in the CD39$^-$CD8$^+$ subpopulation upon POM-1 treatment (Fig. 2D,H) and even more prominently by 37% in CD39$^+$CD8$^+$ T-cells (Fig. 2D,G). This suggests that CD39$^+$CD8$^+$ T-cells in the atherosclerotic lesion, besides inhibiting CD39$^-$CD8$^+$ T-cells in a paracrine fashion,

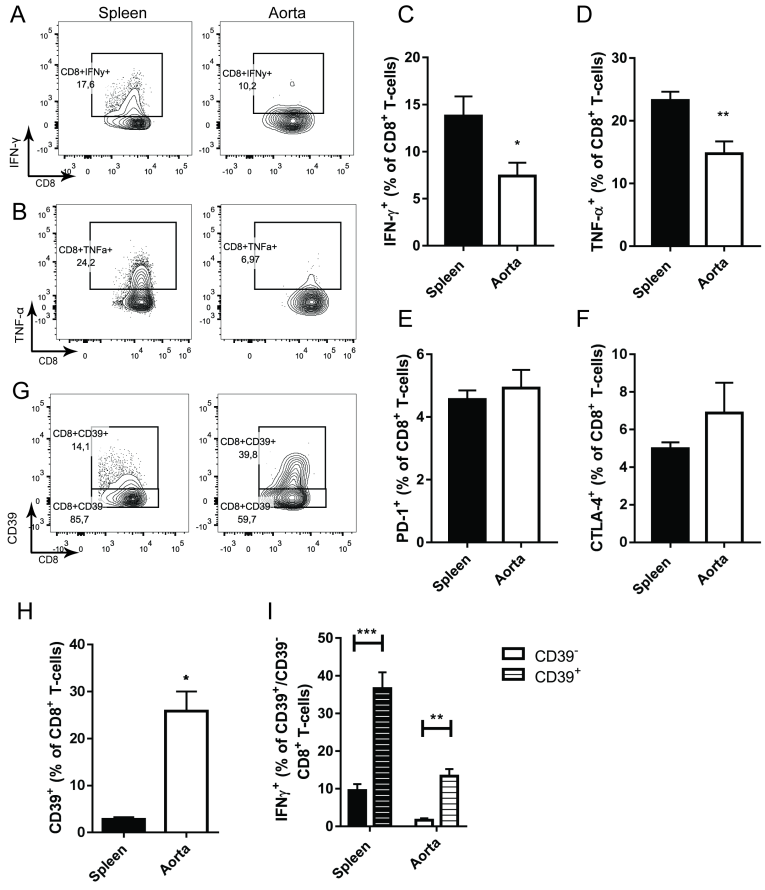

Figure 1: Murine atherosclerotic lesion-derived CD8$^+$ T-cells show a decreased inflammatory phenotype and increased CD39 expression compared to their counterparts in the spleen. (A-D) Flow cytometric analysis of the percentages of IFN-γ^+(A,C) and TNF-α^+ (B,D) CD8$^+$ T-cells derived from aortas and spleens of apoE$^{-/-}$ mice stimulated for 2 hours with PMA and ionomycin. Cells were pregated on live, Thy1.2$^+$CD8$^+$ T-cells. n = 6 (A,C) or n = 5 (B,D) apoE$^{-/-}$ mice of 35-49 weeks old, data are representative of 2 independent experiments. (E-H) Percentages of PD-1$^+$ (E), CTLA-4$^+$ (F) and CD39$^+$ (G,H) CD8$^+$ T-cells derived from aortas and spleens of apoE$^{-/-}$ mice. Cells were pregated on live, Thy1.2$^+$CD8$^+$ T-cells. n = 3-6 apoE$^{-/-}$ mice of 30-37 weeks old, data are representative of multiple independent experiments. (I) IFN-γ production by CD39$^+$ and CD39$^-$CD8$^+$ T-cells in the aortas and spleens apoE$^{-/-}$ mice stimulated for 2 hours with PMA and ionomycin. Cells were pregated on live, Thy1.2$^+$CD8$^+$CD39$^+$ or Thy1.2$^+$CD8$^+$CD39$^-$ T-cells. n = 6 apoE$^{-/-}$ mice of 41-49 weeks old, data are representative of 2 independent experiments. Mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

may maintain a negative feedback loop on their own inflammatory state through the production of adenosine.

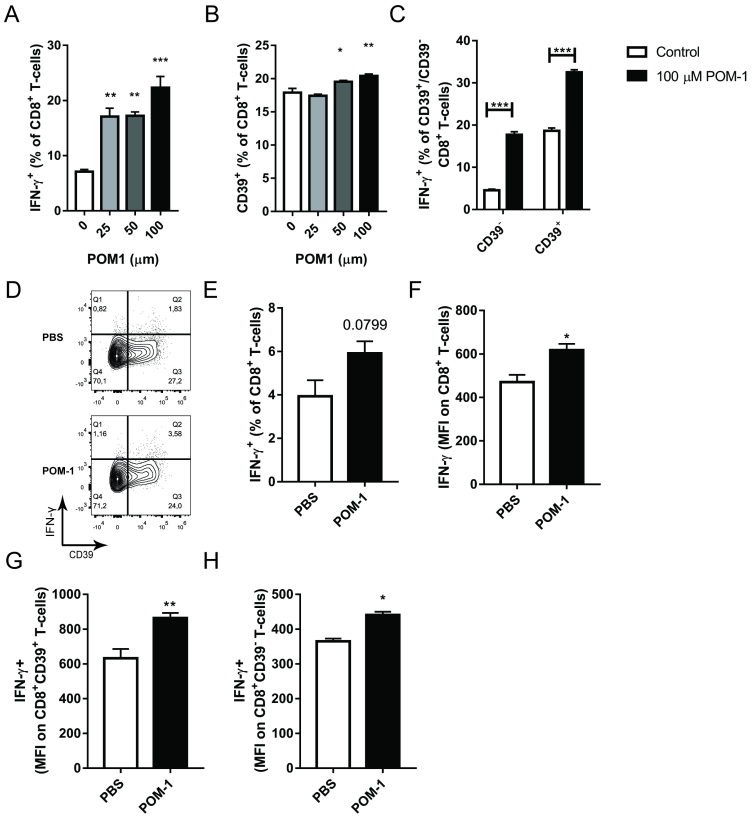

Figure 2: ***In vivo*** **CD39 blockade in atherosclerotic mice partially restores pro-inflammatory cytokine production in both CD39$^+$CD8$^+$ and CD39$^-$CD8$^+$ T-cells.** (A-C) Purified CD8$^+$ T-cells were derived from the spleen of a 31-week old apoE$^{-/-}$ mouse and cultured for 24 hours with the indicated concentrations of POM-1, after which the cells were stained and analyzed by flow cytometry for CD8$^+$IFN-γ^+ (A) and CD8$^+$CD39$^+$ (B) T-cells. (C) IFN-γ production by CD39$^+$ and CD39$^-$ CD8$^+$ T-cells cultured for 24 hours under control conditions or with 100 μM of POM-1. Cells were pregated on live, Thy1.2$^+$CD8$^+$ T-cells. (D-G) apoE$^{-/-}$ mice of 42-47 weeks of age were treated with 10 mg/kg POM-1 (n = 5) or sterile PBS (n = 5). After 24 hours, aortas were isolated and analyzed by flow cytometry. (D) Representative dot plots of PBS or POM-1 treated mice showing CD39 and IFN-γ expression on aortic CD8$^+$ T-cells. (E) Percentages and (F) MFI values of IFN-γ expression by CD8$^+$ T-cells. Cells were pregated on live, CD3$^+$CD8$^+$ T-cells. (G) CD39$^+$CD8$^+$ or (H) CD39$^-$CD8$^+$ T-cells producing IFN-γ. Cells were pre-gated live, CD3$^+$CD8$^+$CD39$^+$ or CD3$^+$CD8$^+$CD39$^-$ T-cells. Mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3. TCR signaling in the aortic microenvironment induces CD39 expression

Recent work has shown that upon T-cell receptor (TCR) activation, reactive oxygen species are generated that activate signaling cascades resulting in increased CD39 expression [16]. Moreover, CD39 has been reported as a useful marker to discriminate antigen-specific from non-specific bystander CD8$^+$ T-cells in a tumor environment [17]. As there is persistent antigen presentation to T-cells in atherosclerotic

lesions [18], we hypothesized that TCR signaling in the aortic microenvironment may cause the observed increase in CD39 expression on the $CD8^+$ T-cells within the lesion, whereas non-specific bystander $CD8^+$ T-cells do not express CD39. To test this, a bone marrow transplantation experiment was performed in which $LDLr^{-/-}$ mice received 70% $CD8^{-/-}$ bone marrow combined with either 30% WT or OT.1 bone marrow. After a 7-week recovery period, mice were fed a Western-type diet for 8 weeks in order to induce atherosclerosis (Fig. 3A). When using this setup, mice in the OT.1 donor group had only $CD8^+$ T-cells that are able to recognize ovalbumin through their TCR and were therefore not able to receive any stimulation from atherosclerosis-specific antigens. In contrast, mice in the WT donor group had $CD8^+$ T-cells that were able to receive TCR stimulation. We opted for this "reverse approach" in which $CD8^+$ T-cells that recognize an irrelevant antigen are compared to WT cells, as the antigen that activates $CD8^+$ T-cells in atherosclerosis is as of yet unknown. Strikingly, the number of $CD39^+CD8^+$ T-cells in the lesions of the mice transplanted with the OT.1 bone marrow was markedly lower when compared to controls (5% vs. 14.6%, Fig. 3B,C). By extension, the $CD8^+$ T-cells in the OT.1 group also showed a significantly reduced proliferative capacity, as measured by Ki-67 staining (15.3% vs. 43% Fig. 3D). Thus, TCR signaling is essential for the increased expression of CD39 on $CD8^+$ T-cells in the atherosclerotic microenvironment, and CD39 may mark antigen-experienced $CD8^+$ T-cells in the plaque.

3.4. CD39 expression is increased on $CD8^+$ T-cells derived from human atherosclerotic lesions compared to their systemic counterparts

We further investigated CD39 expression in human atherosclerotic lesions to determine whether the results described above are relevant to human atherosclerosis. $CD8^+$ T-cells derived from carotid endarterectomy samples showed a 2.7-fold higher expression of CD39 compared to their counterparts in the blood of these patients (Fig. 4A,B, for gating strategy, see Fig. S3). In agreement with the murine data, $CD8^+CD39^+$ T-cells derived from the lesions produced more IFN-γ compared to $CD8^+CD39^-$ cells (30.6% vs. 20.9%, respectively, Fig. 4C-E). Thus, similar to our findings in $apoE^{-/-}$ mice, the percentage of $CD39^+CD8^+$ T-cells in human atherosclerotic lesions is strongly increased compared to the blood, and appears to represent the most activated fraction of $CD8^+$ T-cells in the plaque.

4. Discussion

Atherosclerotic lesions are associated with an influx of $CD8^+$ T-cells as the lesion progresses towards more advanced stages [1]. As the lesion microenvironment is very complex, many factors within the lesion may well affect the phenotype of $CD8^+$ T-cells. It is of particular interest to understand the function of these cells within the plaque as compared to their circulating counterparts, as these cells can affect the lesion development and composition locally. In this work, we show for the first time that $CD8^+$ T-cells display an atherosclerotic microenvironment-specific dysfunction, as aortic-derived

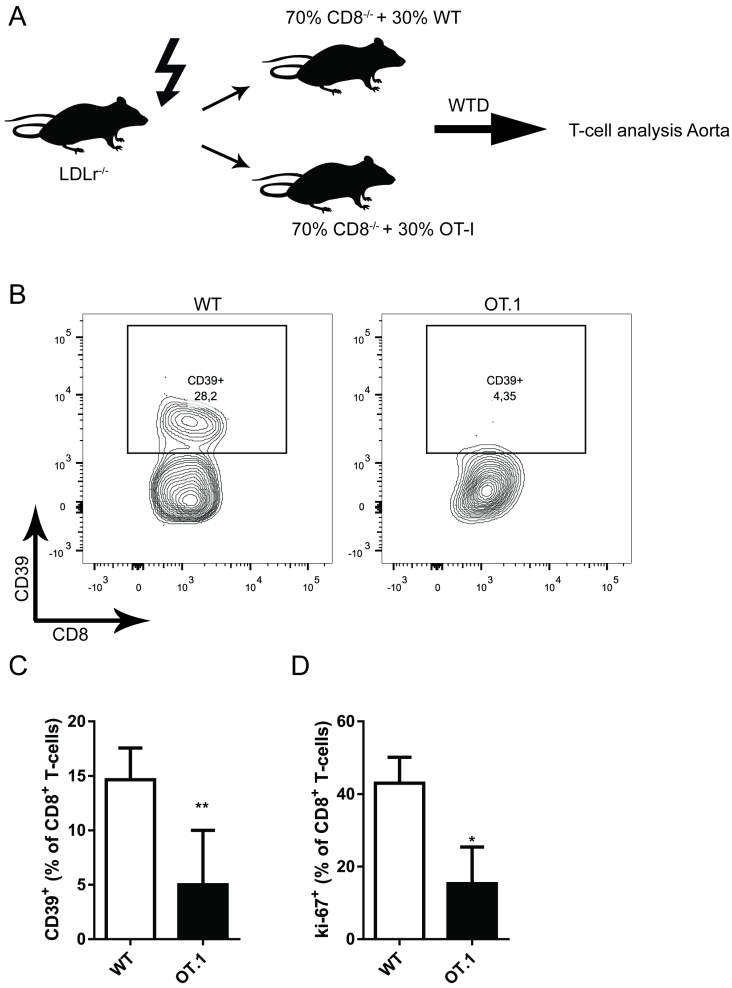

Figure 3: TCR signaling is required to induce CD39 on aortic CD8$^+$ T-cells. (A) Schematic overview of the experimental setup. (B) Representative contour plots depicting CD39 expression on aortic CD8$^+$ T-cells at sacrifice. (C-D) Percentages of CD39$^+$ (C) and Ki-67$^+$ (D) CD8$^+$ T-cells in the aorta of the mice transplanted with WT or OT.1 bone marrow at the time of sacrifice as analyzed by flow cytometry. Cells were pregated on live CD8$^+$ T-cells. WT (n = 5) and OT.1 (n = 11), unequal distribution due to attrition in the experiment. Mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

CD8$^+$ T-cells produce lower levels of cytokines compared with their counterparts in the spleen. Chronic antigen stimulation of CD8$^+$ T-cells is known to result in exhaustion and an associated reduced cytokine production by these cells [19]. Antigen-specific stimulation of T-cells also occurs over prolonged periods of time in atherosclerosis development [20]. We, therefore, hypothesized that CD8$^+$ T-cells specifically become exhausted in the lesion microenvironment, as there are high numbers of APCs in the plaques. Markedly, we found no differences in the expression of the classical

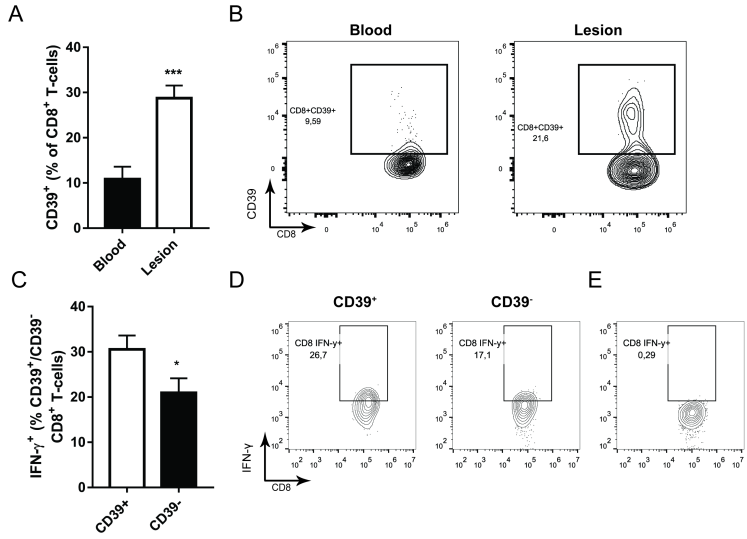

Figure 4: CD39 expression on human atherosclerotic lesion-derived CD8$^+$ T-cells is associated with IFN-γ production. (A-B) Flow cytometry analysis and representative images of CD39 expression on CD8$^+$ T-cells in endarterectomy samples from n = 12 arteria carotis or matched blood. Cells were gated on live CD45$^+$CD3$^+$CD8$^+$ cells. (C-D) Flow cytometry analysis and representative images of IFN-γ production by CD39$^+$ and CD39$^-$CD8$^+$ T-cells in endarterectomy samples from n = 12 arteria carotis stimulated for 4 hours with PMA and ionomycin. Cells were pregated on live CD45$^+$CD3$^+$CD8$^+$CD39$^+$ or live CD45$^+$CD3$^+$CD8$^+$CD39$^-$ cells. (E) Representative image of IFN-γ production by CD45$^+$CD3$^+$CD8$^+$ T-cells in an unstimulated sample, used to determine the gating strategy for IFN-γ. Mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

exhaustion-associated inhibitory receptors PD-1 and CTLA-4 [21]. However, high expression of the ectonucleotidase CD39 is linked to decreased cytokine production and exhaustion of CD8$^+$ T-cells [15, 22], and is also associated with regulatory T-cells [23]. This enzyme is involved in hydrolyzing extracellular ATP into ADP which can be further converted into the immunomodulatory adenosine by ecto-5'-nucleotidases such as CD73 [24]. ATP is a known danger-associated molecular pattern which stimulates pro-inflammatory cytokine production and has been linked to atherosclerosis [25]. We demonstrated an increased expression of CD39 on aortic CD8$^+$ T-cells compared to their splenic counterparts, suggesting a CD39-mediated reduction in cytokine production by lesion-derived CD8$^+$ T-cells. We found a slight decrease in the expression of CD73 on aortic CD8$^+$ T-cells. However, the rate-limiting step in the aforementioned cascade is CD39 [26], thus suggesting CD8$^+$ T-cells in advanced atherosclerotic lesions could have an increased capacity to produce adenosine, although we did not measure this directly. Bai et al. reported that adenosine produced by CD8$^+$CD39$^+$ T-cells can drive the exhaustion of CD8$^+$CD39$^-$ T-cells [16]. In agreement with this, we observed higher IFN-γ production by CD8$^+$CD39$^+$ T-cells compared to their CD39$^-$ counterparts within the aortic microenvironment. As CD39$^-$ cells produce fewer cytokines than their CD39$^+$ counterparts, this may suggest a paracrine regulation of cytokine production of CD39$^-$ cells by CD39$^+$ cells via increased adenosine-mediated

receptor signaling responses. Besides decreasing cytokine production of CD8$^+$ T-cells, adenosine-mediated signaling can inhibit Th1 and Th2 CD4$^+$ T-cell development, as well as their effector functions [27]. Furthermore, adenosine can inhibit the function of type 1 cytotoxic CD8$^+$ T-cells [28], and induce a tolerogenic APC phenotype [29]. This suggests that the increased CD39 expression on lesion-derived CD8$^+$ T-cells may have an immune regulatory, and potentially, an atheroprotective function. Indeed, adenosine signaling via type 1 purigenic receptors was shown to be protective against atherosclerosis development [30]. However, as we did not measure adenosine levels in situ, we cannot exclude that there could be an increase in extracellular levels of ADP and AMP as well. These nucleotides can induce both atherogenic signaling [30], but may also induce phosphorylation of the AMP-activated protein kinase (AMPK) [31]. This phosphorylation results in functional activation of AMPK, which was shown to reduce atherosclerosis development by inducing autophagy and subsequently promoting cholesterol efflux from macrophages, as well as diminishing inflammatory responses [32].

In this study, we show that CD39 expression directly and indirectly affects CD8$^+$ T-cell functionality, by inhibiting the enzymatic activity of CD39 using POM-1. POM-1 has been shown to effectively inhibit ATP hydrolysis by CD39, and thereby diminish the immunosuppressive function of tumor-associated macrophages in ovarian cancer [33]. Furthermore, it was shown to inhibit adenosine generation by regulatory T-cells in a melanoma mouse model [34]. In agreement with this, we observed an increased IFN-γ production by CD8$^+$ T-cells both *in vitro* on splenic-derived CD8$^+$ T-cells and *in vivo* in the aortas of apoE$^{-/-}$ mice upon treatment with POM-1. It must be noted that some off-target effects of the inhibitor may have occurred *in vivo*, but we show these effects *in vitro* on isolated CD8$^+$ T-cells as well, which indicates that the increased cytokine production is most likely due to reduced adenosine production. Interestingly, we observed an increase in cytokine production upon POM-1 treatment by both CD39$^+$ and CD39$^-$CD8$^+$ T-cells, suggesting that the adenosine produced by CD39$^+$CD8$^+$ T-cells may regulate IFN-γ production in both a paracrine and autocrine fashion. It must be noted that even though the levels of IFN-γ production are increased upon POM-1 treatment, they are still not returned to the levels observed in the spleen. This could be due to their prolonged exposure to higher concentrations of adenosine in situ. Alternatively, the short duration of treatment may not be able to completely restore the cytokine production to the levels observed in the spleen, but we cannot exclude other factors that may contribute to the decreased cytokine production of CD8$^+$ T-cells in the aortic microenvironment. Although we did not investigate this directly, increased CD39 expression on CD8$^+$ T-cells may affect the phenotype and function of other immune cells present in the lesion. Indeed, it has been shown that CD39-expressing CD8$^+$ T-cells can suppress CD4$^+$ T-cell function in a simian immunodeficiency virus infection [35] and suppress the proliferative response of Th1 CD4$^+$ T-cells in a mycobacterium infection [36]. Full body knockout of CD39 in apoE$^{-/-}$ mice results in decreased atherosclerosis [37], suggesting an atherogenic role for this enzyme. However, CD39$^{-/-}$apoE$^{-/-}$ mice show impaired platelet activation, enhanced cholesterol efflux and an increase in plasma HDL, which confirms that a full body knockout of CD39 results in a number of complex changes in key factors affecting atherosclerosis development. Similarly, the

blocking of CD39 using POM-1 may affect multiple cell types expressing CD39. Further research that investigates inhibition or knockout of CD39 specifically on $CD8^+$ T-cells in an atherosclerotic context is needed to shed more light on the exact role of $CD8^+CD39^+$ T-cells on lesion initiation and progression. We propose that the increased adenosine produced by these cells functions in an atheroprotective manner, as blocking of CD39 resulted in increased IFN-γ production, which is known to drive atherogenesis [38].

Using a bone-marrow transplantation setup in which atherosclerotic mice are engrafted with bone marrow that gives rise to either wild-type or ovalbumin-restricted $CD8^+$ T-cells, we were able to demonstrate the need for TCR signaling for the upregulation of CD39 on lesional $CD8^+$ T-cells. The aortic microenvironment contains many APCs that are able to locally activate T-cells via their TCR [39]. Upon TCR ligation, reactive oxygen species (ROS) are generated [40], which in turn are able to augment the expression of CD39 [16]. As the atherosclerotic lesion contains many APCs and is characterized by high levels of ROS [41], we suggest that this local microenvironment boosts CD39 expression on the $CD8^+$ T-cells. In agreement with our findings, previous reports have shown that CD39 expression is upregulated on T-cells upon TCR signaling [14, 16, 22, 23]. Moreover, on CD4 regulatory T-cells, the catalytic activity of CD39 was enhanced by TCR ligation [23]. In support of our findings on $CD8^+$ T-cell skewing by the local microenvironment, recent work by Simoni et al. has also shown a role for TCR signaling in inducing CD39 expression on $CD8^+$ T-cells in the tumor microenvironment [17]. Furthermore, Duhen et al. report a unique tumor-microenvironment specific population of $CD8^+$ T-cells co-expressing CD39 and CD103 [42]. Collectively, this suggests that microenvironment specific TCR-signaling is important in inducing CD39 expression on $CD8^+$ T-cells.

Importantly, we show that in human atherosclerotic lesions CD39 is upregulated on $CD8^+$ T-cells as well. Moreover, we observed that the human $CD39^+$ T-cells produce more IFN-γ compared to the $CD39^-$ T-cells, which is in agreement with the phenotype we observed in our murine model. Thus, it can be presumed that the immunomodulatory role we observe for $CD39^+CD8^+$ T-cells in $apoE^{-/-}$ mice can be translated to a clinical setting. This may open up new treatment avenues targeting CD39 expression on $CD8^+$ T-cells in atherosclerosis.

In conclusion, our studies highlight a new role for $CD8^+$ T-cells in advanced atherosclerosis. We propose that the increased adenosine produced by $CD8^+$ T-cells inside the lesions acts in an anti-inflammatory manner. These results suggest that boosting $CD39^+CD8^+$ T-cell function could be an interesting approach for the treatment of atherosclerosis. As CD39 expression is upregulated in an antigen-specific manner, vaccination strategies boosting $CD8^+$ T-cell responses, which have been shown to be effective in a murine model [5], may be a promising treatment avenue.

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Supplementary information

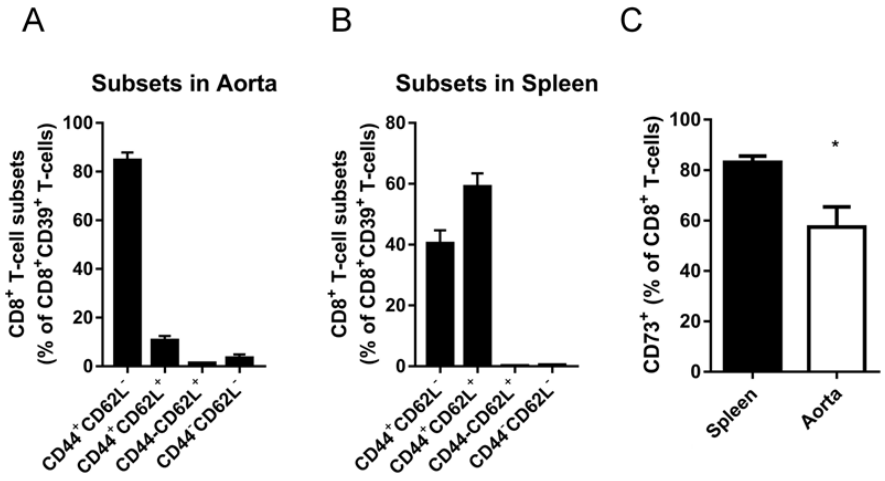

Figure S1: apoE$^{-/-}$-derived CD8^{+} T-cells show CD39 expression within all T cell subsets, and lesion-derived CD8^{+} T-cells show a decrease in CD73 expression compared to their counterparts in the spleen. (A,B) Percentages of different subsets within the CD39^{+}CD8^{+} T-cells classified by their expression of CD44 and CD62L in aortas (A) and spleens (B) of apoE$^{-/-}$ mice. Cells were pregated on live, Thy1.2^{+}CD8^{+} T-cells. n = 5 apoE$^{-/-}$ mice of 37-43 weeks old. (C) Percentages of CD73^{+}CD8^{+} T-cells derived from aortas and spleens of apoE$^{-/-}$ mice analyzed by flow cytometry. Cells were pregated on live, Thy1.2^{+}CD8^{+} T-cells. n = 6 apoE$^{-/-}$ mice of 36 weeks old, data is representative of 2 independent experiments. Mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$.

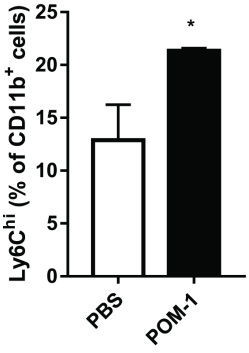

Figure S2: In vivo CD39 blockade in atherosclerotic mice results in increased pro-inflammatory mononuclear cell content the lesion. apoE$^{-/-}$ mice of 38-43 weeks of age were treated with 10 mg kg^{-1} POM-1 (n = 5) or sterile PBS (n = 5). After 24 hours, aortas and blood were isolated and analyzed by flow cytometry. Percentages of inflammatory Ly6C^{hi} mononuclear cells in the aorta are shown, pregated on live, NK1.1^{-}Ly6G^{-}CD11b^{+} cells. Mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$.

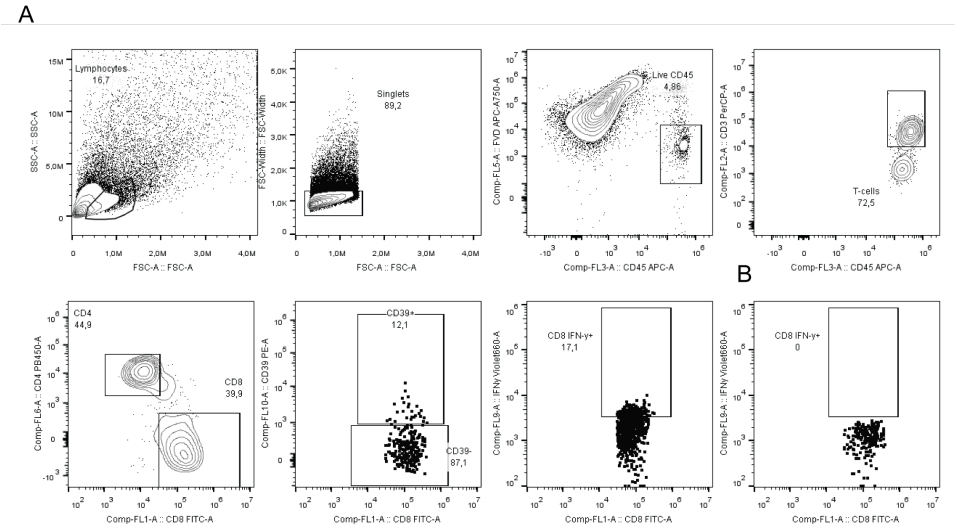

Figure S3: Flow cytometry gating strategy for human atherosclerotic lesions. (A) Flow cytometry gating strategy for determining the expression of CD39 and IFN-γ on $CD8^+$ T-cells in endarterectomy samples stimulated for 4 hours with PMA and ionomycin. Cells were gated on leukocytes, single cells, fixable viability dye-$CD45^+$, $CD3^+$, $CD8^+$, $CD39^+$ and finally IFN-γ^+. (B) Unstimulated control samples were used to determine the placing of the IFN-γ gate.

Supplementary methods

Obtaining single cell suspensions from human material

Single cell suspensions were obtained by cutting the tissue into small pieces, followed by a 2-hour digestion at 37 °C with an enzymatic mix consisting of collagenase IV (Gibco), human serum albumine and DNase (both Sigma), as previously described [13]. Blood samples were centrifuged to remove the serum and subsequently lysed twice for 10 minutes in ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, (0.1 mM Na_2EDTA; pH 7.3) at room temperature and washed once in PBS in order to obtain white blood cells. All samples were frozen on the day of surgery and stored at −80 °C. Cells were defrosted and stained for flow cytometric analysis at the same time.

Bone marrow transplantation experiment

30 $LDLr^{-/-}$ mice of 13-15 weeks of age were injected with 50 µg of an anti-CD8 antibody (clone 2.43, BioXcell) in order to deplete the $CD8^+$ T-cells. After 24 hours, all mice were exposed to a single dose of 9 Gy (0.19 Gy min^{-1} 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International) with a 6 mm aluminum filter to induce bone marrow aplasia. Donor bone marrow was isolated from $CD8^{-/-}$ mice (provided by Dr. Oxenius, ETH Zurich, Switzerland), C57BL/6 (WT) or C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT.1) mice (Jackson Laboratory) by flushing the femurs and tibias with PBS. Single-cell suspensions were prepared by passing the cells

through a 70 µm cell strainer (Greiner Bio-One). Donor bone marrow cells were combined to obtain a mixture of 70% $CD8^{-/-}$ and 30% WT or OT.1 bone marrow, and 1.4 × 107 cells were injected into tail veins of the irradiated $LDLr^{-/-}$ mice. Drinking water was supplemented with antibiotics (83 mg L^{-1} ciprofloxacin, 67 mg L^{-1} polymyxin B sulfate, and 6.5 g L^{-1} sucrose). After a recovery period of 7 weeks on a regular chow diet, animals were placed on a Western-type diet containing 0.25% cholesterol and 15% cacao butter (Special Diet Services) for 8 weeks, after which the mice were sacrificed as described above.

Flow cytometry

Upon sacrifice, spleens and aortas were harvested after in situ perfusion with PBS. Single-cell suspensions of spleens were obtained by using a 70 µm cell strainer (Greiner Bio-One). WBCs from splenocytes were obtained by lysing for 1 min at room temperature in lysis buffer. Aortas were cleaned of perivascular fat, cut up into small pieces, and digested by incubation with digestion mix (collagenase I 450 U ml^{-1}, collagenase XI 250 U ml^{-1} , DNAse 120 U ml^{-1} and hyaluronidase 120 U ml^{-1}; all Sigma-Aldrich) for 30 min at 37 °C while shaking and subsequently strained over a 70 µm strainer. Approximately 100 000 cells (or all available cells from aortic tissue) were stained with the appropriate antibodies (supplementary table I) in PBS containing 2% FCS. For intracellular staining, cells were fixed and permeabilized by using an intracellular staining kit (eBioscience) according to the manufacturer's protocol. To detect cytokine production, cells were stimulated for two or four hours with phorbol 12-myristate 13-acetate (PMA, 50 ng ml^{-1}, Sigma-Aldrich) and ionomycin (500 ng ml^{-1}, Sigma-Aldrich) in the presence of brefeldin A (ThermoScientific) in complete RPMI as stated above at 37 °C and 5% CO_2. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S or BD Biosciences Canto II and FlowJo software (Treestar).

5

Tc17 CD8$^+$ T-cells accumulate in murine atherosclerotic lesions and modulate local inflammatory responses

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ABSTRACT

Aims CD8$^+$ T-cells can differentiate into subpopulations that are characterized by a specific cytokine profile, such as the Tc17 population that produces IL-17. The role of this CD8$^+$ T-cell subset in atherosclerosis remains elusive. In this study, we therefore investigated the contribution of Tc17 cells to the development of atherosclerosis.

Methods and Results Flow cytometry analysis of atherosclerotic lesions from apoE$^{-/-}$ mice revealed a pronounced increase in RORγt+CD8$^+$ T-cells compared to the spleen, indicating a lesion-specific increase in Tc17 cells. To study whether and how the Tc17 subset affects atherosclerosis, we performed an adoptive transfer of Tc17 cells or undifferentiated Tc0 cells into CD8$^{-/-}$LDLr$^{-/-}$ mice fed a Western-type diet. Using flow cytometry, we showed that Tc17 cells retained a high level of IL-17A production *in vivo*. Moreover, Tc17 cells produced lower levels of IFN-γ than their Tc0 counterparts. Analysis of the aortic root revealed a decrease in atherosclerotic lesion size in the Tc17-treated mice compared to Tc0-treated mice, associated with a decrease in plaque macrophage content as well as a reduction in inflammatory CD4$^+$ Th1 cells.

Conclusion These findings demonstrate a lesion-localized increase in Tc17 cells in an atherosclerotic mouse model. Adoptive transfer of these cells into CD8$^{-/-}$LDLr$^{-/-}$ mice reduces lesion size compared to the transfer of Tc0 cells, suggesting a protective role for Tc17 cells in atherosclerosis.

1. Introduction

Atherosclerosis, the most frequent underlying pathology of cardiovascular disease, is characterized by both the buildup of cholesterol as well as chronic inflammation within the wall of large- and medium-sized arteries. T-cells are observed in both early and advanced atherosclerotic lesions [1] and have been shown to contribute to lesion initiation and progression [2–4]. Different subsets of $CD4^+$ helper-T cells have been extensively described and studied in the context of atherosclerosis [5]. A pro-atherogenic function is ascribed to the interferon-γ (IFN-γ)-producing T helper 1 (Th1) subset [6, 7], whereas the interleukin (IL)-10-producing regulatory T cells (Tregs) are atheroprotective [8, 9]. The role of the Th2 subset, characterized by the production of IL-4 and IL-5, is more controversial. Whereas the signature Th2 cytokines IL-4, IL-5, and IL-33 are reported to inhibit atherosclerosis development [2, 10, 11], reduced Th2 responses and IL-4 deficiency were also reported to decrease lesion formation [12–14], suggesting a pro-atherogenic role for Th2 cells as well. Finally, the IL-17-producing Th17 subset is known to drive autoimmunity and atherogenesis via activation of the endothelium, increasing pro-inflammatory cytokine production, and contributing to macrophage recruitment [15–17]. In contrast, loss of suppressor of cytokine signaling (SOCS) 3 in T cells, resulting in increased IL-17 and IL-10 production, reduces atherosclerotic lesion development [18]. This effect is mediated via the induction of an anti-inflammatory macrophage phenotype and a reduction in vascular inflammation. Interestingly, treatment with recombinant IL-17 resulted in reduced expression of vascular cell adhesion molecule-1 (VCAM-1) as well as reduced T-cell infiltration in the lesions, suggesting the aforementioned atheroprotective effects of SOCS3 knockout are at least in part mediated via IL-17.

In a similar vein to their $CD4^+$ T-cell counterparts, $CD8^+$ T-cells can be categorized into subsets based on their cytokine production. Upon activation of $CD8^+$ T-cells, cytokines released by antigen-presenting cells (APCs) can influence the differentiation of the $CD8^+$ T-cells into different subsets. The cytokines IL-2 and IL-12 drive $CD8^+$ T-cells towards a Tc1 phenotype through the induction of the transcription factor T-box–containing protein expressed in T cells (T-bet) [19, 20]. Tc1 cells are known for their cytotoxic function and expression of effector molecules, such as granzymes, perforin, IFN-γ and TNF-α [21, 22]. These cells confer protection against intracellular infections [23, 24] as well as cancer [25]. Alternatively, the release of IL-4 by the APCs polarizes $CD8^+$ T-cells towards a Tc2 phenotype [26]. These cells express the transcription factor GATA3 and are characterized by the production of the cytokines IL-4, IL-5 and IL-13 [21, 26–28]. This cell type is known to propagate allergic reactions and contribute to autoimmune disorders, such as arthritis [29–31]. Finally, exposure to the cytokines IL-6, IL-21, and TGF-β drives $CD8^+$ T-cells to differentiate towards a Tc17 phenotype, by inducing the expression of the transcription factors RAR-related orphan nuclear receptor γt (RORγt) and interferon regulatory factor 4 [32, 33]. Tc17 cells are characterized by their production of IL-17 and have been shown to play a pro-inflammatory role in several autoimmune disorders, such as multiple sclerosis, diabetes and arthritis [21, 33–35].

The roles of CD8$^+$ T-cell subsets in atherosclerosis remain largely unexplored, although there are some studies suggesting that these cells may be involved. Tc1 cells have been implicated in atherogenesis, as IFN-γ-producing CD8$^+$ T-cells potentiated atherosclerosis development in apolipoprotein E deficient (apoE$^{-/-}$) mice [36]. Additionally, IFN-γ produced by CD8$^+$ T-cells was shown to contribute to monopoiesis during early lesion development in low-density lipoprotein receptor-deficient (LDLr$^{-/-}$) mice [37]. Moreover, apoE$^{-/-}$ mice deficient in E3-ligase CBL-B showed an increase in IFN-γ and granzyme B-producing CD8$^+$ T-cells, resulting in enhanced macrophage killing and atherosclerosis [38]. Finally, an increase in IL-17-producing CD8$^+$ T-cells in the circulation of humans has been associated with a higher incidence of myocardial infarction [39], hinting at a role for Tc17 cells in cardiovascular disease. However, direct evidence showing a causal relation between Tc17 cells and atherosclerosis is lacking.

Here, we systematically investigated the presence of different CD8$^+$ T-cell subsets in a murine model of atherosclerosis and observed an increase in the number of Tc17 cells within the lesions. We show that undifferentiated CD8$^+$ T-cells switch to a Tc1 phenotype when transferred into LDLr$^{-/-}$ mice on a Western-type diet (WTD). CD8$^+$ T-cells that are polarized towards Tc17 cells however, produced lower levels of IFN-γ upon adoptive transfer and showed a reduced atherogenicity compared to their Tc1 counterparts.

2. Methods

2.1. Mice

C57Bl/6, CD8$^{-/-}$, LDLr$^{-/-}$ and apoE$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and bred in-house. CD8$^{-/-}$ mice were crossed with LDLr$^{-/-}$ mice to obtain CD8$^{-/-}$LDLr$^{-/-}$ mice in-house, after which genotypes were verified by PCR. The mice were kept under standard laboratory conditions and food and water were provided *ad libitum*. For the development of advanced atherosclerotic lesions in apoE$^{-/-}$ mice, mice were kept on a chow diet for 39-49 weeks before analysis of CD8$^+$ T-cell phenotypes in the lesion. Upon sacrifice, mice were subcutaneously anesthetized with a lethal dose of ketamine (40 mg ml^{-1}), sedazine (8 mg ml^{-1}) and atropine (0.1 mg ml^{-1}). All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

2.2. Cell preparation and flow cytometry

Mice were sacrificed as described above and blood, spleens, and aortas were harvested after in situ perfusion with phosphate-buffered saline (PBS, pH 7.4, Lonza). White blood cells were obtained by lysing blood samples two times for 2 min with lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.3). Single-cell suspensions of

spleens were obtained by using a 70 µm cell strainer (Greiner Bio-One). Splenocytes were lysed for 1 min with lysis buffer to obtain white blood cells. Aortas were cleaned of perivascular fat, cut into small pieces, and digested by incubation with a digestion mix (collagenase I 450 U mL^{-1}, collagenase XI 250 U mL^{-1}, DNAse 120 U mL^{-1}, and hyaluronidase 120 U mL^{-1}; all Sigma-Aldrich) for 30 min at 37 °C while shaking, and subsequently strained over a 70 µm strainer. Cells were stained with the appropriate antibodies (Table S1). For intracellular staining, cells were fixed and permeabilized by using an intracellular staining kit (eBioscience) according to the manufacturer's protocol. To detect cytokine production, cells were stimulated for 3.5 hours with phorbol 12-myristate 13-acetate (PMA, 50 ng ml^{-1}, Sigma-Aldrich) and ionomycin (500 ng ml^{-1}, Sigma-Aldrich) in the presence of brefeldin A (ThermoScientific) in complete RPMI 1640 medium containing 25 mM HEPES (Lonza) supplemented with 5% foetal bovine serum (Greiner), 60 µM β-mercaptoethanol (Sigma), 100 U mL^{-1} mix of penicillin/streptomycin (Lonza), 1% non-essential amino acids (NEAA; Gibco), 1% sodium pyruvate (Sigma), and 2% L-glutamine (Lonza) at 37 °C and 5% CO_2. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S and FlowJo software (Treestar).

2.3. *In vitro* culture of Tc0 and Tc17 cells

Spleens, mesenteric lymph nodes, and iliac lymph nodes were isolated from C57Bl/6 mice after cervical dislocation. CD8$^+$ T-cells were isolated by using a negative selection magnetic CD8$^+$ T-cell isolation kit (Milteny Biotec) according to the manufacturer's protocol. 0.3×10^6 cells were plated per well in a 96-well plate in a total volume of 200 µL complete RPMI (as stated above). In order to obtain undifferentiated Tc0 cells, the medium was supplemented with 20 U mL^{-1} IL-2 (Peprotech), 0.5 ng ml^{-1} IL-7 (Peprotech), 0.5 µg ml^{-1} soluble anti-CD3 (ThermoScientific), 0.5 µg ml soluble anti-CD28 (ThermoScientific) and 10 µg ml^{-1} anti-IFN-γ (BioXcell). For Tc17 differentiation, the medium was supplemented with 20 U mL^{-1} IL-2 (Peprotech), 0.5 ng ml^{-1} IL-7 (Peprotech), 20 ng ml^{-1} IL-6 (Peprotech), 5 ng ml^{-1} TGF-β (BioLegend), 20 ng ml^{-1} IL-1β (Peprotech), 20 ng ml^{-1} IL-23 (R&D systems), 0.5 µg ml^{-1} soluble anti-CD3 (ThermoScientific), 0.5 µg ml^{-1} soluble anti-CD28 (ThermoScientific), 10 µg ml^{-1} anti-IL-4 (BioXcell) and 10 µg ml^{-1} anti-IFN-γ (BioXcell). The cells were incubated for two days at 37 °C and 5% CO_2, after which the medium was refreshed with the same cytokine stimulations, but without anti-CD3 and anti-CD28. The cells were incubated for one more day before analysis by flow cytometry or adoptive transfer.

2.4. Adoptive transfer

Blood samples of 100 µL were drawn via the tail vein in EDTA-containing tubes (Sarstedt) from 18 CD8$^{-/-}$LDLr$^{-/-}$ mice between 8 and 14 weeks of age. Total cholesterol levels were assessed by using an enzymatic colorimetric assay (Roche Diagnostics). The mice were randomized into two groups based on age, weight, and plasma cholesterol levels. From the start of the experiment, mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex,

UK) for 6 weeks. Every week, mice received intravenous injections of matched numbers of between 8.8×10^5 and 2.3×10^6 Tc0 or Tc17 cells, depending on the amount obtained during isolation (on average 1.7×10^6 per injection). During the experiment, transfer efficiency was monitored by drawing blood after 2 and 4 injections of CD8$^+$ T-cells, 5 days after the mice received the last injection. The mice were sacrificed one week after the sixth injection as described above, and organs were isolated as described in Section 2.2.

2.5. Histological analysis

All hearts were embedded in optimal cutting temperature (O.C.T.) compound (Sakura) and horizontally sectioned towards the aortic axis and the aortic arch. Upon reaching of the aortic root, defined by the trivalve leaflets, 10 µm sections were collected. Lesion size analysis was performed on cryosections of the aortic root lesion stained with Oil-red O and hematoxylin (Sigma-Aldrich). Sirius Red staining (Sigma-Aldrich) was performed on corresponding sections to determine collagen content, and Masson's Trichrome staining (Sigma-Aldrich) to determine the necrotic area. Plaque macrophages were stained immunohistochemically by using a rat anti-mouse Monocytes/Macrophages antibody (MOMA, 1:1000, AbD Serotec) as a primary antibody, biotinylated rabbit anti-rat IgG (1:100; Vector) as a secondary antibody, and Vectastain ABC horseradish peroxidase in combination with ImmPACT Nova Red for visualization (Vector). Plaques were stained for VCAM-1 by using purified rat anti-mouse CD106 (1:100, BD Biosciences) as a primary antibody, biotinylated rabbit anti-rat IgG (1:200, Vector) as a secondary antibody and Vectastain ABC horseradish peroxidase in combination with Immpact Nova Red for visualization (Vector). The average plaque size (in µm^2) was calculated from five sequential sections. For all other analyses, three subsequent sections displaying the highest plaque content per mouse were analyzed. All microscopic analyses were performed on a Leica DM-RE microscope using Leica QWin software and were blinded for independent analysis. The relative amount of collagen, macrophages, and necrosis in the atherosclerotic lesions was quantified by dividing the area stained positive for collagen, MOMA or that displaying necrosis by the total lesion surface area, and calculated as a percentage.

2.6. Statistical analysis

The data are presented as individual dot plots with bars denoting the mean, and the number of animals in each group is stated in the text. Data were tested for normal distribution by using a Shapiro-Wilk normality test and analyzed by using a two-tailed Student's *t*-test, Mann–Whitney test, one-way or two-way ANOVA, as appropriate. Statistical analysis was performed using Prism (GraphPad). Probability values of $P < 0.05$ were considered significant.

3. Results

3.1. Increased expression of RORγt by CD8$^+$ T-cells derived from advanced murine atherosclerotic lesions

We investigated the presence of the different CD8$^+$ T-cell subsets in the atherosclerotic lesions of apoE$^{-/-}$ mice with advanced atherosclerosis using flow cytometry. We focused on the difference in phenotype between CD8$^+$ T-cells derived from the aortic lesions and their counterparts in the spleen, as these cells can locally affect the lesion development and composition. We observed a significant decrease in the percentage of CD8$^+$ T-cells that produce IFN-γ within the lesions compared to their counterparts in the spleen (11.7% vs 39.3%, Fig. 1A). This is in line with our previous research, showing a reduced number of cytokine-producing CD8$^+$ T-cells in the lesions of these mice, probably due to the immunosuppressive effects of increased CD39-expression on these cells [40]. We were unable to detect any IL-4 secretion by aortic CD8$^+$ T-cells, whereas we did detect low levels of this cytokine in the splenic CD8$^+$ T-cells (Fig. 1B). Conversely, we observed no production of IL-5 by splenic CD8$^+$ T-cells, whereas there was a low expression of this cytokine in their aortic counterparts (Fig. 1C). Finally, we observed only very low expression levels of IL-17A in the CD8$^+$ T-cells derived from both sites, with no significant differences between the different sites (Fig. 1D). Therefore, we looked into the expression of the key transcription factors associated with the different Tc subsets in the lesions of these mice: T-bet, GATA3, and RORγt for Tc1, Tc2, and Tc17 cells, respectively. Interestingly, we observed a significant 45-fold increase in the percentage of CD8$^+$ T-cells that are positive for RORγt (Fig. 1G), as well as a 19-fold increase in the percentage of GATA3-expressing CD8$^+$ T-cells in the aorta compared to the spleen (Fig. 1F), whereas the percentage of T-bet-expressing CD8$^+$ T-cells showed a 3-fold decrease (Fig. 1E). Of note, we only observed IFN-γ production by the T-bet positive CD8$^+$ T-cells in the aorta, but not by the RORγt or GATA3-expressing CD8$^+$ T-cells (Fig. S1), confirming functionally distinct lineages. As there was such a pronounced increase in the RORγt-expressing lesional CD8$^+$ T-cells, we set out to further explore the role of Tc17 cells in atherosclerosis.

3.2. *In vitro* characterization of Tc0 and Tc17 cells

To evaluate the role of Tc17 cells in atherosclerotic lesion development, we decided to perform an adoptive transfer of Tc17 cells into CD8$^{-/-}$LDLr$^{-/-}$ mice. First, we cultured Tc0 and Tc17 cells *in vitro*, based on previously published protocols [33, 41, 42]. CD8$^+$ T-cells were isolated from wild-type mice and activated by using anti-CD3 and anti-CD28 antibodies. Undifferentiated control CD8$^+$ T-cells (Tc0) were cultured for three days in medium supplemented with IL-2, IL-7, and anti-IFN-γ. Tc17 cells were differentiated for three days in medium supplemented with IL-2, IL-7, IL-6, IL-1β, TGF-β, IL-23, anti-IL-4, and anti-IFN-γ. Flow cytometry analysis revealed that our approach led to a robust Tc17 phenotype, with a 19-fold increase in the percentage of cells positive for IL-17A in the Tc17 cells compared to the Tc0 cells (24.8% vs 1.3%, Fig. 2A), associated with a 5-fold increase in RORγt-expressing cells (13.2% vs. 2.7%, Fig. 2B),

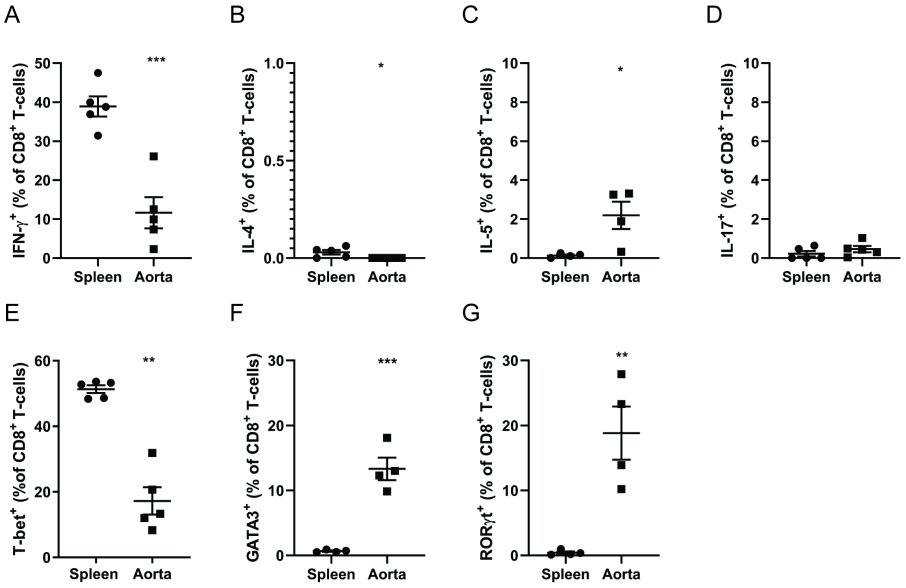

Figure 1: Murine atherosclerotic lesions display an increased expression of the Tc17-associated transcription factor RORγt as well as the Tc2-associated transcription factor GATA3 within the CD8$^+$ T-cell compartment compared to the spleen. Flow cytometric analysis of IFN-γ$^+$ (A), IL-4$^+$ (B), IL-5$^+$ (C), IL-17A$^+$ (D), T-bet$^+$ (E), GATA3$^+$ (F) and RORγt$^+$ (G) CD8$^+$ T-cells in the aortas and spleens of apoE$^{-/-}$ mice stimulated for 3.5 hwith PMA and ionomycin. Cells were pre-gated on live, Thy1.2$^+$CD8$^+$ T-cells. Individual data points and mean ± SEM of n = 5 (A, B, D, E) or n = 4 (C, F, G) apoE$^{-/-}$ mice of 39 to 49 weeks old, data are representative of n = 3 independent experiments. Significance was determined by using an unpaired *t*-test (A, C, F, G) or a Mann-Whitney test (B, D, E). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and a reduction in T-bet-expressing cells (7.3% vs. 25.1%, Fig. 2D). Moreover, both Tc0 and Tc17 produced low amounts of IFN-γ (4.5% and 4.9%, respectively, Fig. 2C), indicating that these cells do not display a Tc1 phenotype. We observed a low production of IL-5 by both subsets, although the Tc0 subset produced 3-fold more IL-5 compared to the Tc17 subset (2.4% vs. 0.8%, Fig. 2E). The percentage of GATA3$^+$cells was low in both groups and did not differ between the two subsets (Fig. 2F), indicating the cultured cells do not display a Tc2 phenotype.

3.3. Adoptively transferred Tc17 cells migrate to the atherosclerotic lesion and affect the local CD4$^+$ T-cell population

To determine the effect of Tc17 cells on the development of atherosclerotic lesions, we performed an adoptive transfer of cultured Tc17 cells in LDLr$^{-/-}$ mice that were also deficient in CD8 and therefore had no endogenous CD8$^+$ T-cell population. The control group received a transfer of undifferentiated Tc0 cells. The adoptive transfer resulted in an increase in the CD8$^+$ T-cell population in the blood of both treatment groups over time (Fig. 3A). At four weeks post the first adoptive transfer, the circulating CD8$^+$ T-cells in the Tc17-treated group showed a substantially (4-fold) increased production of IL-

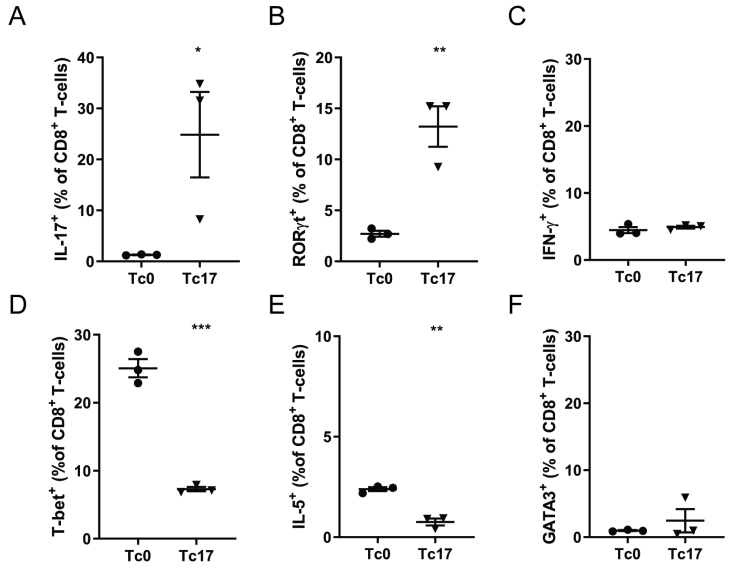

Figure 2: Tc0 and Tc17 cells demonstrate phenotypical differences in cytokine production and transcription factor expression. Flow cytometric analysis of IL-17A$^+$ (A), RORγt$^+$ (B), IFN-γ^+ (C), T-bet$^+$ (D), IL-5$^+$ (E) and GATA3$^+$ (F) CD8$^+$ T-cells isolated from C57Bl/6 mice and polarized for 3 days towards Tc0 or Tc17 cells. Cells were stimulated for 3.5 h with PMA and ionomycin and pre-gated on live, Thy1.2$^+$CD8$^+$ T-cells. Individual data points and mean ± SEM of n = 3, representative of n = 3 experiments. Significance was determined by using an unpaired *t*-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

17A compared to those in the Tc0-treated group (6.4% vs 1.7%, Fig. 3B), suggesting a stable Tc17 phenotype. Interestingly, we observed plasticity in the Tc0 subset, as these cells showed an increase in IFN-γ production from 4.5% at baseline to 53.6% four weeks after the first transfer. The Tc17 cells also showed an increased IFN-γ production from 4.9% to 44.9% (Fig. 2C, Fig. 3C). Therefore, we analyzed the relative amount of splenic IFN-γ+CD8$^+$ T-cells at sacrifice. Again, we observed that in the Tc0-treated group a large fraction of the cells produce IFN-γ (75.9%) compared to those in the Tc17-treated group (30.5%, Fig. 4A), although in both groups the percentage of IFN-γ^+cells were are notably higher than directly after *in vitro* differentiation (Fig. 2C). In line with our expectations, the Tc17-treated group still displayed more IL-17A$^+$cells as compared to the Tc0 group (7.6% vs 2.2%, Fig. 4B), albeit less compared to the *in vitro* cytokine production levels at the moment of injection (Fig. 2A). Moreover, at sacrifice, 86.1% of CD8$^+$ T-cells in the Tc0 group expressed T-bet, whereas in the Tc17 group this comprised 46.1% of the total CD8$^+$ population (Fig. 4C), suggesting the majority of the injected Tc0 cells had converted to a Tc1 phenotype. We observed a non-significant 1.2-fold increase in RORγt expression in the Tc17-treated group compared to the controls (Fig. 4D). Of note, the adoptively transferred CD8$^+$ T-cells were able to proliferate *in vivo*, as we observed 30.2% and 20.0% Ki-67 expression in the Tc0 and Tc17 group, respectively (Fig. 4E). Besides the spleen and blood, we were able to detect CD8$^+$ T-cells in the aortic lesions of both Tc0- and Tc17-recipient mice at the time of sacrifice (Fig. 4F), illustrating that

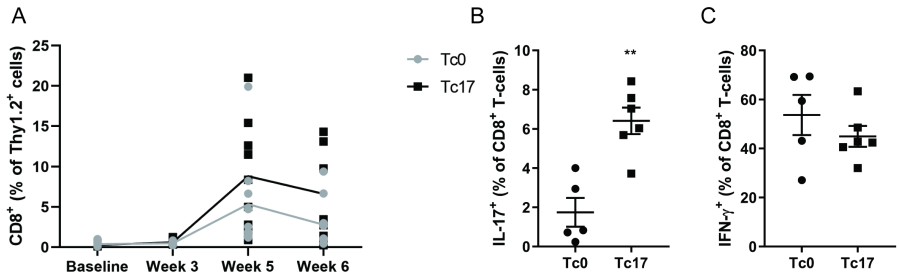

Figure 3: CD8$^+$ T-cell populations increase over time upon adoptive transfer in both treatment groups. (A) Analysis of percentages of CD8$^+$ T-cells in the blood of the CD8$^{-/-}$LDLr$^{-/-}$ mice after 2 and 4 injections of Tc0 or Tc17 cells and at sacrifice. Cells were pre-gated on live, Thy1.2$^+$cells, mean ± SEM. (B) IL-17A and (C) IFN-γ production by the CD8$^+$ T-cells in the blood after 4 injections, as analyzed by flow cytometry. Cells were stimulated for 3.5 h with PMA and ionomycin and pre-gated on live, Thy1.2$^+$CD8$^+$ T-cells. Individual data points and mean ± SEM, n = 9 mice per group. Significance was determined by using a two-way ANOVA with Bonferroni's multiple comparisons (A) or by using an unpaired *t*-test (B, C). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the adoptively transferred CD8$^+$ T-cells migrate into the plaques. However, as the total number of lymphocytes within murine aortas is low, these numbers did not allow us to distinguish the different CD8$^+$ T-cell subsets. Interestingly, we did observe changes in the CD4$^+$ T-cell compartment in the lesion, showing a trend towards decreased IFN-γ production (20.9% vs 13.7%, P = 0.11, Fig. 4G), as well as a significant decrease in T-bet expression (9.4% vs 4.7%, Fig. 4H) in the Tc17-treated group, suggesting a skewing away from the inflammatory Th1 phenotype.

3.4. Adoptive transfer of Tc17 cells reduces atherosclerotic lesion development compared to Tc0 transfer in CD8-deficient atherosclerotic mice

We next assessed how the adoptive transfer affected atherosclerosis development. The weight of the mice and the plasma cholesterol levels were similar between both groups (Fig. S2). Plaque size was assessed in the aortic root lesions of the hearts. Interestingly, neutral lipid staining of the lesions revealed a 38% smaller lesion size in the mice that received the adoptively transferred Tc17 cells compared to those that received the Tc0 cells (Fig. 5A). The reduction in lesion size is most likely due to a decrease in total macrophage accumulation, as the absolute MOMA-positive area was decreased 2.2-fold in the Tc17-treated group (Fig. 5B). The relative plaque composition appeared to be similar in the Tc17- and Tc0-treated groups, as no change in the percentage of MOMA positive or collagen positive area was observed (Fig. 5B, C). Analysis of the VCAM-1$^+$area in the caps of the lesion revealed a 1.5-fold reduction in the Tc17-treated group, although this did not reach significance (P = 0.21, Fig. 5D). No differences were observed in both absolute and relative necrotic areas in the lesions between the two groups (Fig. S3).

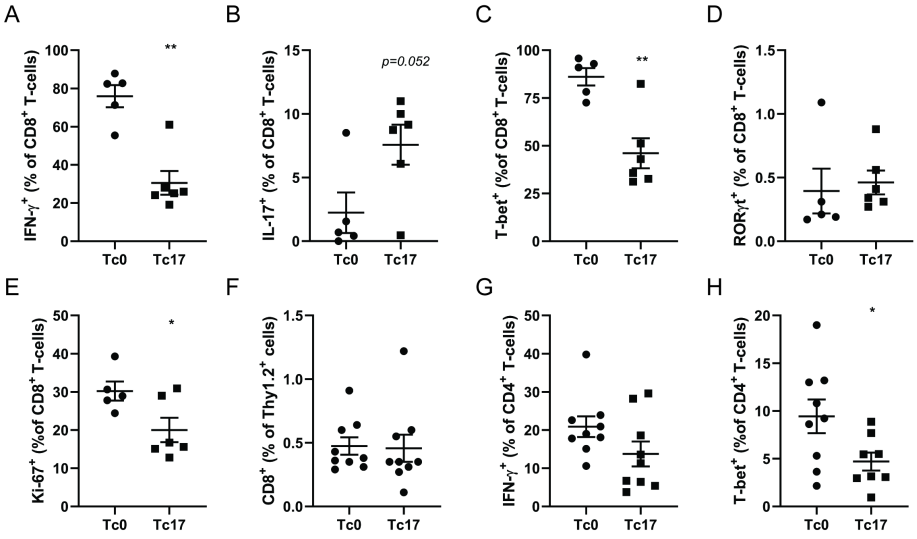

Figure 4: Adoptive transfer of Tc17 cells in CD8$^{-/-}$LDLr$^{-/-}$ mice skews the CD4$^+$ T-cells towards a less inflammatory phenotype in the aortic microenvironment. Flow cytometric analysis of IFN-γ^+ (A), IL-17A$^+$ (B) T-bet$^+$ (C) RORγt$^+$ (D), and Ki-67$^+$ (E) CD8$^+$ T-cells in the spleens of the CD8$^{-/-}$LDLr$^{-/-}$ mice that received the adoptive transfer of Tc0 or Tc17 cells at the time of sacrifice. Cells were pre-gated on live, Thy1.2$^+$CD8$^+$ T-cells. (F) percentages of CD8$^+$ T-cells in the aortas of the CD8$^{-/-}$LDLr$^{-/-}$ mice at the time of sacrifice, analyzed by flow cytometry. Cells were pre-gated on live, Thy1.2$^+$cells. Flow cytometry analysis of IFN-γ^+ (G) and T-bet$^+$ (H) CD4$^+$ T-cells in the aortas of the CD8$^{-/-}$LDLr$^{-/-}$ mice at the time of sacrifice. Cells were pre-gated on live, Thy1.2$^+$CD4$^+$ T-cells. Individual data points and mean ± SEM, n = 9 mice per group. Cells were stimulated for 3.5 h with PMA and ionomycin. Significance was determined by using a Mann-Whitney test (A, B, D, G) or by using an unpaired *t*-test (C, E, F, H, I). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

CD8$^+$ T-cells play an important role in the adaptive immune response, responding to intracellular pathogens. Recently, CD8$^+$ T-cell subsets such as Tc1, Tc2, and Tc17, which are characterized by their cytokine production resulting from different environmental cues, have been reported to also play a role in various autoimmune disorders [21]. We report a large increase in Tc17 cells in the atherosclerotic lesion microenvironment specifically and show that adoptive transfer of Tc17 cells results in a reduced atherosclerosis development compared to adoptive transfer of undifferentiated Tc0 cells, which differentiate into IFN-γ-producing Tc1 cells *in vivo*.

It is of particular interest to investigate the phenotype and function of CD8$^+$ T-cells within the lesion, as we have previously reported that CD8$^+$ T-cells can locally affect the lesion development and composition [43]. However, it is difficult to determine the presence of the different CD8$^+$ T-cell subsets within the lesional microenvironment based on their cytokine production, as the production of inflammatory cytokines produced by CD8$^+$ T-cells within the lesions is reduced [40]. Indeed, here we report a reduced percentage of IFN-γ^+lesion-derived CD8$^+$ T-cells compared to their counterparts in the

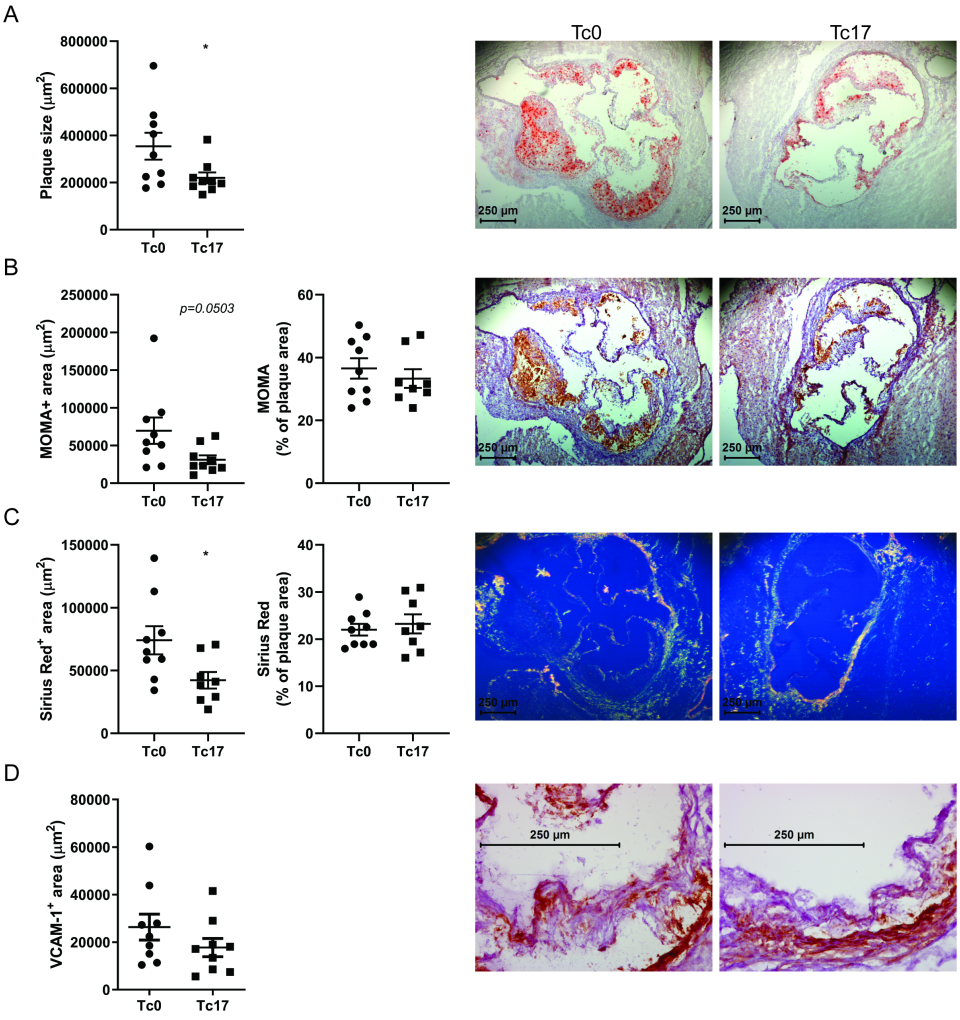

Figure 5: Adoptive transfer of Tc17 cells results in a decrease in lesion size, absolute collagen content and absolute macrophage content compared to the transfer of Tc0 cells in CD8$^{-/-}$LDLr$^{-/-}$ mice. (A) Quantification of lesion size in the aortic roots by Oil-red O staining and representative pictures of the lesions in CD8$^{-/-}$LDLr$^{-/-}$ mice treated with Tc0 or Tc17 cells. (B) Quantification of absolute and relative monocyte/macrophage content in the aortic root lesions by MOMA staining and representative pictures of the lesions. (C) Quantification of absolute and relative collagen content in the aortic root lesions by Sirius Red staining and representative pictures of the lesions. (D) Quantification of absolute VCAM-1$^+$area in the caps of the aortic root lesions by VCAM-1 staining and representative pictures of the lesions. Individual data points and mean ± SEM, n = 9 mice per group. Significance was determined by using a Mann-Whitney test (A, B) or by using an unpaired *t*-test (B, C, D). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

spleen and were hardly able to detect any IL-4, IL-5 or IL-17A production above background levels in the aorta of old apoE$^{-/-}$ mice. Therefore, we set out to measure the transcription factors associated with the Tc1, Tc2 and Tc17 subsets instead. There is

a reduced percentage of $CD8^+$ T-cells expressing T-bet in aortic lesions compared to splenic $CD8^+$ T-cells, suggesting that the pro-inflammatory Tc1 subset is not enriched in the lesion environment. However, we observed a modest increase in the percentage of cells expressing GATA3 in the lesions, implying an increase in Tc2 cells compared to lymphoid tissues. Strikingly, the percentage of cells expressing RORγt was strongly increased within the lesion microenvironment, which indicates a relative enrichment of Tc17 cells at the site of disease. It has previously been reported that 3 months of high-fat diet feeding in $apoE^{-/-}$ mice results in increased IL-17A production by splenic T-cells [44], although the cell-type responsible for this increase was not specifically identified. We observed only very low percentages of IL-17+$CD8^+$ T-cells in the spleens of $apoE^{-/-}$ mice, which were kept on a chow diet. Nonetheless, this study suggests that the inflammatory stimuli associated with the development of atherosclerosis may drive Tc17 skewing. Moreover, another analysis of the entire T-cell compartment showed an increase in IL-17A production in the aorta compared to the spleens of $apoE^{-/-}$ mice fed a WTD for 15 weeks [16]. As there are enhanced levels of the Tc17-polarizing cytokines IL-1β and IL-6 in the plaque [45, 46], the atherosclerotic environment in the lesion may indeed drive the local T-cells to differentiate towards a Tc17 phenotype, or stimulate increased recruitment of these cells.

In vitro polarization of isolated $CD8^+$ T-cells from wild-type mice resulted in successful differentiation towards a Tc17 phenotype, as described previously using a similar differentiation protocol [41]. Some basal levels of IFN-γ were produced by both the Tc0 and Tc17 cells, which is in agreement with available data [33, 41]. There was an increased expression of T-bet in the Tc0 subset compared to the Tc17 subset, which we hypothesize is due to the natural tendency of $CD8^+$ T-cells to differentiate towards an inflammatory effector phenotype upon the addition of IL-2, anti-CD3 and anti-CD28 antibodies [47]. Indeed, we found that the addition of anti-IFN-γ to the Tc0 conditions induced a great reduction in the T-bet expression, strengthening this hypothesis. However, upon adoptive transfer, Tc0 cells upregulated their expression of T-bet and IFN-γ production, indicating a switch towards the Tc1 phenotype. The Tc17 cells retained IL-17A production *in vivo*, though at lower levels than after *in vitro* differentiation. Moreover, they also increased their IFN-γ production, although significantly less pronounced than the Tc0 cells. Finally, the RORγt expression was downregulated in these cells. Indeed, previous work using antigen-specific Tc17 cells has shown that these cells can convert to IFN-γ-producing cells, although they retain some of their IL-17A production [41]. Similar plasticity has been reported for $CD4^+$ Th17 subsets [48, 49]. To date, the molecular mechanisms underlying these switches in phenotype remain unknown. However, the pro-inflammatory environment in the atherosclerotic mouse model may contribute to the increased production of IFN-γ, as hypercholesterolemia results in increased inflammatory responses [50, 51]. As $CD8^+$ T-cells activated by using anti-CD3 and CD28 antibodies tend to differentiate towards an effector phenotype [47, 52], it is likely that absence of anti-IFN-γ, that was present *in vitro*, as well as the systemic inflammatory signaling induced by the WTD-feeding in our mouse model, drives the switch towards a Tc1 phenotype. Indeed, the Tc0 cells showed a similar phenotype *in vivo* to that which we observed for the splenic $CD8^+$ T-cells in the atherosclerotic $apoE^{-/-}$ mice. Tc17 cells appeared more resistant to a Tc1 shift but still gained the ability to produce IFN-

γ. This may be explained by different transcriptional programs that are at work within this subset. IL-12, a cytokine known to be upregulated in atherosclerotic mice [53], is able to induce repressive epigenetic modification of the SOCS3 promoter. As SOCS3 is an essential mediator of IL-17 production, IL-12 can stimulate the conversion of Tc17 cells towards a mixed Tc1/Tc17 phenotype, associated with an increased IFN-γ production [54]. This is in agreement with our work, in which we observed maintenance of the Tc17 cytokine profile, but additional acquired characteristics of Tc1 cells. Of note, the Tc0 cells were more proliferative than their Tc17 counterparts. Possibly, the Tc0 cells resemble a more naïve phenotype as they are less fixed in their transcriptional program towards a certain phenotype. This enables them to proliferate more vigorously upon antigen recognition *in vivo*, compared to their more differentiated counterparts [55].

The injected CD8$^+$ T-cells were able to infiltrate the lesions *in vivo*, supporting the notion that at least part of the differences in the lesions observed between the Tc17- and Tc0-treated groups are due to local CD8$^+$ T-cell interactions. The Tc17-treated group showed a reduced plaque size compared to the Tc0-treated group. This suggests that the Tc17 cells could exert an atheroprotective effect, compared to the Tc0/Tc1 cells described here. Tc17 cells have been reported to have an impaired cytolytic function [32], however, we have previously shown a protective function for Fas ligand-induced cell death by CD8$^+$ T-cells [43], making a reduced cytolytic activity an unlikely explanation for the results observed here. There are reports suggesting a protective role for IL-17 in atherosclerosis. Increased IL-17 production reduced lesion development and neutralization of IL-17 accelerated atherosclerosis [18]. This protective effect is mediated by limiting inflammatory macrophage functions and reduced VCAM-1-mediated inflammatory cell recruitment towards the lesion. In agreement with this, we also observed a trend towards reduced VCAM-1 expression associated with a decrease in absolute macrophage content in the Tc17 treated group. This suggests that the protective effects of the Tc17 cells we observed here could be mediated through the increased IL-17A production.

Additionally, as the Tc0 cells produced more IFN-γ compared to the Tc17 cells, the differences in atherosclerotic lesion development could also result from the inflammatory effects of IFN-γ. IFN-γ is able to augment macrophage activation [56], which may, in turn, contribute to increased atherosclerosis development. IFN-$\gamma^{-/-}$apoE$^{-/-}$ mice have been shown to display a large reduction in atherosclerosis compared to controls, associated with a decrease in lesion cellularity but an increase in lesional collagen content [36]. In another study, administration of IFN-γ to apoE$^{-/-}$ mice resulted in a two-fold increase in lesion size, mediated by an increase in both T-cells as well as APCs [57]. CD8$^+$ T-cell-derived IFN-γ has previously been shown to have limited impact on lesion size and stability [58], but this study was performed in lymphocyte-deficient apoE$^{-/-}$ mice, which may overlook the effect of CD8$^+$ T-cell-derived IFN-γ on CD4$^+$ T-cells. We observed an increase in the total macrophage content of the lesions in the Tc0 group compared to the Tc17 group, which could be mediated by the increased IFN-γ that is produced in these mice. In fact, we observed an increase in T-bet expressing Th1 CD4$^+$ T-cells in the lesion microenvironment of the Tc0-treated mice. IFN-γ is an important regulator of T-bet expression within CD4$^+$ T-cells [59–61], suggesting that the increase

in Th1 cell population within the lesions of the Tc0-treated mice could be due to the increase in IFN-γ levels. In addition to the inflammatory effects of the adoptively transferred cells, this increase in the Th1 cell population within the lesions further promotes inflammation and atherogenesis, as these cells also display atherogenic functions [6]. Thus, both the increase in IL-17A as well as the decrease in IFN-γ production by the Tc17 cells compared to the Tc0 cells may have contributed to the protective effects observed here.

5. Conclusion

In conclusion, we have shown an enrichment of Tc17 cells in the plaque microenvironment of atherosclerotic mice. Adoptive transfer of Tc17 cells leads to a decrease in lesion size and is accompanied by a decrease in the Th1 cell population and reduced vascular inflammation compared to adoptive transfer of Tc0 cells. These findings demonstrate the presence of different Tc subsets within atherosclerotic lesions and warrant further research into the therapeutic options of skewing $CD8^+$ T-cell responses as a potential treatment for atherosclerosis.

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Supplementary information

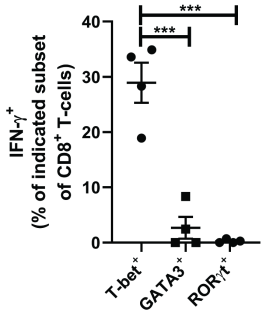

Figure S1: IFN-γ production in murine atherosclerotic lesions is restricted to T-bet-expressing CD8$^+$ T-cells. Flow cytometric analysis of IFN-γ production by T-bet+, GATA3$^+$ and RORγt$^+$CD8$^+$ T-cells in the aortas of apoE$^{-/-}$ mice stimulated for 3.5 hwith PMA and ionomycin. Cells were pre-gated on live, Thy1.2$^+$CD8$^+$ T-cells and the indicated transcription factors. Individual data points and mean ± SEM of n = 4 apoE$^{-/-}$ mice that were 46 to 49 weeks old. Significance was determined by using a one-way ANOVA with Bonferroni's multiple comparisons. $^*p < 0.05$, $^{**}p < 0.01$.

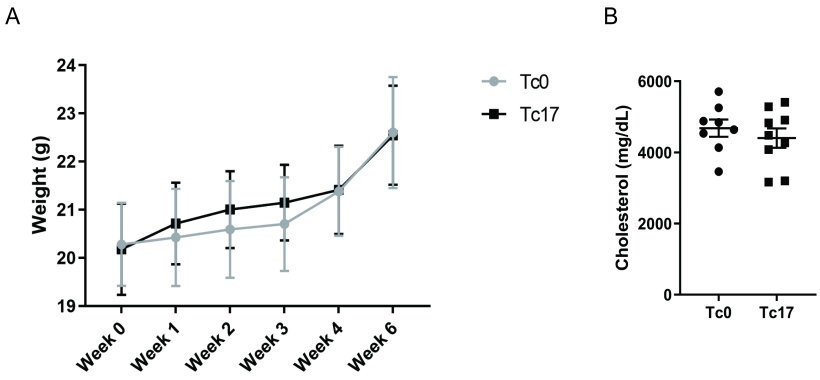

Figure S2: Adoptive transfer of Tc0 or Tc17 cells into CD8$^{-/-}$LDLr$^{-/-}$ mice does not result in any significant differences in weight or serum cholesterol. (A) Body weights of CD8$^{-/-}$LDLr$^{-/-}$ mice that received adoptive transfer of Tc0 or Tc17 cells once weekly, mean ± SEM. Significance was determined by using a two-way ANOVA with Bonferroni's multiple comparisons (B) Serum cholesterol levels at sacrifice. Individual data points and mean ± SEM, n = 9 mice per group. Significance was determined by using an unpaired *t*-test.

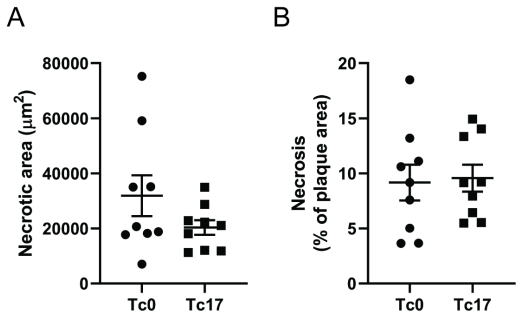

Figure S3: Adoptive transfer of Tc0 or Tc17 cells into CD8$^{-/-}$LDLr$^{-/-}$ mice does not change absolute or relative necrotic areas in the lesions. Quantification of absolute and relative necrotic core content by Masson's Trichrome staining in the aortic root lesions of CD8$^{-/-}$LDLr$^{-/-}$ mice treated with Tc0 or Tc17 cells. Individual data points and mean ± SEM, n = 9 mice per group. The data was tested for significance by using unpaired *t*-tests.

Table S1: Antibodies used for flow cytometric analysis.

Antibody	Fluorochrome	Clone	Supplier
CD4	APC	GK1.5	GK1.5
CD4	PerCP	RM4-5	BD Biosciences
CD4	V500	RM4-5	BD Biosciences
CD8	eFluor450	53-6.7	eBioscience
CD8	FITC	53-6.7	eBioscience
CD8	PE-Texas Red	5H10	Invitrogen
EOMES	APC	Dan11mag	eBioscience
GATA3	PE	16E10A23	eBioscience
IFN-γ	eFluor450	XMG1.2	eBioscience
IL-17A	PE	TC11-18H10.1	Biolegend
IL-17A	PEdazzle594	TC11-18H10.1	Biolegend
IL-5	APC	TRFK5	Biolegend
Ki-67	FITC	SolA15	eBioscience
RORyt	BV650	Q31-378	BD Biosciences
Thy1.2	PeCy7	53-2.1	Biolegend
Thy1.2	PerCP-cy5.5	53-2.1	Biolegend
T-bet	PeCy7	eBio4B10	eBioscience
Fixable Viability	Efluor780	-	eBioscience

6

Orchestrating immune responses: how size, shape and rigidity affect the immunogenicity of particulate vaccines

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ABSTRACT

Particulate carrier systems are promising drug delivery vehicles for subunit vaccination as they can enhance and direct the type of T-cell response. In order to develop vaccines with optimal immunogenicity, a thorough understanding of parameters that could affect the strength and quality of immune responses is required. Pathogens have different dimensions and stimulate the immune system in a specific way. It is therefore not surprising that physicochemical characteristics of particulate vaccines, such as particle size, shape, and rigidity, affect multiple processes that impact their immunogenicity. Among these processes are the uptake of the particles from the site of administration, passage through lymphoid tissue, and the uptake, antigen processing and activation of antigen-presenting cells. Herein, we systematically review the role of the size, shape, and rigidity of particulate vaccines in enhancing and skewing T-cell responses and attempted to provide a "roadmap" for rational vaccine design.

1. Introduction

The implementation of vaccines has proven to be an affordable and effective strategy to prevent disease. Concerted vaccine efforts in the 20th century have resulted in the reduced occurrence or even elimination of infectious diseases [1, 2]. The first generation of successful vaccines was composed of weakened (attenuated) or inactivated pathogens. Despite their enormous success, some problems arise when using these types of vaccines. Firstly, there is a risk of genetic exchanges with other viruses, which may restore the virulence of live attenuated vaccines [3]. Secondly, due to their complex nature, these vaccines can induce adverse effects such as fever [4–6]. To circumvent these issues, subunit vaccines, containing only the antigen(s) against which the immune response must be targeted, have become more commonly used. These types of vaccines lead to superior safety profiles at the expense of decreased immunogenicity, due to the lack of pathogen-associated molecular patterns (PAMPs). Therefore, these vaccines require the addition of adjuvants [7] and/or the use of a particulate delivery system. The advantages of particulate delivery systems entail the protection of the integrity of antigens until they are delivered to antigen presenting cells (APCs) [8] and co-localisation of adjuvant and antigen to the same APCs, which limits systemic exposure to the adjuvant and thereby minimises adverse effects [9]. Furthermore, uptake of particulate matter by APCs induces an inflammatory response, contributing to the adjuvanticity [10].

Several classes of particulate vaccines have been developed which have been reviewed in great detail [11]. Interestingly, not only the composition of the particle affects its immunogenicity, but a growing number of reports has covered the effect of particle size on vaccine efficiency. More recently, several publications reported the effect of particulate vaccine shape or rigidity on immunogenicity. This is probably due to the fact that techniques to alter and characterise particle shape and rigidity were developed later than those for particle size. There are several ways to alter size, shape, and rigidity. The size of particles is controlled by manufacturing conditions such as extrusion for vesicles [12], centrifugation for vesicles or solid particles [13, 14], and emulsification conditions for polymeric particles [15]. The shape of particles can be altered by mechanical stretching [16] or by producing particles in a mould [17]. Rigidity, a measure of the particle's ability to retain its shape under mechanical stress can be manipulated for instance by varying the density of cross-linking in polymer hydrogel particles, by incorporating cholesterol in liposomes, or by increasing shell layer thickness in capsules [18–20].

In this review, we discuss how particle size, shape, and rigidity affect biodistribution, cellular uptake, antigen presentation and the resulting immune response in murine models (unless stated otherwise), where appropriate as a function of the route of administration. We acknowledge that more parameters, such as surface charge, particle composition, biodegradability or the inclusion of adjuvants are important characteristics that affect immunogenicity. However, the effect of these parameters has been extensively described elsewhere [21–30]. In addition, vaccines equipped with targeting ligands and adjuvants may induce immunological effects solely based on their physicochemical parameters [31].

2. Particle size

2.1. Particle distribution

Vaccination aims to mimic a pathogenic infection and induce immunological memory for possible future encounters. For a vaccine to elicit an immune response, effective delivery of antigens from the site of injection to secondary lymphoid tissue, where APCs, B- and T-cells reside, is the first requirement. Antigens can directly drain to lymphoid organs (such as spleen or lymph nodes, LNs) through the interstitial fluid and the collecting lymphoid vessels. Alternatively, particulate vaccines can be taken up by APCs at the site of injection and subsequently travel through the lymphatic system to interact with T and B cells that reside in the LNs [32, 33].

The size of particulate vaccines plays a crucial role in their transport to the LNs. Upon intradermal injection, interstitial flow (drainage of fluids from the interstitial space) transports small, non-liposomal nanoparticles (< 50 nm) more efficiently into lymphatic capillaries and draining LNs than particles larger than 100 nm. Smaller particles are hypothesised to be convected much easier through the interstitial flow, whereas larger particles require active transport by tissue-resident dendritic cells (DCs) to shuttle them to the LN. These smaller particles show increased retention in the LNs, due to efficient uptake by LN-resident DCs [34–36]. Of note, for larger-sized particles (> 50 nm), the efficiency of DC migration towards the draining LNs creates an extra parameter that might affect the quantity of antigen that is able to reach the LNs [37].

The effect of size on antigen distribution is also evident for liposomal formulations. Oussoren and colleagues reported a negative correlation between lymphatic uptake and liposome size in rats upon s.c. injection [38]. Interestingly, small 40 nm sized liposomes were poorly retained by the LNs compared to larger (> 400 nm) liposomes. This was due to more efficient phagocytosis of larger liposomes, as macrophage-depleted LNs showed reduced LN localisation of large liposomes [39]. Small liposomes are possibly less affected, since they can be taken up by multiple cell types in the LNs via endocytic pathways other than phagocytosis, such as pinocytosis. This, of course, will also affect the immunogenicity of these particles as a lower percentage will reach APCs.

Different routes of administration impose different barriers for the antigen to reach secondary lymphoid tissue. Thereby, different tissues contain different subsets of DCs, such as Langerhans cells in the skin, $CD103^+$ DCs in connective tissue and mucosal DCs in the gut. The type of DC to which the antigen is delivered may influence the skewing of the immune response, but this is outside the scope of this review [40]. A study using orally dosed biodegradable polylactic acid (PLA) microparticles ranging from 1 – 26 µm in diameter showed that the uptake of these particles into intestinal lymphoid structures referred to as Peyer's patches, increased with increasing particle size up to 11 µm, and decreased again hereafter [41]. Microspheres smaller than 5 µm were subsequently translocated via the lymphatic system from the Peyer's patches to the spleen, whereas larger particles remained in the Peyer's patches in the jejunum. The authors suggested that uptake by phagocytes of particles larger than 10 µm was less likely to occur, explaining the decrease in splenic localisation when microparticle size exceeds this limit.

Extending the size into the nanometre range, it was shown that oral administration of nanometre-sized particles results in higher uptake in the rat intestine than microparticles [42, 43]. Nanoparticles are taken up more efficiently by the intestinal epithelial cells and are able to penetrate deeper into the Peyer's patches, which make them more efficient than microparticles for oral delivery [14]. Thus, it appears that for optimal gut barrier passage, particulate vaccines should be designed to have a size in the nanometre range.

Concerning nasal delivery, it has been reported that migration of non-liposomal particles across the nasal mucosa of rats increases with decreasing particle size, resulting in stronger immunoglobulin G (IgG) and IgA responses, which are markers for general and mucosal immune responses, respectively [44]. Possibly, nanoparticles can permeate the epithelial lining more efficiently than microparticles, resulting in enhanced immunity, as shown in rats and mice [13, 45, 46].

Overall, it can be concluded that smaller-sized particles (< 50 nm) can directly drain and penetrate deeper into the LNs. However, larger-sized particles are retained more efficiently in the LNs, which emphasises the need for studies that find the optimal particle size to ensure efficient lymphatic drainage as well as retention. Furthermore, the route of administration can affect the distribution and should be considered in vaccine design as well.

2.2. Cellular uptake

An important step towards inducing a potent immune response is the uptake of antigen-containing particles by APCs. APCs are continuously probing their environment for the presence of pathogens or danger-related signals, which enables them to internalise pathogens or other antigens and process them into peptides. Extracellular fluid, which may contain small antigens, is continuously taken up by APCs through macropinocytosis. Larger particles are generally internalised via phagocytosis due to binding to receptors on the plasma membrane of APCs, which triggers actin assembly and drives particle engulfment. All resulting vesicles travel to endosomes within the APC where their content is processed [47, 48].

Conceivably, due to their exceptional capacity for macropinocytosis, DCs appear to preferentially take up nanoparticles. Studies in DC lines and DCs derived from human mononuclear cells have shown an inverse correlation between particle size and internalisation for particles of different compositions ranging from 20 µm to 150 nm [49–51]. Shima *et al.* have shown that 40, 100 and 200 nm sized poly(γ-glutamic acid)particles also show excellent uptake by DCs in vivo in the LNs upon s.c. administration. Interestingly, they report that the number of DCs that have taken up the 40 nm particles is twice as high as the number of DCs that have taken up the 200 nm particles, while the relative amount of antigen taken up was three times as high for 200 nm particles compared to the 40 nm particles. This suggests that smaller-sized nanoparticles are taken up more efficiently, but larger-sized nanoparticles can deliver a greater amount of antigen to APCs [52]. In a study comparing uptake of polystyrene particles ranging from

20 nm – 1 µm in lung-draining LN upon intranasal administration, it was reported that smaller (< 50 nm) particles were preferentially taken up by LN-resident DCs [53].

Examining the behaviour of particles in the extremely small size ranges, le Guével *et al.* produced gold nanoparticles of 12 nm and nanoclusters (clusters of gold atoms) of 2 nm in size [54]. Comparing the number of particles per human-derived DC, the nanoclusters showed a higher uptake compared to the nanoparticles. However, only the nanoparticles induced DC maturation and subsequent Th1-mediated immunity. Of interest, the nanoclusters have a higher diffusion capacity than the nanoparticles. This suggests the nanoparticles are taken up by receptor-mediated endocytosis, which is less efficient than diffusion, resulting in lower uptake, but the particles taken up via this process are able to induce immunity.

Of note, the mechanism of antigen delivery has been reported to differ between nano- and microparticles. Here we discuss the consequences of particle size on uptake, however, it must be noted that attachment of microparticles to the APCs, without endocytosis of the delivery system, appears to be sufficient to deliver the antigen to the APCs [55, 56].

2.3. Antigen presentation and APC activation

Following antigen uptake by APCs, these cells need to become activated via the recognition of PAMPs by pattern recognition receptors (PRRs) [57]. Effective processing leading to robust antigen presentation is required to induce potent immune responses. After uptake, antigen loaded particles are deposited in the endosome, where the particle and antigen are broken down by enzymatic degradation upon acidification of the endosome, resulting in short peptide sequences. These small protein fragments are loaded upon major histocompatibility complex (MHC) class II molecules, leading to $CD4^+$ T-cell activation. Alternatively, particles can be modified to facilitate endosomal escape, after which the antigenic peptide can reach the cytosol and, after proteasomal degradation, can be loaded upon MHC class I molecules, which can activate $CD8^+$ T-cells. This process, referred to as cross-presentation, can occur via two pathways: the presently described 'phagosome to cytosol pathway' and via the 'vacuolar pathway' in which antigens are loaded onto MHC class I molecules within the phagosome, which is not necessarily TAP-dependent [58–60].

Nanoparticles appeared to be efficient at inducing class I antigen presentation *in vitro*, whereas microparticles induced almost no MHC class I antigen presentation [61]. Particles larger than 500 nm were delivered into phagosomes, which subsequently fused with early endosomes, whereas smaller (< 200 nm) particles localised rapidly into late endosomes which fused with lysosomes. MHC class II complexes were recruited to both compartments, but delivery to the prelysosomal (early) compartment was shown to be more efficient in processing and presenting an encapsulated antigen. Consequently, the larger particles (> 500 nm) produced enhanced $CD4^+$ T-cell activation compared to smaller particles [12]. It has been suggested that the accumulation of nanoparticles within the lysosomes may have caused lysosomal overload, which

resulted in defective lysosomal degradation, which may explain the reduced MHC class II antigen-presenting capacity [62].

Considering cross-presentation, it was suggested that particles in the nanometre size range induced MHC class I presentation via the phagosome-to-cytosol pathway, whereas the larger micrometre-sized particles were processed via the vacuolar pathway, which yielded relatively fewer MHC class I complexes [60]. Together, these studies provide strong evidence for a size-dependent effect of both liposomal and non-liposomal particles on endosomal antigen processing and subsequent presentation.

2.4. Skewing immune responses

Pathogens can infect host cells via various routes, occupy different (intracellular or extracellular) compartments and cause acute or chronic infections. Therefore, clearance of pathogens requires a specific approach. $CD8^+$ T-cells play a seminal role in detecting and clearing intracellular pathogens as they recognise infected cells through specific epitopes presented upon MHC class I molecules, upon which they exert inflammatory and cytotoxic functions [63, 64]. $CD4^+$ T-cells recognize MHC class II via their T-cell receptor (TCR) and can be subdivided into different classes, the principal of which are T helper 1 (Th1), Th2 and T-regulatory (Treg) cells, characterised by the expression of T-bet, GATA-3, and FoxP3, respectively. Th1 cells produce inflammatory cytokines and are major producers of interferon-γ (IFN-γ) and TNF-α, which are pivotal for cell-mediated immunity (e.g. macrophage activation, $CD8^+$ T-cell help). The Th2 subset is characterised by a different cytokine profile, including cytokines such as interleukin 4 (IL-4), IL-5, IL-10 and IL-13, and is associated with the induction of humoral (antibody-mediated) immunity. Finally, Treg cells are a tolerogenic subset that suppresses inflammatory responses through secretion of anti-inflammatory cytokines (e.g. IL-10, TGF-β) [65].

As clearing a pathogen requires a specific type of immune response, skewing of the response after immunisation is an important aspect that particles can influence. As the size of a particulate vaccine affects the extent of MHC class I or MHC class II presentation, this directly influences effective $CD8^+$ and $CD4^+$ T-cell priming. However, the size of particles also appears to influence the type $CD4^+$ T-cell that is induced. Nanoparticles (ranging in size from $100-600$ nm) induce the most prominent activation of DCs (as measured by CD80 expression) compared to micro-sized particles. As a result, nanoparticles generated the highest antigen-specific $CD8^+$ T-cell response and a higher proportion of IgG2a antibodies relative to IgG1 antibodies, which indicates skewing towards a Th1 phenotype. Other studies showed that particles of $40-50$ nm in size are most potent in inducing IFN-γ mediated Th1 immunity compared to particles in the 100 nm range, which induced stronger IL-4 responses. It has been suggested that smaller (<100 nm) particles may enter APCs through one of the mechanisms used by viruses, such as clathrin-coated pit-mediated uptake, which may induce a stronger Th1 immune response [66, 67]. This suggests there is an optimal particle size of around 50 nm that triggers Th1 responses.

Microparticles (2 – 8 µm) were not taken up but instead attached to the surface of the APC, releasing their antigen into the cell in both mouse and rat models. This favoured IL-4 secretion and showed a higher IgG1/IgG2a ratio and higher antibody titres. Thereby, microparticles upregulated MHC class II expression, whereas nanoparticles induced more MHC class I. This suggests that nanoparticles induce a Th1 type immune response while microparticles induce a Th2 type response [49, 55, 68]. There appears to be an upper size limit for effective Th2 response; 5 µm poly(lactic-co-glycolic) acid (PLGA) microspheres containing hepatitis B surface antigen in pulmonary immunisation in rats induced higher antibody titres compared to larger (12 µm) PLGA particles, which could be due to less efficient uptake or adherence of particles that are larger in size than DCs [56]. Thus, the optimal size for inducing Th2 responses is approximately 1 – 5 µm.

Thus far we have seen a trend that smaller solid (polymeric or gold) particles induce stronger Th1 and CD8-mediated responses, whereas larger particles seem to skew towards a Th2 and B-cell mediated response (Fig. 1). Lipid vesicles, however, have been reported to show an opposite trend; small (< 200 nm) liposomes appear to induce Th2 mediated immunity, whereas larger liposomes skew towards Th1 responses [12, 69, 70].

The reason for the apparently contradictory effects observed for liposomal and non-liposomal particles could be explained by the differences in lysosomal degradation rates. Tran and colleagues studied the intracellular trafficking of 50 nm, 500 nm or 3 µm particles and showed that OVA conjugated to 50 nm polystyrene beads was rapidly exposed to an acidic environment in the lysosome [71]. This led to fast degradation of the antigen in the lysosome and, therefore, inefficient presentation. Furthermore, antigens bound to 500 nm and 3 µm particles remained in a less acidic environment within the phagosomes for a longer period of time, resulting in more efficient MHC class I presentation.

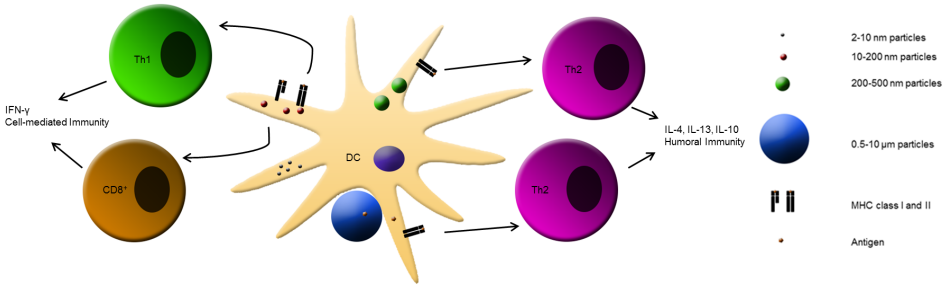

Figure 1: Schematic overview of the effect of non-liposomal particle size on inducing T-cell immunity. Ultra-small particles (2 – 10 nm) are taken up very efficiently by APCs but are poorly immunogenic. 10 – 200 nm nanoparticles are most efficient at inducing Th1 and CD8-mediated immunity, whereas larger 200 – 500 nm nanoparticles tend towards Th2 mediated responses. Microparticles adhere to the cell membrane and release antigen into the cell, which is presented upon MHC class II molecules and skews the immune response towards Th2 mediated responses.

3. Particle shape

3.1. Particle distribution

Besides particle size, shape is an important parameter influencing the immune response. A common way of characterising particle shape is by using the ratio between the height and width of the particle, denoted as the aspect ratio (AR). Huang *et al.* reported that the shape of mesoporous silica nanoparticles affects the biodistribution of these particles after intravenous (i.v.) administration in mice [72]. Both short-rod-shaped particles (185 nm, low AR) and long-rod particles (720 nm, high AR) were trapped in the spleen and liver. However, compared to the short-rod particles, the long-rod particles were more prominent in the spleen. Furthermore, short-rod particles were cleared faster from the body by urine and faeces than long-rod particles. Likely, the shape of the particles affects the ability for uptake by tissue-residenT-cells, which in turns affects the biodistribution and retention ability in the tissues. However, a size effect cannot be excluded in this experiment [72]. Injected filomicelles, micelles with a tubular shape (high AR), remained in circulation in rats and mice for up to one week [73]. Short tubular micelles were cleared from the circulation within two days. Filomicelles longer than 3 µm could not be taken up by human macrophages, whereas shorter filomicelles could be taken up via phagocytosis. The authors suggest that longer circulation time of the long filomicelles can be explained by the theory that they are stretched out by the blood flow, thereby minimising interactions with phagocytes and the blood vessel wall. Shorter cylinders will be less affected by the blood flow and interact more with phagocytes, resulting in more efficient uptake and thus faster clearance from the circulation. However, it must be noted that these particles were injected i.v. and therefore, this study focused on the uptake from the circulation, instead of lymphatic trafficking [73]. Upon oral administration of mesoporous silica nanoparticles, different effects were observed; decreasing ARs (5, 1.75 and 1) of the particles resulted in increased absorption by the small intestine, whereas urinary secretion was decreased [74]. Indeed, particles with the smallest AR showed the highest content in the spleen compared to the other particles, which were mainly deposited in the liver, lungs and kidneys. These results suggest that upon oral administration, spherical particles will exhibit a more favourable biodistribution profile than non-spherical ones, emphasising that the route of administration is an important parameter influencing the effect of particle properties on immunogenicity.

3.2. Cellular uptake

Similar to particle size, particle shape also plays a major role in the uptake of particulate vaccines by APCs. Non-spherical long-rod polystyrene particles stretched from 3 µm spheres were shown to exhibit negligible phagocytosis in a macrophage cell line, as observed by time-lapse imaging [75]. Moreover, spherical particles of similar size were internalised efficiently by macrophages. Spheres and rods of 1 µm in size showed the same differential phagocytosis as 3 µm spheres and rods. The authors suggested that macrophages cannot take up the rod-like particles, as the shape is mostly flat and

only contains curvatures on extreme ends, which hinders phagocytosis. Niikura *et al.* tested macrophage uptake of gold particles of different shapes; spheres of 20 nm and 40 nm in diameter, 40 nm × 10 nm rods (AR = 4) and 40 × 40 × 40 nm cubes [76]. Interestingly, rod-shaped particles appeared to be taken up more efficiently by macrophages than spherical or cubic particles (with cubic particles being the least effective), but in fact, the spherical particles had more efficient uptake per weight. Sharma *et al.* produced initially spherical polystyrene particles that were stretched to either prolate ellipsoids (high AR) or oblate ellipsoids (lower AR) [77]. The phagocytosis efficiency was in the order of oblate ellipsoids>>spheres>prolate ellipsoids. Even though oblate ellipsoids did not have the highest cell attachment, almost 90% of the attached particles were internalised, compared to 50% of the prolate ellipsoids and 70% of spheres. The combination of relatively high attachment and internalisation gives oblate ellipsoids a clear advantage for phagocytosis. Champion *et al.* reported that the particle shape at the point of cell contact dictated whether or not phagocytosis was initiated [78] (Fig. 2). Polystyrene particles were fabricated in the shapes of spheres, oblate ellipsoids, prolate ellipsoids, elliptical discs, rectangular discs or flying saucer shapes. The orientation of the particle towards the phagocyte was of great importance in Fc receptor-mediated phagocytosis by macrophages. Actin polymerisation in the shape of a cup occurs beneath the particle, which then forms an actin ring that forces the membrane along the particle surface until it is engulfed. When this initial actin attachment forms on the flat side of a particle, the formation of the actin ring is not supported. The contact angle between the membrane normal and the particle, therefore, is an important determinant of the internalisation efficiency. Particles for which this angle is small are phagocytosed more efficiently, as only gradual expansion of the actin ring is required, which is a metabolically intensive process. If the contact angle is too large, the cell will spread across the surface of the particle but cannot internalise it. Therefore, the uptake of (near) spherical particles is always favourable, whereas that of rod-shaped particles depends on the likelihood of the particle approaching at a favourable contact angle, thereby negatively influencing the uptake of such particles (Fig. 2). Indeed, Huang *et al.* manufactured mesoporous silica nanoparticles of different lengths and ARs; 100 nm spherical (AR = 1), 240 nm short rod (AR = 2) and 450 nm long rod (AR = 4) [72]. Incubation with human melanoma cells showed the formation of well-organised F-actin bundles for the particles with ARs 1 and 2. However, F-actin was disorganised for cells incubated with the particles with an AR of 4. This may explain why near-spherical particles are taken up more effectively as described in the aforementioned papers.

Yi and Gao created a theoretical model for membrane wrapping of particles of different shapes [79]. Keeping rigidity constant, they found that longer and thinner rods require more energy for cellular wrapping than more spherical particles. Furthermore, non-spherical particles undergo an orientation change during wrapping, which also contributes to increased energy expenditure. Both *in vitro* and *in silico* models suggest that spherical and slightly ellipsoidal nanoparticles are most efficiently taken up due to favourable energy expenditure during actin membrane wrapping. It can also be noted that small spheres inherently require less polymerisation of the actin cytoskeleton, compared to larger spheres; hence, less energy is expended in this process. This might explain the preferential uptake of smaller compared to larger spheres.

3.3. Antigen presentation and APC activation

The effect of particle shape on antigen presentation is currently poorly described. In one study, rod-shaped gold particles (40 nm long, AR = 4) coated with West Nile virus induced production of IL-1β and IL-18 in bone marrow-derived DCs; these cytokines are secreted upon inflammasome activation. It is known that lysosomal rupture can induce inflammasome activation and indeed, rod-shaped particles were able to escape from the lysosome into the cytosol, suggesting lysosomal rupture could have occurred. In contrast, spherical and cubical particles induced production of tumour necrosis factor-α (TNF-α), IL-6 and IL-12, which are not associated with inflammasome activation [76]. Mathaes *et al.* reported that both nano- (150 nm) and micro-sized (1.5 μm) spherical PLGA particles induced stronger activation of DCs as measured by upregulation of CD83 and CD86 than similar sized, non-spherical, stretched particles [50]. As these molecules provide important co-stimulatory signals during antigen presentation, this finding may suggest that spherical particles result in more efficient antigen presentation. However, more research is required to study this relationship.

3.4. Skewing immune responses

Recent observations suggest particle shape directly influences the type of immune response. In the aforementioned study by Niikura *et al.*, spherical 40 nm gold particles coated with antigen derived from West Nile virus, induced superior levels of West Nile-specific IgG as compared to cubical and rod-shaped particles of similar size [76]. Kumar *et al.* performed an elegant study in which they used spherical polystyrene ovalbumin conjugated particles of 190 and 520 nm in diameter, which they stretched into rod-shaped particles of 380 and 1530 nm in length [16]. They found that the 190 nm spheres were most potent at inducing IgG2a antibody responses, whereas the 1530 nm rods induced the highest IgG1 antibody responses. Moreover, they showed that the small spheres were most potent at inducing IFN-γ responses, whereas IL-4 was consistently produced in all groups. From this, it can be concluded that the smaller-sized nanoparticles are more effective than larger particles are inducing Th1 and $CD8^+$ T-cells, and this effect is most pronounced when these small particles are spherical. In contrast, the larger-sized nanoparticles are more potent at inducing Th2 responses, which are most effective when using rod-shaped particles.

4. Particle rigidity

4.1. Particle distribution

Apart from size and shape, rigidity can also affect the biodistribution and elimination rate of particles. Merkel *et al.* produced red blood cell mimics (RBCM); hydrogels containing particles of a similar shape, size (6 μm) and rigidity as compared to RBCs [80]. By altering the rigidity, they observed that circulation time was inversely correlated with rigidity, with the most rigid RBCMs being eliminated much faster than the least rigid

ones. This was likely due to the less rigid particles being able to reach areas with constricted blood flow, increasing circulation times. Similarly, "soft" polyethylene glycol (PEG) based hydrogel nanoparticles were formulated which had a longer distribution half-life (rate of particle distribution from plasma into tissues) and elimination half-life (rate of particle clearance from plasma) than rigid particles [81]. Analysis of tissues after 30 min and 12 hours showed that soft particles were found at a higher concentration in almost all tissues (spleen, kidney, heart, lungs, brain, and blood) except for the liver. The differences in biodistribution were attributed to the longer circulation time of the soft nanoparticles, which led to increased retention in organs with high blood flow. Possibly, the soft nanoparticles were degraded in the liver, explaining their reduced retention in this tissue. Moreover, it was found that more rigid particles accumulated in the capillaries in the lungs while the less rigid particles avoided lung filtration and instead were found mostly in the spleen, suggesting that more rigid particles become trapped in the first tissue with microvasculature they encounter [80].

It was found that liposomes containing phosphatidylcholine (PC) with a high transition temperature (i.e., high rigidity) injected i.v. remained in the blood for a longer period of time than similar liposomes containing PC with a low transition temperature. This was combined with a decreased uptake in the liver and spleen [82, 83]. Similar results were observed by Senior *et al.* who hypothesised that longer circulation was due to less interaction between the liposomes and high-density lipoprotein in the blood [84]. There is evidence that ApoA-I and ApoA-II on high-density lipoprotein react with PC and cholesterol-containing liposomes, which results in faster clearance [85]. After i.m. injection, rigid cationic liposomes remained at the site of injection longer than less rigid ones. This corresponded with higher amounts of non-rigid liposomes found in the draining LNs [86]. In contrast, Kaur *et al.* observed no effects of cholesterol content (which also affects rigidity) in cationic liposomes on drainage from the site of injection or transport to LNs after i.m. injection [87].

It appears that similar to particles of increasing size, lymphatic trafficking of particles with increasing rigidity will be hindered by a decreased ability to navigate through narrow lymphatic vessels.

4.2. Cellular uptake

To understand the importance of particle rigidity on cellular uptake, one must first examine the interplay between the cell membrane and the particle. The first theoretical model of adhesive wrapping of a vesicle by the cell membrane was created by Yi *et al.* [88]. They theorised that the degree of wrapping was dependent on adhesion energy between the vesicle and the cell surface, vesicle size, the surface tension of the cell membrane upon contact with the vesicle, and the difference in rigidity of the cell membrane and the vesicle. They concluded that rigid particles are in general more easily wrapped by the cell membrane due to cell membrane deformation by the particles and that flexible particles spread out more across the cell membrane. This was supported by molecular dynamics simulations by Sun *et al.* [89]. Experimentally, Beningo and Wang reported that macrophages preferentially phagocytosed $1-6\mu$m-sized rigid

particles over softer particles due to rigid particles stimulating actin filament assembly in macrophages [90]. The previously described RBCM hydrogels also showed minimal (< 10%) uptake by human umbilical vein endothelial cells, probably due to a combination of low rigidity and large (6 µm) particle size [80]. Similarly, Anselmo *et al.* found that PEG-based rigid particles had significantly higher uptake than flexible particles (both spheres of 200 nm) in an endothelial brain cell line (bEnd.3), an epithelial tumour cell line (4T1) and macrophages (J774) [81]. As previously stated, phagocytosis by macrophages is important for retention of particles in the LNs, which improves the overall immunogenicity of the particles [39]. Similarly, cationic gel-state liposomes with higher cholesterol contents (i.e., lower rigidity) showed reduced uptake by THP-1 macrophages [87] and gel-state liposomes consisting of high transition temperature lipids had increased APC uptake compared to fluid-state liposomes made up of low transition temperature lipids [86].

Generally, it can be stated that rigid particles are most efficiently taken up, whereas more flexible particles are deformed by the membrane, resulting in increased energy expenditure and consequently reduced uptake (Fig. 2).

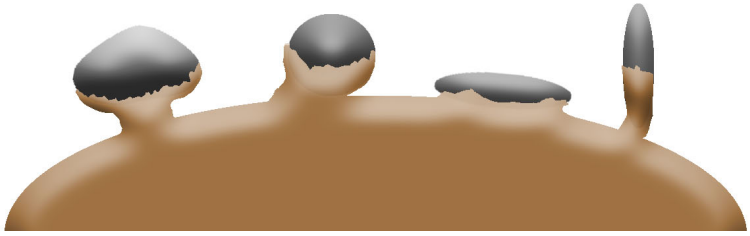

Figure 2: Cellular uptake of a flexible sphere, rigid sphere and rigid rod approaching the cellular membrane at a perpendicular or tangential angle. The rigid sphere is taken up more efficiently than the flexible sphere; while the membrane envelopes both particles, the flexible particle deforms leading to slower uptake. For rod-shaped particles, the angle at which the particle approaches the cell is important; when the particle arrives at a tangential angle, it would require too much energy to form an actin cup around the particle leading to no uptake. Therefore, an orientation change is needed, which also requires high energy expenditure. The rigid rod approaching the cell at a perpendicular angle requires much less energy to be taken up.

4.3. Antigen presentation and APC activation

Once a particle has been taken up by a cell, intracellular processing can also be affected by rigidity. Hartmann *et al.* produced microcapsules of about 4 µm with varying shell thickness that altered their rigidity [91]. By observing uptake and acidification of the microcapsules in HeLa cells, they found that more rigid capsules had longer endosomal processing times and reached the lysosome later than more flexible capsules. Unfortunately, it was not reported how this affects the efficiency of antigen processing. Cui *et al.* prepared capsules of around 1 µm composed of polyglycolic acid (PGA) cross-linked to the adjuvant CpG [92]. They altered rigidity by increasing the cross-linker concentration. Incubation with plasmacytoid DC (pDCs) showed increased particle association to pDCs with increasing rigidity. The authors also showed a rigidity-dependent increase in pDC activation as measured by CD86 and CD40 levels. Thus, the effect of rigidity on

uptake by macrophages and DCs may have important implications for immunity. In the case of liposomes, Christensen *et al.* showed that more rigid cationic liposomes injected i.m. resulted in increased activation of DCs in draining lymph nodes, as measured by CD40 and CD86 upregulation [86].

To our knowledge, no reports have been published that specifically examine antigen presentation as a function of particle rigidity. However, since antigen presentation is largely dependent on particle uptake by APCs, and it was shown above that rigid particles are more likely to be taken up, we suggest rigid particles shall have more efficient antigen presentation. Thereby, it can be speculated that the shorter endosomal processing time of rigid particles shall enhance antigen stability and lead to more efficient MHC presentation compared to flexible particles. This will mainly affect MHC class II epitopes as they require endosomal processing, whereas MHC class I epitopes are derived from the cytosol.

4.4. Skewing immune responses

Several studies have shown that particle rigidity can affect the skewing of the immune response. In two studies by the same group, the immune response was measured in mice after immunisation with liposomes composed of phospholipids with different transition temperatures. They found that liposomes containing high transition temperature lipids elicited higher antibody responses [93, 94]. A similar rigidity effect on antibody [95, 96] and T-cell responses [97, 98] has been found by other groups. There is some evidence that reducing liposome rigidity by the addition of cholesterol or by selecting lipids with lower phase transition temperatures leads to reduced Th1 responses after i.m. immunisation. In contrast to the studies mentioned above, the authors state that there is no measurable effect of particle rigidity on Th2 or antibody responses. However, this was hypothesised to be due to reduced APC uptake of non-rigid liposomes from the site of injection [86, 87].

Arnal and colleagues reported that the presence of virulence factors in Bordetella pertussis increased rigidity; a non-infectious mutant deficient of filamentous haemagglutinin (FHA) had lower rigidity [99]. The authors postulate that FHA increases the rigidity of the cell, specifically by creating rigid nanodomains that could enhance the adhesion of B. pertussis to cells. Studying different strains of *Lactobacillus* and *Bifidobacterium*, Mokrozub *et al.* found that *Lactobacillus* strains with elastic cell walls were more effectively digested by macrophages *in vitro* and enhanced their ability to produce nitric oxide and accumulate reactive oxygen species. However, the more rigid strains had higher IL-12 and IFN-γ production (indicative of a Th1 immune response). In the case of *Bifidobacterium* strains, uptake of the more rigid strains increased macrophage effector functions while also enhancing IFN-γ production [100]. The authors hypothesise that strains with more rigid cell walls remain viable within macrophages longer, prolonging cytokine production. For viruses, it was shown in two separate papers by Kol *et al.* that rigidity differs between the immature (non-infectious, viral budding) and mature (infectious, entry into cells) stage, for both murine leukaemia virus (MLV) and human immunodeficiency virus (HIV) (about 100 nm in size). In the case of MLV, the mature form

of the virus is more rigid. Conversely, immature HIV is much more rigid than mature HIV, suggesting that a more flexible viral structure is beneficial for cell entry [101, 102].

It can be concluded that, for lipid vesicles, immunisation with more rigid particles results in higher antibody and T-cell responses. Studies that examine the effect of particle rigidity on the immune response would be extremely valuable to further understanding and the role of this parameter in vaccine design.

5. Summary and conclusions

Here we reviewed how the immunogenicity of particulate vaccines is directed by their shape, size, and rigidity. Clearly, the choice of the optimal physicochemical parameters depends on multiple factors, such as the route of administration, which immune cells are targeted and what type of immune response is preferred. Importantly, in most of the studies discussed here, not only the shape, size or rigidity of the particles differ, but other parameters are also (indirectly) altered. Particle shape and rigidity are especially closely related, since highly deformable particles can alter their shape during circulation or cellular uptake. Additionally, differences in rigidity measurements and calculations can result in different definitions of "soft" or "rigid" particles. We strongly plead for systematic investigations where only one particle parameter is changed and all others are kept constant. This will help to accurately define the relationship between particle size or shape and the immunogenicity of particulate vaccines.

This review suggests it is important to take the physicochemical characteristics of particulate vaccines into account in order to induce maximal antigen responses. For instance, what may be a favourable characteristic for, e.g., transport towards the LN may not be ideal for inducing the desired skewing of the immune response. Therefore, the choice of particle size, shape and rigidity must involve a careful consideration of the effects of these on all of the events influencing the immunogenicity. Figure 3 could function as a "roadmap" and when the desired immunologic outcome is known, it might provide a model for rational vaccine development.

For instance, the development of a $CD8^+$ T-cell activating vaccine (e.g. cancer vaccines) may be most efficient when a small (< 200 nm), rigid, elliptical non-liposomal nanoparticle is used. The elliptical shape, as well as rigidity, will ensure efficient uptake by APCs and a size smaller than 200 nm will skew the immune response towards Th1 and $CD8^+$ T-cell immunity. Alternatively, the development of a Th2-directed vaccine (e.g. hepatitis B vaccines), could benefit most from a small (< 200 nm), spherical, slightly more flexible liposomal particulate formulation. Since liposomes show opposite trends compared to non-liposomal formulations in skewing immune responses, this will ensure a Th2-directed response. Thereby, the uptake of this particle by APCs will still be efficient due to the small size. As rigid bacterial strains and liposomes are known to skew the immune response towards type 1 immunity, it could be desirable to use a more flexible particle. However, more energy is needed for uptake of soft particles, which may have a negative influence on the antigen presentation. Thus, depending on the application, one could also choose to use a larger, non-liposomal particle. Micro-sized particles are

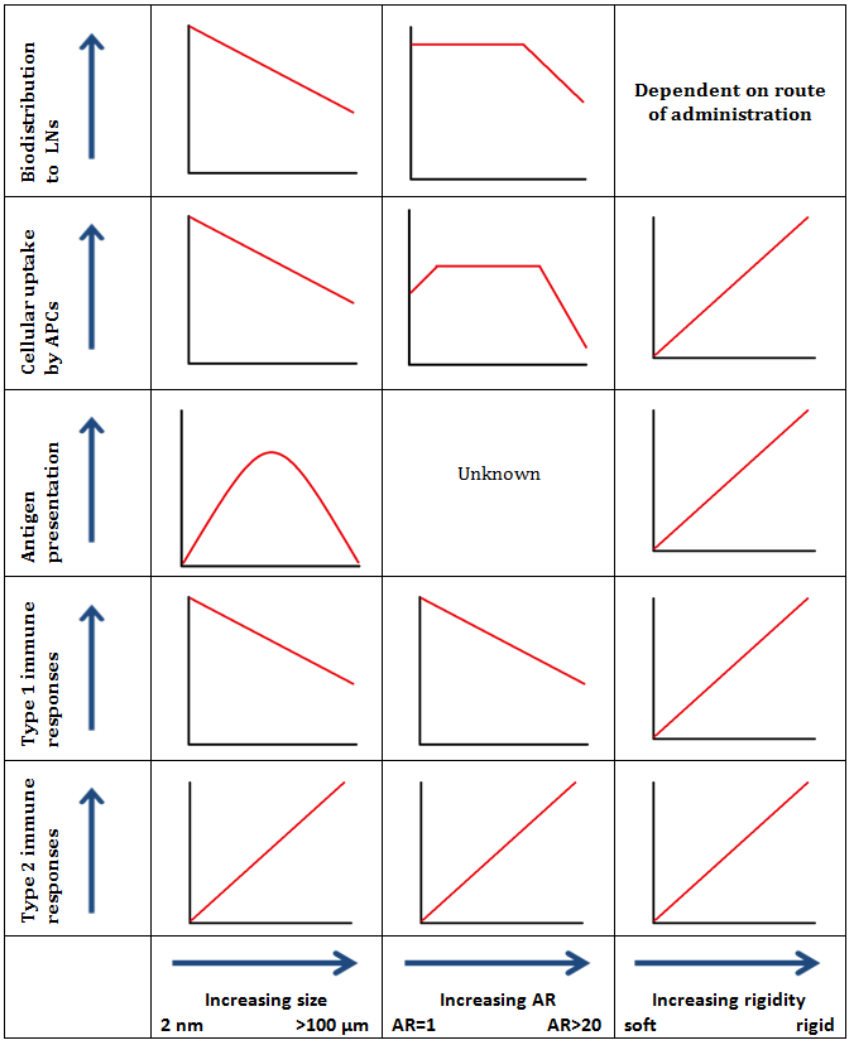

Figure 3: Summary of the general trends of the effects of shape, size and rigidity on different parameters affecting the immunogenicity of particulate vaccines. The height of the red line represents the efficiency per parameter. Size ranges from left to right from ultra-small (2 nm) to large (> 100µm) microparticles. Shape ranges from left to right from spherical (AR = 1) to long filomicelles (AR >> 20). Rigidity ranges from left to right from very elastic particles to non-deformable particles

known to tether to the membrane of APCs and deliver their antigens without being internalised. This also results in skewing towards a Th2-mediated immunity.

Overall, it appears that small, rigid, near spherical nanoparticles are the most favourable particles to reach APCs in the LNs and induce strong immune responses. The exact size can be tailored based on the type of particle used, to skew towards either Th1 or Th2 mediated immunity. Due to the scarcity of research, particularly

in the field of shape and rigidity, more studies will inevitably contribute to a more thorough understanding of how these parameters influence the immune response. This knowledge, in turn, will be of great importance for the rational design of more efficient vaccines, especially for diseases for which there is currently no vaccine, such as HIV, cancer or tuberculosis.

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; neutral, poloxamer 407-stabilised poly(propylene sulphide)	BALB/c and C57BL6 mouse	i.d.	Size	SAINNEHNI	[35]
Spherical; neutral; PEG-poly(propylene sulphide)	BALB/c mouse	s.c.	Size	Distribution	[34]
Spherical; anionic; polystyrene	C57BL/6 mouse	i.c.	Size	Distribution	[36]
Spherical; anionic; egg PC:egg phosphatidylglycerol(PG):cholesterol (10:1:4 molar ratio) liposomes	Wistar rat	s.c.	Size	Distribution	[38]
Spherical; neutral; egg PC:cholesterol (6:1 molar ratio)	Wistar rat	s.c.	Size	Distribution	[39]
Spherical; anionic; polystyrene	Sprague-Dawley rat	oral	Size	Distribution	[42]
Spherical; anionic; PLGA (50:50, MW 100000)	Sprague-Dawley rat	*In situ* intestinal tissue loop	Size	Distribution	[43]
Spherical; anionic; PLA (MW 7000)	BALB/c mouse	oral	Size	Distribution	[41]
Spherical; anionic; PLGA (75:25, MW 98000)	CD1 mouse	oral	Size	Distribution	[14]
Spherical; neutral; PLA-PEG (31:69, MW 28000)	Sprague-Dawley rat	intranasal	Size	Distribution	[13]
Spherical; cationic; N-trimethyl chitosan:tripolyphosphate (10:1.7 weight ratio)	BALB/c mouse rat	intranasal	Size	Distribution	[46]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; anionic; γ-PGA-g-phenylalanine (MW 380 000, 50:50)	C57BL/6j mouse	s.c.	Size	Distribution, cell uptake, APC activation	[52]
Spherical; anionic; carboxyl-modified polystyrene	C57BL/6j mouse	s.c.	Size	Distribution, cell uptake, APC activation	[53]
Spherical; anionic; poly(sulfobutyl-vinyl alcohol)-g-PLGA (50:50 MW 221 000)	BALB/c mouse	oral, intranasal, i.p.	Size, administration, route	Distribution, immune response	[45]
Spherical; anionic; PLGA (50:50, MW 24 000)	C57BL/6 mouse	i.p.	Size	Cell uptake, APC activation, immune response	[49]
Spherical and elliptical; anionic; polystyrene	JAWSII DC cell line	-	Size, shape	Cell uptake, APC activation	[50]
Spherical and elliptical; anionic; polystyrene	JAWSII DC cell line	-	Size, shape	Cell uptake, APC activation	[50]
Spherical; anionic and cationic; polystyrene	Human monocyte derived DCs	-	Size	Cell uptake	[51]
Spherical; anionic; PLA (MW 45 000)	Wistar rat	i.m.	Size	Cell uptake, antigen presentation, immune response	[55]
Spherical; neutral; glutathione-conjugated gold)	Human monocyte derived DCs	-	Size	Cell uptake, APC activation, immune response	[54]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; anionic; PLGA (50:50, MW 43 500 and 4500)	Sprague-Dawley rat	pulmonary	Size, weight	Cell uptake, immune response	[56]
Spherical; anionic; PLGA (50:50 MW 5000 and 75:25 MW 4000)	C57BL/6 mouse	s.c.	Size	Cell uptake, immune response	[61]
Spherical; anionic; monopalmitoyl glycerol:cholesterol:dicetyl phosphate (DCP) (5:4:1 molar ratio) liposomes	BALB/c or CBA/ca mouse derived macrophages	-	Size	Antigen presentation, cell uptake	[12]
Spherical; anionic; carboxyl-modified polystyrene	BALB/c and C57BL/6 mouse	i.d.	Size	Cell uptake, immune response	[66]
Spherical; anionic; carboxyl-modified polystyrene	C57BL/6 mouse	i.d.	Size	Distribution, immune response	[67]
Spherical; anionic; soy lecithin:glycerol monostearate (7:1 weight ratio)	C57BL/6 mouse	s.c.	Size	Distribution, antigen presentation, cell uptake, immune response	[68]
Spherical; anionic; monopalmitoyl glycerol:cholesterol:DCP (5:4:1 molar ratio) liposomes	BALB/c mouse, ferret	oral, i.m.	Size, administration route	Immune response	[69]
Spherical; cationic; dimethyldioctadecylammonium (DDA): trehalose dibehenate (TDB) (5:1 weight ratio) liposomes	BALB/c mouse	i.m.	Size	Distribution, cell uptake, immune response	[70]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Elliptical (AR 1.5 and 5); cationic; tetraethyl orthosilicate mesoporous silica	ICR mouse	i.v.	Shape	Distribution	[72]
Filamental; neutral; PEG-polyethylethylene or PEG-polycaprolactone	Sprague-Dawley rat, C57BL/6 mouse	i.v.	Shape, size	Distribution, cell uptake	[73]
Elongated (AR > 20); anionic; polystyrene	Rat alveolar macrophage	-	Shape, size	Cell uptake	[75]
Spherical, cubic and rod-like (AR 4); anionic; gold	C3H/HeN Jc1 mouse	i.p.	Shape, size	Cell uptake, APC activation, immune response	[76]
Spherical, oblate elliptical, prolate elliptical; anionic; polystyrene	RAW264.7 macrophage	-	Shape, size	Cell uptake	[77]
Spherical, oblate elliptical, prolate elliptical, elliptical disk, rectangular disk, UFO-shaped; anionic; polystyrene	Rat alveolar macrophage	-	Shape, size	Cell uptake	[78]
Spherical, short rod (AR 2), long rod (AR 4); cationic; tetraethyl orthosilicate mesoporous silica	A375 human melanoma	-	Shape	Cell uptake	[72]
Spherical, rod-shaped (AR 3 and 7); anionic; carboxyl-modified polystyrene	BALB/c mouse	s.c.	Shape, size	Cell uptake, immune response	[16]
Discoid; anionic; 2-hydroxyethyl acrylate and 2-carboxyethyl acrylate cross-linked with PEG-diacrylate hydrogel	BALB/c mouse	i.v.	Rigidity	Distribution, cell uptake	[80]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; anionic; PEG-diacrylate and 2-carboxyethyl acrylate hydrogel	BALB/c mouse	i.v.	Rigidity	Distribution, cell uptake	[81]
Spherical; anionic; polyacrylamide	Murine BMDC	-	Rigidity	Cell uptake	[90]
Spherical; cationic; poly(sodium 4-styrenesulphonate):poly(allylamine hydrochloride) (MW 70000 and 56000) and dextran sulphate:poly-L-arginine (MW 40000 and 15-70.000) capsules	A549 cells, HeLa cells, SH-SY5Y cells, HUVECs and human monocyte-derived macrophages and DCs	-	Rigidity	Cell uptake	[91]
Spherical; anionic; PGA (MW 3-15000 and 45000) capsules	PBMCs	-	Rigidity	Cell uptake, APC activation	[92]
Spherical; neutral and anionic; several liposomes containing egg PC, sphingomyelin (SM), DSPC, phosphatidylinositol, phosphatidic acid, DPPG, PS and monosialoganglioside	ICR mouse	i.v., i.p., s.c.	Rigidity	Distribution	[83]
Spherical: neutral; liposomes containing egg PC, egg SM, DSPC and cholesterol	T.O. mouse	i.v. and i.p.	Rigidity	Distribution	[84]

Table 1: Overview of studies referenced in this review. Particle characteristics, animal or cell model, administration route (where appropriate), studied parameters and studied effects are specified for each article.

Particle characteristics	Model	Administration route	Parameter studied	Effect observed	Ref
Spherical; cationic; DDA:TDB (1.98:0.25 molar ratio) and dimethyldioleoylammonium:TDB liposomes	C67BL/6 and BALB/c mouse	i.m.	Rigidity	Distribution, cell uptake, APC activation, immune response	[86]
Spherical; cationic; DDA:TDB:cholesterol (8:1:0, 8:1:2 and 8:1:4 molar ratio) liposomes	C67BL/6 and BALB/c mouse	i.m.	Rigidity	Distribution, cell uptake, immune response	[87]
Spherical; anionic; various PC:cholesterol:DCP:dinitrophenyl-aminocaproyl phosphatidylethanolamine (PE)(2:1.5:0.2:0.1 molar ratio)	AKR mouse	i.p.	Rigidity	Immune response	[93]
Spherical; neutral, anionic or cationic; various PC and PE, DCP and stearylamine liposomes	AKR mouse	i.p.	Rigidity	Immune response	[94]
Spherical; anionic; PC:cholesterol:PG, DPPC:cholesterol:DPPG, DSPC:cholesterol:DSPG liposomes of various molar ratios	Cpb:SE mouse	s.c.	Rigidity	Immune response	[95]
Spherical; anionic; various PC/PE, DPPG/DCP and cholesterol (7:2:1 molar ratio) liposomes	W/Fu rat	s.c.	Rigidity	Immune response	[96]
Spherical; anionic; cholesterol:DCP:DLPC or cholesterol:DCP:DSPC (1:2:7 molar ratio) liposomes	BALB/c mouse	s.c.	Rigidity	Immune response	[97]
Spherical; neutral; various PC:cholesterol (7:2 molar ratio) liposomes	Hamster	i.p.	Rigidity	Immune response	[98]

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7

Liposome-based vaccination against an ApoB100-derived $CD8^+$ T-cell epitope does not affect atherosclerosis development in $LDLr^{-/-}$ mice

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ABSTRACT

Aims Atherosclerosis is a chronic disease of the vessel wall, characterized by the buildup of plaques consisting of lipids and inflammatory cells. We have previously shown that CD8$^+$ T-cells, which make up an important part of the inflammatory component of the lesions, confer a protective effect on atherosclerosis. The objective of this study was to boost the protective function of CD8$^+$ T-cells in atherosclerosis via vaccination against ApoB100, the main protein component of the cholesterol-carrier LDL.

Methods and Results Prediction of ApoB100-derived MHC-I binding peptides revealed several peptides that could induce strong CD8$^+$ T-cell responses *in vivo*. The QSFDLSVK peptide was effective at inducing immune responses in a dendritic cell vaccination study and therefore selected to be encapsulated in liposomes containing the lipids DOPC and DOTAP. LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks to induce atherosclerosis, and vaccinated with the liposomal formulation containing the peptide followed by two boost injections with the peptide adjuvanted with poly(I:C) and an anti-CD40 antibody. Unexpectedly, the treatment affected neither plaque burden nor stability. Plaque macrophage content and serum cholesterol levels were unaffected as well. Ten weeks following vaccination, we were unable to measure any antigen-specific immunity towards our peptide. Since OVA-specific CD8$^+$ T-cell responses were readily detected using the SIINFEKL peptide in the same prime-boost strategy in a subsequent experiment, we conclude that the QSFDLSVK peptide could not be efficiently cross-presented, and thus failed to induce CD8$^+$ T-cell responses. Interestingly, we did observe an increase in weight upon vaccination against the QSFDLSVK peptide.

Conclusion In short, a pro-inflammatory vaccination against the ApoB100-derived peptide QSFDLSVK formulated in DOPC/DOTAP liposomes does not affect atherosclerosis development in LDLr$^{-/-}$ mice, suggesting that using different antigens to boost CD8$^+$ T-cell responses may be more relevant for the treatment of atherosclerosis.

1. Introduction

Cardiovascular diseases are among the leading causes of morbidity and mortality worldwide [1]. Atherosclerosis, the main underlying pathological process that drives cardiovascular disease, is characterized by lipid accumulation in the form of low-density lipoprotein (LDL) as well as chronic inflammation within the vessel wall of medium- and large-sized arteries [2]. Currently, patients are mainly treated with statins, drugs that lower the LDL cholesterol levels in blood. However, statins reduce the risk of a cardiovascular event by only 20-25% [3], emphasizing the need for additional therapeutic strategies. Targeting the immune component of atherosclerosis might be an interesting option to pursue, as a recent large clinical trial has demonstrated that modulating inflammatory responses via blockade of IL-1β improves cardiovascular outcome [4]. Among the inflammatory cell-buildup within the atherosclerotic plaque, $CD8^+$ T-cells are abundant and comprise up to 50% of all lymphocytes in advanced human plaques [5, 6]. $CD8^+$ T-cells have been reported to show both pro- and anti-atherogenic properties, depending on their inflammatory status and interactions with other cell types within the atherosclerotic lesion [7].

We have previously shown a protective role for $CD8^+$ T-cells in the advanced stages of atherosclerotic lesion development, by limiting lesional macrophage content and $CD4^+$ T-cell responses via FasL-induced cell death [8]. Moreover, we reported that activation of $CD8^+$ T-cells specifically in the lesion microenvironment decreases inflammatory cytokine production through upregulation of the ectonucleotidase CD39 [9]. Therefore, increasing the number of $CD8^+$ T-cells in the atherosclerotic lesion, for instance through vaccination, may be a potential strategy to stabilize atherosclerotic plaques. Although the antigens that drive the adaptive immune response in atherosclerosis are still a matter of debate [10], there is a strong body of evidence suggesting a role for LDL- and ApoB100-derived peptides. Several studies have shown that antibodies direct against ApoB100 are generated in both atherosclerotic patients and mouse models [11–14]. Moreover, T-cells from human atherosclerotic lesions can recognize oxidized LDL [15], whereas blocking T-cells from recognizing ApoB100 (the primary protein in LDL) reduces atherosclerosis formation in mice [16].

Previous work has demonstrated an atheroprotective effect of multiple immunizations with the ApoB100-derived p210 peptide conjugated to cationic bovine serum albumin and using Alum as an adjuvant [17], which was mediated by $CD8^+$ T-cells [18]. Interestingly, Alum preferentially primes a type 2 immune response [19] and has not been described as a potent inducer of cytotoxic $CD8^+$ T-cells. Here, we aimed to raise the number of atheroprotective $CD8^+$ T-cells using a cationic liposomal formulation specifically designed to induce protective $CD8^+$ T-cell responses in a murine model of atherosclerosis. Liposomes are a promising antigen-delivery system to use *in vivo*, as they enhance the uptake of the encapsulated antigen in dendritic cells (DCs) [20]. Furthermore, by altering the physicochemical properties of the liposomes, the ensuing immune response can be skewed in the desired direction [21]. For instance, cationic liposomes are known to induce strong $CD8^+$ T-cell responses [22], possibly due to their ability to facilitate endosomal escape [23], resulting in increased presentation of their peptide load on ma-

jor histocompatibility complex class I (MHC-I) molecules. Encouraging results using cationic liposomes for inducing CD8$^+$ T-cells in cancer treatment have been reported previously [24–27], which is why we opted to use DOPC:DOTAP liposomes in our study. We show that vaccination with an ApoB100-derived peptide encapsulated in these liposomes, unexpectedly, does not affect the atherosclerotic burden in LDLr$^{-/-}$ mice.

2. Materials & Methods

2.1. Materials

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Göttingen, Germany). The ApoB100-derived peptides (LSIQNYHVF, VMWLMDSFI, VTYLMALI, STNVYSNL, VPYAFKSL, SAINNEHNI, ANILNSEEL, TNLKYSPL, QSFDLSVK and TTKQSFDL) were synthesized by GenScript (Piscataway, New Jersey, USA). SIINFEKL was purchased from Tebu-bio (Heerhugowaard, the Netherlands) Poly(I:C) was purchased from Invivogen (Toulouse, France) and monoclonal anti-CD40 antibody (rat IgG2a clone FGK4.5) was obtained from BioXcell (Hanover, New Hampshire, USA).

2.2. Liposome preparation

Empty or peptide-containing cationic liposomes (DOTAP:DOPC, 1:1 molar ratio) were produced by making use of the thin film dehydration-rehydration method as previously described [28]. Briefly, DOTAP and DOPC were mixed in a 1:1 molar ratio to reach a final concentration of 10 mg lipids in 1 mL chloroform. A lipid film was formed by rotary evaporation. The lipid film was hydrated in the presence of glass beads with a 2 mL solution of 0.04% NH_4OH for non-loaded (empty) liposomes or with a 2 mL of a solution of 0.04% NH_4OH containing 500 µg QSFDLSVK for the loaded liposomes. The liposomal dispersion was snap-frozen and freeze-dried in a Christ alpha 1–2 freeze-dryer (Osterode, Germany) overnight. After freeze-drying overnight, the lipid cake was rehydrated with 10 mM phosphate buffer, pH 7.4 (PB), in three consecutive steps: twice 250 µL was added followed by vortexing and 30 min equilibration after each addition, and as a third step the remaining 500 µL was added, followed by 1 h equilibration. The liposomes were next sized using high-pressure extrusion at room temperature with a LIPEX Extruder (Northern Lipids Inc., Canada). To obtain monodisperse liposomes, the liposome mixture was passed four times through a 400 nm-pore and a 200 nm-pore sized membrane (Nuclepore, Millipore, Kent, UK). The liposomes were purified and concentrated using Vivaspin 2 centrifugation concentrators (PES membrane, molecular weight cut-off 300 kDa) which were centrifuged at 1500 RPM and 4 °C for 5–6 hours until the suspension was concentrated five-fold. The filtrate containing the free peptide was removed and the concentrated liposomes were washed with 1 mL PB and again centrifuged until the volume was concentrated five-fold.

2.3. Liposome characterization

The Z-average diameter and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS). Zeta-potential was determined by using laser Doppler electrophoresis, both using a NanoZS Zetasizer (Malvern Ltd., Malvern, UK). Samples were diluted 100-fold in PB to a total volume of 1 mL. Peptide encapsulation efficiency was measured using reversed-phase ultra-pressure liquid chromatography (UPLC) on a Waters ACQUITY UPLC (Waters, MA, USA). 5 µL of the sample was injected into a 1.7 µm BEH C18 column (2.1 × 50 mm, Waters). The column temperature was set to 40 °C and the sample temperature set to 4 °C. The mobile phases were Milli-Q water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). For detection, the mobile phases were applied in a linear gradient from 5% to 95% solvent B over 6 min at a flow rate of 0.370 mL min^{-1}. Peptides were detected by absorbance at 214 nm using an ACQUITY UPLC TUV detector (Waters).

2.4. Animals

LDLr$^{-/-}$ mice and C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and bred in-house. Animals were kept under standard laboratory conditions; food and water were provided *ad libitum*. All animal work was performed in compliance with the Directive 2010/63/EU of the European Parliament and the Dutch government guidelines. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

2.5. Bone marrow-derived dendritic cells (BMDCs)

Bone marrow was isolated from the tibias and femurs of C57BL/6 mice. A single-cell suspension of bone marrow cells was obtained by using a 70 µm cell strainer (Greiner Bio-One B.V., Alphen aan den Rijn, NL). The cells were cultured in IMDM (Lonza, Breda, NL) supplemented with 2 mM L-glutamine, 8% (v/v) FCS, 100 U mL^{-1} penicillin/streptomycin (Lonza), and 50 µM β-mercaptoethanol (Sigma, Zwijndrecht, NL) at 37 °C and 5% CO_2 in 95 mm Petri dishes with 20 ng mL^{-1} GM-CSF (Immunotools, Friesoythe, Germany) for 10 days.

2.6. Cell preparation and flow cytometry

Mice were sacrificed by injection of a lethal dose of ketamine (40 mg mL^{-1}), sedazine (8 mg ml^{-1}) and atropine (0.1 mg ml^{-1}) and blood, spleens, heart lymph nodes (hLN), inguinal lymph nodes (iLN), and aortas were harvested after in situ perfusion with phosphate-buffered saline (PBS, pH 7.4, Lonza). White blood cells were obtained by lysing the blood twice for 2 min with lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na2EDTA; pH 7.3). Single-cell suspensions of spleens and LNs were obtained by using a 70 µm cell strainer (Greiner Bio-One). Splenocytes were lysed for 1 min with lysis buffer to obtain white blood cells. For peptide restimulations, approximately

1×10^6 cells were plated out in 96-well U-bottom plates (Greiner Bio-One) in RPMI 1640 Medium (Lonza, Basel, Switzerland) supplemented with fetal bovine serum (10%, Greiner Bio-One), L-glutamine (2%, Lonza), penicillin/streptomycin (1%, Lonza), sodium pyruvate (1%, Sigma-Aldrich) and β-mercaptoethanol (60 µM, Sigma-Aldrich) at 37 °C and 5% CO_2. Cells were incubated for 5 hours in the presence of 3 µg mL^{-1} Brefeldin A (ThermoFisher Scientific, MA, USA) and 10 µm, 5 µM, 1 µM or 10 nM of the ApoB100-derived peptides. Cells were stained with the appropriate antibodies for flow cytometric analysis. For intracellular staining, cells were fixed and permeabilized by using an intracellular staining kit (BD bioscience) according to the manufacturer's protocol. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S and FlowJo software (Treestar).

2.7. Dendritic cell vaccination studies

BMDCs were cultured as described above and stimulated overnight with 100 ng mL^{-1} of LPS and 50 µM of the ApoB100-derived peptides LSIQNYHVF, VMWLMDSFI, VTYLMALI, STNVYSNL, VPYAFKSL, SAINNEHNI, ANILNSEEL, TNLKYSPL, QSFDLSVK and TTKQSFDL. The next day, 18 LDLr$^{-/-}$ mice were injected i.v. with 1E6 DCs loaded with a combination of the different peptides (n = 12), LPS-treated DCs (n = 3) or PBS (n = 3) or the ovalbumin-derived SIINFEKL peptide as controls. After one week, the immune response was boosted by intraperitoneal (i.p.) injection with 200 µg of the peptides, 50 µg anti-CD40 antibody, and 50 µg poly(I:C) in PBS. One week after the boost injections, mice were sacrificed by cervical dislocation and spleens were removed for flow cytometric analysis.

2.8. Atherosclerosis studies

LDLr$^{-/-}$ mice of between 10 and 13 weeks of age were randomized into 4 groups of 10 mice each, based on age, weight, and plasma cholesterol levels. At the start of the experiment, the mice were immunized intravenously (i.v.) with either 100 µL PBS, 3.5 nanomole of free QSFDLSVK peptide in PBS, 0.9 mg DOPC:DOTAP liposomes, or 3.5 nanomole QSFDLSVK peptide encapsulated in 0.9 mg DOPC:DOTAP liposomes. From the moment of immunization, mice were fed a Western-type diet (WTD) containing 15% cocoa butter, 10% maize starch, 20% casein, 40.5% sucrose, 0.25% cholesterol and 5.95% cellulose (Special Diet Services, Witham, Essex, UK) for 10 weeks to induce atherosclerotic lesion formation. One week and four weeks after the start of the experiment, the PBS and empty liposome-treated mice received a boost injection with 50 µg poly(I:C) and 50 µg anti-CD40 antibody, while the free peptide and peptide liposome-treated mice were boosted with 200 µg QSFDLSVK peptide, 50 µg poly(I:C) and 50 µg anti-CD40 antibody in 200 µL PBS via i.p. injection. In weeks 3 and 6, 100 µL blood was obtained via venipuncture of the tail vein for the determination of plasma cholesterol levels. After 10 weeks, mice were sacrificed and organs were harvested as described above. Total cholesterol levels were assessed using an enzymatic colorimetric assay (Roche Diagnostics, Almere, The Netherlands).

2.9. Liposomal vaccination studies

$LDLr^{-/-}$ mice that were 10 weeks of age were divided into two groups of three mice each. At the start of the experiment, the mice were immunized i.v. with either 3.5 nanomole QSFDLSVK peptide or SIINFEKL peptide encapsulated in DOPC:DOTAP liposomes. One week after the initial vaccination, the mice received a boost injection with 50 µg poly(I:C), 50 µg anti-CD40 antibody, and 200 µg QSFDLSVK or SIINFEKL via i.p. injection. One week after the boost vaccination, mice were sacrificed via cervical dislocation and spleens were harvested.

2.10. Histological analysis

All hearts were embedded in optimal cutting temperature (O.C.T.) compound (Sakura, Alphen aan den Rijn, The Netherlands) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, consecutive 10 µm sections were collected. Analysis of lesion size was performed on aortic root lesions stained with Oil-Red O and hematoxylin (Sigma-Aldrich). Corresponding sections were stained with Sirius Red (Sigma-Aldrich) to determine collagen content. Plaque macrophages were stained immunohistochemically by using a rat-anti-mouse monocytes/macrophages antibody (MOMA, 1:1000, AbD Serotec) as a primary antibody, biotinylated rabbit anti-rat IgG (1:100; Vector) as a secondary antibody, and Vectastain ABC horseradish peroxidase in combination with ImmPACT Nova Red for visualization (Vector). The average plaque size (in μm^2) was calculated from 5 sequential sections. For all other stainings, three subsequent sections displaying the highest plaque content per mouse were analyzed. All microscopic analyses were performed on a Leica DM-RE microscope using Leica QWin software and were blinded for independent analysis. The percentages of collagen and macrophages in the atherosclerotic lesions were determined by dividing the area in μm^2 stained positive for collagen or MOMA-2 by the total lesion surface area and calculated as a percentage.

2.11. Statistical analysis

Data are presented as individual values or as mean ± SEM, the number of animals in each group is stated in the text. Data were tested for normal distribution and analyzed using a one-way ANOVA or two-way ANOVA, as appropriate. Statistical analysis was performed using Prism (GraphPad). Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. Identification of peptide candidates to induce ApoB100-specific CD8$^+$ T-cells

We set out to investigate which peptides derived from the ApoB100 protein would be likely bind strongly to the murine MHC-I molecules and thus show potential for CD8$^+$ T-cell activation, using the Immune Epitope Database Analysis Resource (IEDB). We restricted our predictions to the H2-K^b and H2-D^b alloantigens, as these are expressed in mice on a C57Bl/6 background. The IEDB T Cell Epitope Prediction Tool [29] uses a combination of the artificial neuronal network (ANN) [30], stabilized matrix method (SSM) and Scoring Matrices derived from Combinatorial Peptide Libraries (Comblib_Sidney2008) [31] to predict which peptides in a given sequence can bind strongly to MHC-I molecules. Peptide length was set to octamers for H2-K^b and to nanomers for H2-D^b to ensure optimal binding [32]. We selected 8 peptide sequences for synthesis that showed high predicted binding scores in which we included both H2-K^b- and H2-D^b-binding peptides throughout all regions of the ApoB100 protein (Table 1). Furthermore, the P210-derived peptides QSFDLSVK and TTKQSFDL, which were described to be recognized by CD8$^+$ T-cells in atherosclerotic mice [18], were synthesized as well (Table 1).

To evaluate whether these peptides are able to induce antigen-specific CD8$^+$ T-cell responses in an atherosclerotic mouse model, we performed a DC vaccination study. Bone-marrow-derived DCs were activated with LPS and pulsed with the peptides and subsequently injected into LDLr$^{-/-}$ mice. One week later, the mice were boosted with 200 µg of each of the peptides, 50 µg anti-CD40 antibody and 50 µg poly(I:C). One week after the boost injections, splenocytes were obtained and restimulated with the peptides in the presence of Brefeldin A. We observed significant dose-dependent increases in IFN-γ production by CD8$^+$ T-cells from vaccinated mice stimulated with the peptides QSFDLSVK, TNLKYSPL, VTYLMALI, LSIQNYHVF, and STNVYSNL compared to the effect of these peptides on CD8$^+$ T-cells derived from mice treated with LPS-stimulated DCs (Fig. 1). Based on these results, we identified TNLKYSPL and QSFDLSVK as the best peptide candidates for further studies, as they are able to induce the highest antigen-specific CD8$^+$ T-cell responses in our mouse model. When considering the physicochemical properties of the peptides, QSFDLSVK was the best candidate for encapsulation in liposomes. We were unable to dissolve TNLKYSPL in a range of solvents, including water, methanol, chloroform and acetonitrile, which hampered the possibility to further study this peptide in a liposomal vaccination setting. QSFDLSVK, however, is water-soluble and therefore easier to work with.

3.2. Preparation of liposomes

Cationic DOPC:DOTAP liposomes, empty or loaded with the QSFDLSVK (Abbreviated to QSF) peptide, were prepared using the film dehydration-rehydration method. The resulting empty and loaded liposomes had a very similar size (146 vs. 147 nm, respec-

Table 1: ApoB100-derived peptides selected from the epitope binding prediction.

Allele	Sequence		Length	Peptide	Method	Percentile rank
	Start	End				
H-2-D^b	3070	3078	9	SAINNEHNI	ann/smm/ comblib_ sidney2008	0.1
H-2-K^b	1631	1638	8	TNLKYSPL	ann/smm	0.15
H-2-K^b	384	391	8	VTYLMALI	ann/smm	0.15
H-2-K^b	1332	1339	8	STNVYSNL	ann/smm	0.15
H-2-K^b	4254	4261	8	VPYAFKSL	ann/smm/ comblib_ sidney2008	0.2
H-2-D^b	571	579	9	ANILNSEEL	ann/smm/ comblib_ sidney2008	0.3
H-2-D^b	4436	4444	9	LSIQNYHVF	ann/smm/ comblib_ sidney2008	0.3
H-2-D^b	4103	4111	9	VMWLMDSFI	ann/smm/ comblib_ sidney2008	0.6
H-2-K^b	3131	3138	8	TTKQSFDL	ann/smm	11.5
H-2-K^b	3134	3141	8	QSFDLSVK	ann/smm	19.5

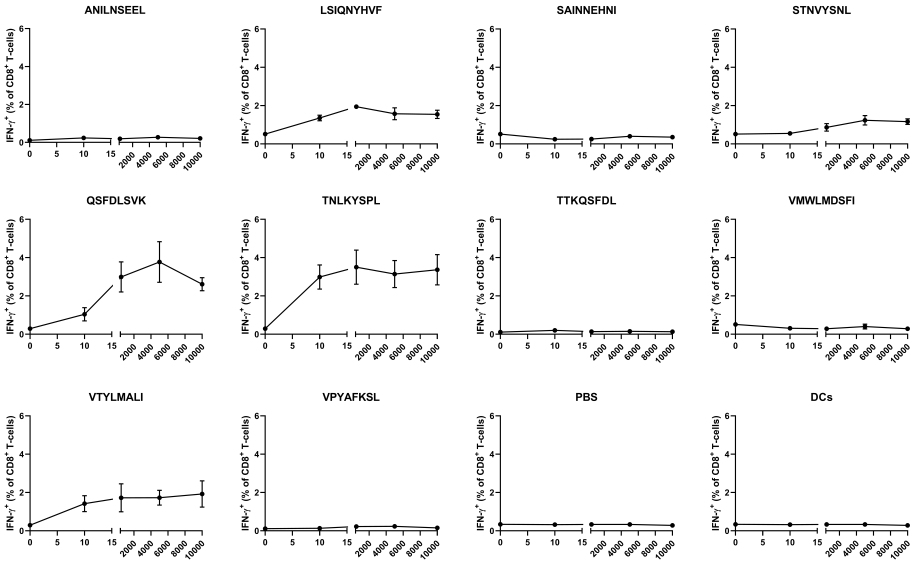

Figure 1: Dendritic cell vaccination with ApoB100-derived peptides in LDLr$^{-/-}$ mice identifies two peptide candidates that induce cytokine production in CD8$^+$ T-cells upon restimulation. Splenocytes were isolated from the vaccinated mice and 1×10^6 cells were stimulated with the indicated concentrations of peptides in the presence of Brefeldin A for 5 hours. Subsequently, cells were stained and analyzed by flow cytometry. Percentages of CD8$^+$ T-cells that are positive for IFN-γ are plotted for each tested peptide. Two-way ANOVA shows a significant difference between the DC control group and QSF***, TNL***, VTY***, LSI***, and STN*. Mean ± SEM, n = 3 per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3: Physicochemical properties of liposomal formulations. Data are averages ± SD of four independent batches. Z_{ave} is the Z-average particle diameter, PDI is the polydispersity index.

	Z_{ave} (nm)	PDI	Zeta potential (mV)	QSF loading efficiency (%)
Empty	147 ± 6	0.15 ± 0.01	30.7 ± 0.7	-
QSF-loaded	146 ± 6	0.16 ± 0.01	31.2 ± 3.6	13.2 ± 5.1

tively), PDI (0.15 vs. 0.16, respectively) and zeta potential (30.7 vs. 31.2 mV, respectively). The peptide loading efficiency was on average 13% (Table 3).

3.3. Vaccination using DOPC:DOTAP liposomes with encapsulated ApoB100-derived peptides does not affect atherosclerosis development

To investigate the effect of our liposomal formulation containing the QSF peptide on atherosclerosis development, $LDLr^{-/-}$ mice were fed a WTD and immunized i.v. with either 100 µL PBS, 3.5 nanomole of free QSF peptide in 100 µLl PBS, 0.9 mg DOPC:DOTAP liposomes in 100 µL PBS, or 3.5 nanomole QSFDLSVK peptide encapsulated in 0.9 mg DOPC:DOTAP liposomes in 100 µL PBS. The mice were boosted twice with 50 µg anti-CD40 antibody, 50 µg poly(I:C) and 200 µg of the peptide. Ten weeks after the start of the treatment, the mice were sacrificed and immunological responses as well as atherosclerotic lesion formation were assessed. The atherosclerotic lesion size was assessed in the aortic root lesions using Oil-Red-O staining. No significant differences were observed in lesion size between the different treatment arms (Fig. 2A). Moreover, analysis of collagen content in the lesions, a measure for the stability of the plaques, revealed no significant differences (Fig. 2B). Finally, we stained the aortic root lesions for MOMA2 using immunohistochemistry, which visualizes the monocyte and macrophage content in the plaques, and observed no differences between the groups (Fig. 2C). As expected, all mice gained weight during the experiment due to their consumption of a WTD. However, the QSF liposome treated mice showed a significantly stronger gain in weight compared to the control-treated mice, resulting in an average weight of 34 vs. 31 grams at the end of the experiment, respectively (Fig. 3A). These differences were not associated with increases in cholesterol levels, as these were found not to differ in the plasma throughout the experiment (Fig. 3B) or in the serum at the end of the experiment (Fig. 3C).

As we did not observe changes in plaque size or composition as a result of vaccination, we next evaluated the immunological effects of our vaccination. We only observed limited, non-significant increased percentages of $CD8^+$ T-cells in blood (64% vs 61%, p = 0.42), iLN (47% vs 45%. p = 0.55), hLN (46% vs. 41%, p = 0.38) and spleens (38% vs. 36% p = 0.54) in the QSF liposome-treated group compared to PBS controls (Fig. 4A).

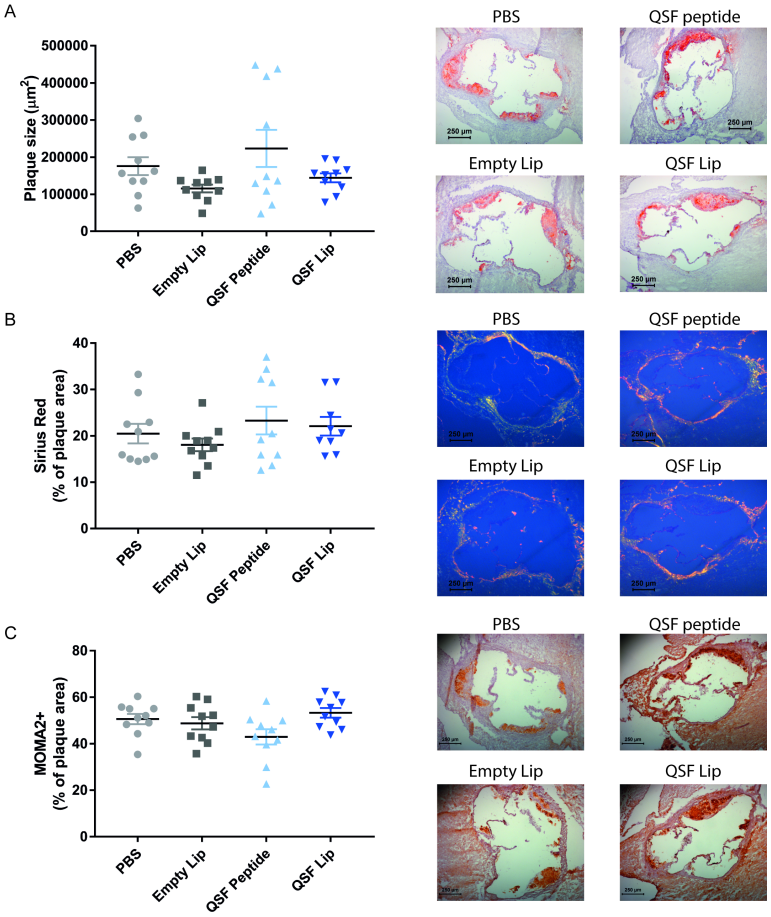

Figure 2: No differences in plaque size or composition in the aortic root lesions of LDLr$^{-/-}$ mice on a WTD vaccinated with PBS, empty liposomes, QSF peptide or QSF liposomes. (A) Quantification of lesion size in the aortic root by Oil-Red-O staining and representative pictures from each group. (B) Quantification of collagen content by Sirius Red staining in the aortic roots of LDLr$^{-/-}$ mice and representative pictures from each group. (C) Macrophage quantification in the aortic roots of the LDLr$^{-/-}$ mice by MOMA-2 staining and representative images of MOMA-2 staining. Data points represent individual values, lines denote mean ± SEM, n = 10 per group, significance was determined by one-way ANOVA and Bonferroni's multiple comparisons tests.

We analyzed the expression of CD62L and CD44 on these CD8$^+$ T-cells, to determine if these cells were naïve (CD62L$^+$CD44$^-$), effector memory (TEM, CD62L$^-$CD44$^+$), or central memory (TCM, CD62L$^+$CD44$^+$) cells. We observed no significant differences, but in the blood there appeared to be a small decrease in the percentage of TCM cells (40% vs. 48%, p = 0.29) and a small increase in the percentage of TEM cells (35% vs. 29% p = 0.48) in the QSF liposome treated group compared to PBS controls (Fig. 4B). Similarly, no differences were observed in the expression of the proliferation-associated transcription factor Ki-67 in these CD8$^+$ T-cells. Analysis of the transcription factors

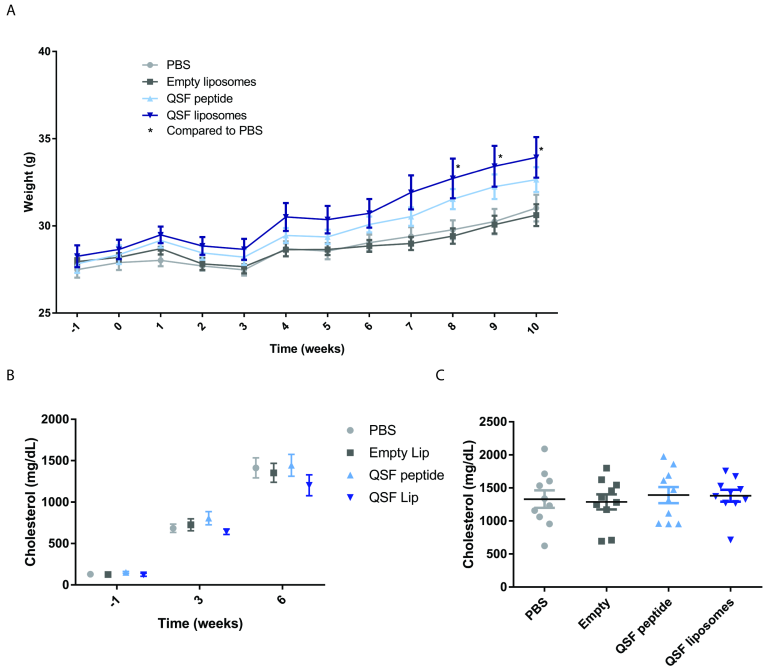

Figure 3: QSF liposome vaccinated mice show a significant increase in weight at the end of the experiment, but no differences in cholesterol levels. (A) Body weights of the LDLr$^{-/-}$ mice over time during the vaccination experiment, WTD feeding and vaccination were initiated at time point 0. (B) Plasma cholesterol levels in the LDLr$^{-/-}$ mice at baseline, week 3 and week 6. (C) Serum cholesterol levels in the LDLr$^{-/-}$ mice at the time of sacrifice. Mean ± SEM, n = 10 per group, significance was determined by either a one-way or two-way ANOVA and Bonferroni's multiple comparisons tests, as appropriate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

associated with different CD8^{+} T-cell subsets (T-bet, GATA3, RORγt, and FoxP3) were also not found to differ between the groups (data not shown). Apart from the effects of the treatment on the CD8^{+} T-cells, we also analyzed other immune cell subsets. We observed no differences in the number of inflammatory (Ly6Chi) and patrolling (Ly6Clo) monocytes in the blood (Fig. 4C).

We previously reported that CD8^{+} T-cells may affect the number of macrophages and CD4^{+} T-cells in the lesion, whereas such differences were not observed systemically. However, when analyzing the aortic lesions in this study, we observed no difference in the presence of CD11b^{+} APCs (Fig. 4D), nor in the expression levels of T-bet and GATA3 (data not shown), or in the ratio between Th1 and Th2 cells within the CD4^{+} T-cell compartment (Fig. 4E).

Although we found no significant difference in the bulk CD8^{+} T-cell population, vaccination might have induced changes in the number of antigen-specific CD8^{+} T-cells. This was analyzed by stimulating splenocytes with the QSF peptide, after which cytokine production was measured using flow cytometry. However, we observed no differences in IFN-γ production (Fig. 4F), TNF-α production, nor IL-10 production (data

not shown) between the different groups or between the stimulated samples and non-peptide treated controls.

3.4. DOPC:DOTAP liposomes are able to induce antigen-specific CD8$^+$ T-cell responses towards the SIINFEKL peptide, but not the QSFDLSVK peptide in LDLr$^{-/-}$ mice

As we observed no differences in both atherosclerotic lesion formation and immunological responses upon vaccination with the QSF peptide, we set out to determine whether or not there was any immune response induced using our vaccination strategy. We vaccinated LDLr$^{-/-}$ - mice with 3.5 nanomole of the QSF peptide in the liposomes described above or with 3.5 nanomole of the model antigen SIINFEKL, derived from ovalbumin (abbreviated as OVA peptide), in the same liposomes as a positive control. The injected liposomes were similar in size (146 vs. 143 nm, respectively), PDI (0.14 vs. 0.14, respectively) and zeta-potential (29.4 vs. 30.2 mV, respectively). The peptide loading efficiencies were 7.8% for QSF and 9.6% for the OVA peptide (Table 5). One week after the boost vaccination with 50 µg anti-CD40 antibody, 50 µg poly(I:C) and 200 µg of the respective peptides, the mice were sacrificed and the splenocytes were restimulated with a range of different concentrations of the peptides. Interestingly, we observed antigen-specific CD8$^+$ T-cell responses towards the OVA peptide in the OVA-vaccinated group upon restimulation. Flow cytometry revealed an increase from 0.2% to 10.9% in IFN-γ^+CD8$^+$ T-cells (Fig. 5A) and from 0.1% to 1.5% in TNF-α^+CD8$^+$ T-cells (Fig. 5B) upon restimulation with the lowest concentration (10 nM) of the peptide. However, no significant increases in either IFN-γ or TNF-α production were observed upon restimulation with the QSF peptide in the QSF-vaccinated group (Fig. 5A, B). So, whereas the liposomal formulation containing OVA peptide is effective in inducing CD8$^+$ T-cell-mediated antigen-specific responses in LDLr$^{-/-}$ mice, the QSF peptide in the same liposomes lacks immunogenicity in our model.

Table 5: Physicochemical properties of liposomal formulations containing the QSF peptide or OVA peptide. Data are averages ± SD of n = 3 measurements. Z_{ave} is the Z-average particle diameter, PDI is the polydispersity index.

	Z_{ave} (nm)	**PDI**	**Zeta potential** (mV)	**QSF loading efficiency (%)**
OVA-loaded	143.3 ± 4.6	0.137 ± 0.02	30.2 ± 1.2	9.6
QSF-loaded	145.9 ± 7.7	0.138 ± 0.003	29.4 ± 0.15	7.8

4. Discussion

Previous studies have identified T-cell responses and autoantibodies against (oxidized forms of) LDL and its main protein constituent, ApoB100, in both humans and animal

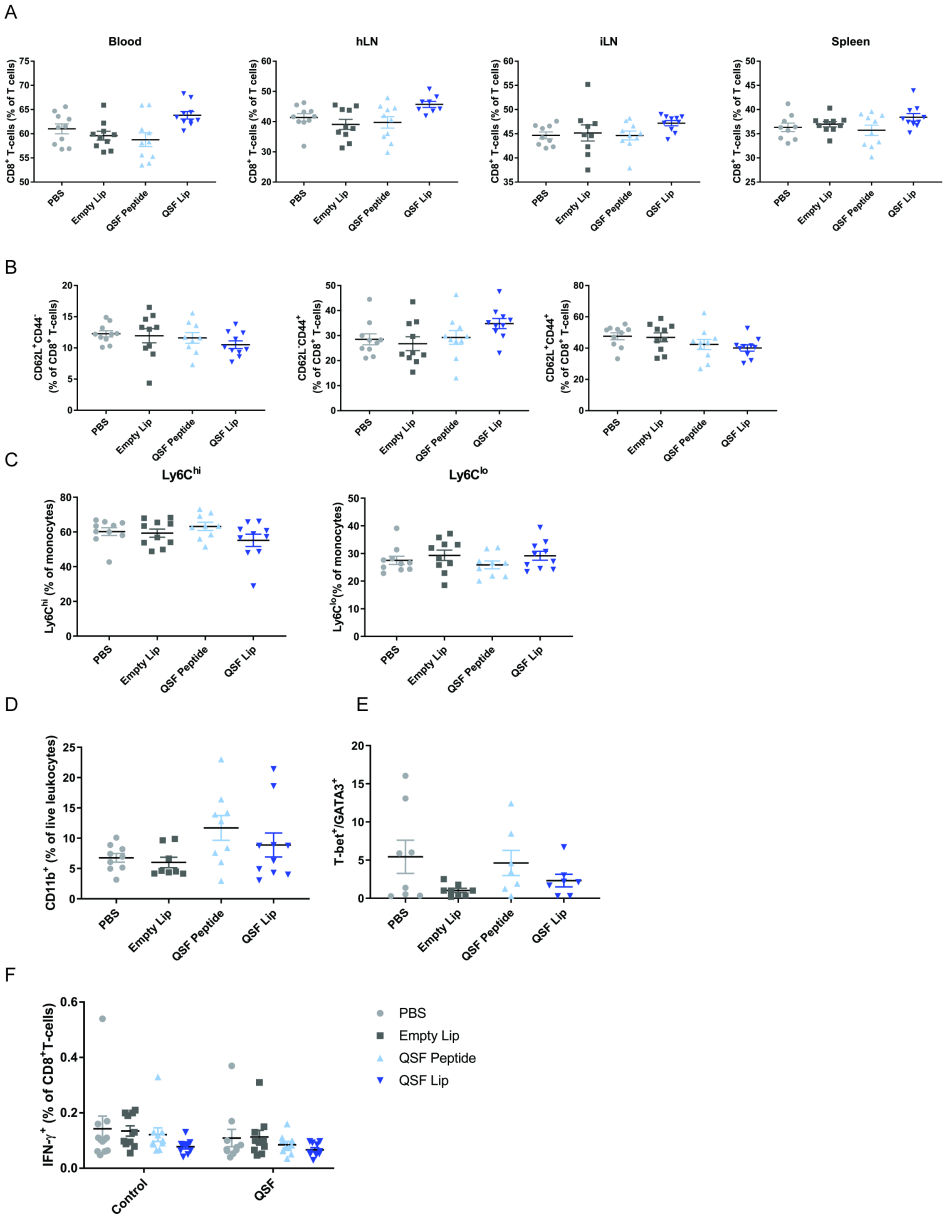

Figure 4: Flow cytometric analysis of CD8$^+$ T-cell responses in LDLr$^{-/-}$ mice on a WTD vaccinated with PBS, empty liposomes, QSF peptide or QSF liposomes. (A) Percentage of CD8$^+$ T-cells in the blood, hLN, iLN, and spleens of the LDLr$^{-/-}$ mice at the time of sacrifice. (B) Flow cytometric analysis of percentages of naïve CD44$^-$CD62L$^+$, TEM CD62L$^-$CD44$^+$, and TCM CD62L$^+$CD44$^+$ CD8$^+$ T-cells in the blood of the LDLr$^{-/-}$ mice at the time of sacrifice. (C) Percentages of Ly6C^{hi} and Ly6C^{lo} monocytes in the blood at the time of sacrifice. Monocytes were gated as viable, NK1.1$^-$Ly6G$^-$CD11b$^+$ cells. (D-E) Flow cytometry analysis of the percentage of CD11b$^+$ cells in the aorta (D) and the ratio between the percentages of aortic CD4$^+$ T-cells expressing T-bet and GATA3 at sacrifice (E). (F) CD8$^+$IFN-γ^+ T-cells in the splenocytes derived from the LDLr$^{-/-}$ mice at sacrifice, stimulated for 5 hours in the presence or absence of 10 µm QSFDLSVK peptide and Brefeldin A. Data points represent individual values, lines denote mean ± SEM, n = 10 per group, significance was determined by one-way or two-way ANOVA and Bonferroni's multiple comparisons tests, as appropriate. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

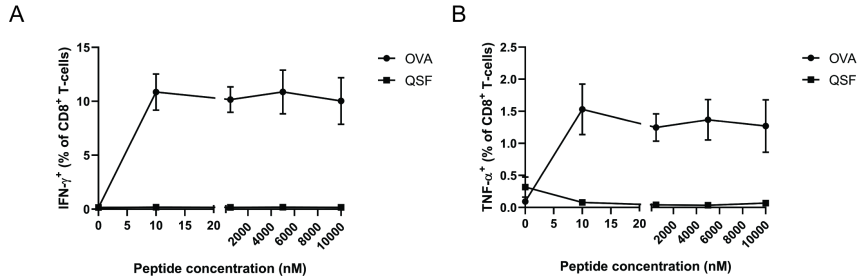

Figure 5: Liposomal vaccination against the QSFDLSVK peptide does not induce antigen-specific CD8$^+$ T-cell responses in LDLr$^{-/-}$ mice. Splenocytes were isolated from the mice that were vaccinated with either QSF or OVA peptide and 1×10^6 cells were restimulated with the indicated concentrations of the corresponding peptide in the presence of Brefeldin A for 5 hours. Subsequently, cells were stained and analyzed by flow cytometry. Percentages of CD8$^+$ T-cells that are positive for IFN-γ (A) and TNF-α (B) are plotted for both groups of mice. Mean ± SEM, n = 3 per group.

models of atherosclerosis [11–13, 15]. As protective roles for CD8$^+$ T-cells in atherosclerosis have been demonstrated previously [8, 17], we aimed to specifically boost antigen-specific CD8$^+$ T-cells, to induce an atheroprotective immune response that will result in cytolytic activity towards plaque-resident cells that present parts of this protein on their MHC-I molecules. However, we were unable to detect any antigen-specific CD8$^+$ T-cell responses using our liposomal vaccination strategy targeted towards the ApoB100-derived QSFDLSVK peptide. Of note, this peptide could be recognized by CD8$^+$ T-cells using a different vaccination strategy. As no differences were observed in immune responses in the peptide-liposome treated groups compared to the controls, it is not surprising that no differences were observed in atherosclerotic lesion size, plaque stability or plaque macrophage content at the end of the experiment.

There are several possible explanations as to why our vaccination was unsuccessful. Firstly, the choice of the antigen is obviously crucial for the efficacy of a vaccine. From our peptide predictions and DC vaccination study, we identified QSFDLSVK as a likely peptide candidate against which CD8$^+$ T-cells can mount a response. This is in agreement with previous publications, as vaccination with the ApoB100-derived peptide p210, which contains the QSF sequence, was previously shown to induce CD8$^+$ T-cell responses and reduce atherosclerosis development in apoE$^{-/-}$ mice [17]. Indeed, immunization of apoE$^{-/-}$ mice with p210 increased the number of CD8$^+$ T-cells that recognize the peptide sequence QSFDLSVK, which exerted increased cytolytic activity towards APCs [18]. Moreover, vaccination of mice against the ApoB100-derived peptides P2 and P45 induced CD8$^+$FoxP3$^+$ regulatory CD8$^+$ T-cells, which protect against atherosclerosis development via increased IL-10 production [33], confirming that CD8$^+$ T-cells are able to mount an immune response against ApoB100-derived peptides. We have successfully encapsulated the QSF peptide in liposomal particles consisting of DOPC and DOTAP, which were approximately 150 nm in size. Positively charged carriers of this size have been shown to be preferentially taken up by DCs and to elicit a significant CD8$^+$ T-cell response [25, 34–36]. In our LDLr$^{-/-}$ model, we observed no antigen-specific immunity towards the QSF peptide at the end of the

atherosclerosis experiment. However, we did observe antigen-specific $CD8^+$ T-cell responses in the DC vaccination experiment. This shows that there are $CD8^+$ T-cells that are able to react towards the QSF peptide in our mouse model. This discrepancy in immunological responses between the DC vaccination study and the liposomal vaccination study suggests that the failing of our vaccination strategy is likely not caused by the choice of a non-functional peptide epitope.

Secondly, the efficacy of a vaccine depends on the choice of the antigen delivery system. We observed that our liposomal delivery system is capable of inducing $CD8^+$ T-cell responses towards the model peptide SIINFEKL after two weeks, but vaccination with the QSF peptide in these liposomes did not result in any detectable antigen-specific $CD8^+$ T-cell responses even one-week after the boost injection. Thus, this indicates a problem with the liposomal delivery of the QSF peptide specifically, rather than a general incapability of peptide-loaded DOPC:DOTAP liposomes to induce a $CD8^+$ T-cell response.

In this study, we used a peptide consisting of 8 amino acids, the minimal length required to induce a $CD8^+$ T-cell response. For a DC vaccination strategy, the use of minimal peptides is favorable, as they can be loaded directly onto the MHC-I molecules without the need for processing. However, minimal peptides induce lower immune responses *in vivo* compared to longer peptides [37]. In this study, we opted for the shorter peptide in combination with an anti-CD40 antibody to bypass $CD4^+$ T-cell help [38]. However, this may have negatively affected the processing of the peptide and the ensuing immune responses. Indeed, previous work has shown that synthetic long peptides (SLPs), peptides which include flanking residues around the epitope of interest, offer superiority over the use of minimal peptides containing only the epitope recognized by the $CD8^+$ T-cell receptor [39, 40]. It has been suggested that a minimal peptide in the absence of a continuous danger signal, may induce T-cell tolerance towards this antigen [41]. However, as we delivered the peptide in a liposomal formulation, a danger signal should have been present, as the liposomes themselves act as immunostimulators [42]. APCs loaded with minimal peptides are less efficient in sustaining antigen presentation over longer periods of time compared to SLPs [43], which may have contributed to the negative results observed here. Alternatively, there could have been a problem in one of the steps of the cross-presentation process, by which the antigen gains access to the cytosol. Possibly, the acidity of the endosomes in which the liposomes are taken up may affect the stability of the QSF peptide more than that of the OVA peptide. Alternatively, the QSF peptide may have been more susceptible to degradation by enzymes present within the endosomes. For instance, cathepsins are known to preferentially cleave after small hydrophobic or basic amino acid residues [44], and could, therefore, cleave after the L and V residues. Cathepsin S may be able to cleave after the D and S residues [45]. Moreover, cathepsin B would be able to cleave off the terminal lysine residue by its exopeptidase activity [46]. Thus, the QSF peptide may have been degraded in the endosomes, preventing presentation upon MHC-I molecules. An alternative explanation could lie in the affinity of the peptides for binding to MHC-I. According to the IEDB database, SIINFEKL binds to MHC-I with an IC50 in the nanomolar range, whereas this is in the micromolar range for the QSF peptide. Thus, perhaps there is limited endosomal escape by both peptides, but as SIINFEKL binds stronger to the

MHC-I molecules, this peptide is still able to induce immunity, whereas the QSF peptide cannot. In the DC vaccination study, the peptide was freshly dissolved and loaded onto the DCs on the day of injection, thus endosomal processing played no role in this study.

Alternatively, the peptide stability may already have been negatively affected before the administration *in vivo*. The liposomal formulation process can take up to eight days between the dissolution of the peptide and the injection of the liposomes, during which the peptide may have become less stable. After the liposomal formulation process, the QSF peptide was successfully detected using UPLC, albeit at a low encapsulation efficiency. From this, it can be concluded that at least some of the QSF peptide is within the liposomes in the original form, although it cannot be excluded that some of the original peptide had undergone chemical modifications that were not detected by the UPLC method.

Interestingly, we observed a significant increase in weight in the QSF liposome treated mice compared to the PBS controls. The induction of immunity towards ApoB100 could be expected to reduce plasma cholesterol levels [47] and therefore reduce the weight of the mice. However, we found no differences in plasma cholesterol levels between the different treatment groups and as we did not observe any immunological effects demonstrating the effectiveness of our vaccine, it is unlikely that there would be effects on ApoB100 levels. Blast analysis revealed that the QSFDLSVK sequence is aligned for 87% to sequences encoding LPS binding protein (LBP) and dual oxidase maturation factor 2 (DUOXA2). LBP is expressed mainly in the liver and aids in the binding of bacterial lipopolysaccharide (LPS) to the CD14 receptor [48]. Increased levels of LBP and the resulting increased inflammatory responses have been linked to increased weight loss in cancer patients [49]. Therefore, cytotoxic activities against LBP-expressing cells could be a factor that affects weight gain. However, other studies report a role for LBP in adipogenesis and weight gain [50, 51]. Finally, a mutation in the DUOXA2 gene, expressed mainly in endocrine tissue and the liver, results in hypothyroidism [52], which is known to affect body weight. Thus, the differences in weight observed in this work could possibly be explained by off-target immunity of our vaccination directed towards the cells that present epitopes of the aforementioned proteins via MHC-I that share sequence homology with the peptide against which we vaccinated. Alternatively, our therapy may have exerted off-target pharmacological effects on other systems that we did not investigate, which could have caused the observed weight differences.

5. Conclusion

Collectively, our data show that vaccination against the ApoB100-derived peptide QSFDLSVK formulated in DOPC:DOTAP liposomes did not result in the induction of CD8$^+$ T-cell responses and did not affect atherosclerosis development in LDLr$^{-/-}$ mice. However, DC vaccination using the same peptide did induce antigen-specific CD8$^+$ T-cells, and vaccination with the same liposomes encapsulating SIINFEKL could induce OVA-specific CD8$^+$ T-cells. We suggest that our choice of peptide in combination with

this liposomal formulation is responsible for the lack of immune responses, probably due to problems occurring during one of the steps of the cross-presentation process. Further exploration into activating $CD8^+$ T-cells to induce a strong cytolytic response towards antigen-presenting cells in atherosclerosis using a different peptide antigen in the form of an SLP may be of great value for atherosclerosis research.

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8

General Discussion and Perspectives

1. Immunomodulatory treatments for atherosclerosis

Cardiovascular diseases (CVD) remain a major concern for global health, as they contribute to 31% of all deaths worldwide [1]. The main underlying pathogenic process that drives CVD is atherosclerosis, a chronic inflammatory process that results in the buildup of cholesterol-rich plaques within the arteries [2]. As lipids are such an important driving force behind the disease process, current therapeutic interventions focus on the lowering of the plasma cholesterol levels via the use of statins [3] or by inhibiting protein convertase subtilisin/kexin type 9 (PCSK9) [4]. However, treatment with these lipid-lowering drugs is not effective in all patients [5], and even in patients that achieve low cholesterol levels, cardiovascular events still occur [6]. This illustrates that there is a need for innovative treatments that improve cardiovascular outcomes. Recently, there has been increased interest in targeting the immune component of atherosclerosis. The REMOVAL (REducing with MetfOrmin Vascular Adverse Lesions) trial assessed the potential beneficial effect of treatment with metformin, an anti-diabetic drug, on cardiovascular outcomes in patients with type 1 diabetes [7]. Metformin not only can help restore blood glucose levels via activation of AMP-activated protein kinase, but also inhibits nuclear factor κB-mediated inflammatory signaling [8]. Treatment with metformin reduced the maximal common carotid artery intima-media thickness [7], a measure for atherosclerotic plaque size, suggesting that targeting the immune system may be of use in cardiovascular risk management. Indeed, observational studies have shown that in psoriatic arthritis patients, treatment with monoclonal antibodies that block the function of the inflammatory cytokine TNF-α reduces the development of carotid atherosclerotic plaques [9]. There is also a reduced incidence of cardiovascular events in rheumatoid arthritis patients treated with TNF-α-blocking medication [10]. However, blocking the function of TNF-α in cardiovascular patients using either etanercept in the RENEWAL (Randomized Etanercept Worldwide Evaluation) trial [11], or infliximab in the ATTACH (anti-TNF Therapy Against Congestive Heart Failure) trial [12], did not improve and even adversely affected heart failure. This could indicate that specifically targeted treatments as compared with generalized immune suppression may be needed to increase the effectiveness of anti-inflammatory therapies for the treatment of CVD. Alternatively, this could suggest that anti-inflammatory treatments are only beneficial in a certain subset of patients. In agreement with this, the outcome of the CIRT trial (Cardiovascular Inflammation Reduction Trial) suggests that the effectiveness of anti-inflammatory treatment differs based on the inflammation levels of each patient [13], which therefore may be an important inclusion criterion to consider in similar studies. Besides the blocking of TNF-α, there is the currently ongoing ASSAIL-MI trial (ASSessing the Effect of Anti-IL-6 Treatment in Myocardial Infarction), which investigates the effect of blocking the inflammatory cytokine IL-6 as a potential treatment strategy for atherosclerosis. In addition, the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) trial studied the effect of treating patients who previously experienced a myocardial infarction with a monoclonal antibody that blocks the function of the inflammatory cytokine IL-1β, on top of state-of-the art lipid treatment. This resulted in a significantly lower rate of recurrent cardiovascular events compared to placebo treatment, although treatment was also associated with a higher incidence of fatal infection, due to generalized suppression of the immune system [14].

This study shows that there is potential in inhibiting the immune system in atherosclerosis, but emphasizes the need for specifically targeted treatments in order to prevent adverse effects related to immune suppression.

$CD8^+$ T-cells can exert their effector functions in an antigen-specific manner, as they release their cytotoxic molecules upon stimulation of their T-cell receptor (TCR) by binding to an MHC-I molecule that presents their cognate antigen. Because of the highly specific nature of this interaction, targeting these cells is of particular interest for modulating the immune system in atherosclerosis, while preventing non-specific immune inhibition. As $CD4^+$ T-cells have already been studied extensively in the context of atherosclerosis [15–17], some progress towards utilizing or targeting $CD4^+$ T-cells for the treatment of atherosclerosis has been made [18–20], demonstrating proof-of-concept for the use of T-cell therapy in this disease. However, research into the role of $CD8^+$ T-cells in atherosclerosis has lagged behind, and much less is known about their contribution to the pathogenesis of atherosclerosis and particularly how these cells function within the lesion microenvironment. Therefore, it is unclear if they can be targeted for the treatment of atherosclerosis.

In this thesis, we aimed to (1) determine the phenotype and function of $CD8^+$ T-cells in atherosclerotic lesions and (2) assess whether therapeutic targeting of $CD8^+$ T-cells can provide a means of modulating the inflammatory responses in atherosclerosis.

2. $CD8^+$ T-cells exert a protective function in advanced atherosclerotic lesions

Experimental studies have provided conflicting evidence regarding the role of $CD8^+$ T-cells in atherosclerosis development. As reviewed in **Chapter 2**, the presence of $CD8^+$ T-cells in atherosclerotic lesions has been established in both beginning and advanced human atherosclerotic plaques [21, 22]. Early studies using genetic knockout mouse models provide contrasting data regarding the role of $CD8^+$ T-cells in the pathogenesis of this disease, suggesting either a limited function [23, 24] or an atheroprotective one [25]. More recent studies have suggested both pro- and anti-atherogenic functions for $CD8^+$ T-cells, depending on what subset is studied and what stage of the lesions is evaluated. For instance, pro-inflammatory cytokine-producing $CD8^+$ T-cells aggravate lesion development in the initial stages of atherosclerosis development due to increased monopoiesis [26]. This effect is most likely mediated via the inflammatory functions of TNF-α and granzyme B, as the adoptive transfer of IFN-γ-deficient $CD8^+$ T-cells does not affect atherosclerosis development compared to transfer of wild-type $CD8^+$ T-cells, suggesting that the role of $CD8^+$ T-cell derived IFN-γ in the pathogenesis of atherosclerosis is limited. Regarding atheroprotective roles for $CD8^+$ T-cell subsets, immunization of $apoE^{-/-}$ mice with an LDL-derived peptide was shown to induce protective $CD8^+$ T-cell responses, by inducing cytolytic activity towards antigen-presenting cells (APCs) [27, 28]. Adoptive transfer of $CD8^+CD25^+$ regulatory T-cells into $apoE^{-/-}$ mice resulted in reduced lesion development, via inhibition of pro-inflammatory $CD4^+$ and $CD8^+$ T-cell responses [29]. Moreover, a subset of Qa1-restricted regulatory $CD8^+$

T-cells has been described to reduce atherosclerotic lesion development in apoE$^{-/-}$ mice via inhibition of T follicular helper cell function, reducing the formation of germinal centers and immunoglobulin production [30].

We also discussed the published work regarding CD8$^+$ T-cell function in human atherosclerosis in this chapter. Increased CD8$^+$ T-cell numbers in the blood are associated with the incidence of coronary events [31]. Later work has shown that this appears to hold true for the number of activated CD8$^+$ T-cells in particular [32, 33]. In contrast, regulatory CD8$^+$ T-cells were shown to exert anti-atherogenic functions in human patients [34], again stressing the need to differentiate between distinct CD8$^+$ T-cell subsets in order to understand their role in atherogenesis.

Finally, we suggest in this review chapter that targeting the right subset of CD8$^+$ T-cells, for instance via vaccination, might be of therapeutic value for the treatment of atherosclerosis. Indeed, boosting CD8$^+$ T-cell responses can induce increased lysis of APCs and reduce atherosclerotic plaque formation [24, 26]. Moreover, boosting the function of the regulatory CD8$^+$ T-cell responses may prove to be an effective strategy, as these cells have been shown to limit atherosclerosis development [26]. However, several challenges remain to be met before CD8$^+$ T-cell vaccination can be successfully employed in atherosclerosis. First of all, antigens have to be identified against which the CD8$^+$ T-cell response can be directed. LDL-derived proteins are potential candidate antigens, as previous studies have shown the induction of T-cell responses towards ApoB100 epitopes [27, 28, 35]. Secondly, it is important to identify the optimal adjuvant, as these immune-boosting compounds can affect atherosclerosis development [36]. Finally, it is of the utmost importance to avoid inducing the pro-atherogenic CD8$^+$ T-cell subsets discussed above.

Of the papers reviewed in **Chapter 2**, most of the experimental studies have focused mainly on initial lesion development, whereas from a clinical point of view, it is more relevant to study advanced and/or unstable lesions. Patients usually experience symptoms related to severe stenosis (such as chest pain and shortness of breath) when lesions are advanced, and seek medical advice at this stage of the disease. Thus, from a drug development perspective, it is most valuable to understand the function of CD8$^+$ T-cells in the progressed stages of atherosclerosis, as this is when pharmacological intervention is possible.

In **Chapter 3** we set out to investigate how CD8$^+$ T-cells affect the stability and composition of advanced atherosclerotic lesions. First, we investigated whether there was any significant correlation between the percentage of CD8$^+$ T-cells present in human atherosclerotic lesions and other immune cells that are known to be involved in atherogenesis. Interestingly, we observed a significant negative correlation between CD8$^+$ T-cell and macrophage percentages in human endarterectomy samples. This relationship could be indicative of a regulatory effect of CD8$^+$ T-cells on macrophages. In order to study this further, we depleted CD8$^+$ T-cells in the advanced stages of lesion development in LDLr$^{-/-}$ mice. The mice were fed a Western-type diet (WTD) for 10 weeks in order for lesions to establish and were kept on the WTD for another 6 weeks, during which they received injections of a monoclonal antibody to deplete CD8$^+$ T-cells, or an

isotype control antibody. We found that this treatment did not result in any differences in lesion size between the two groups. However, the absence of $CD8^+$ T-cells did result in an increase in macrophage content in the lesions, which is in agreement with the association we found between these two cell types in the human lesions. Moreover, depletion of $CD8^+$ T-cells resulted in a reduced lesion stability, as the collagen content of the lesions was decreased, whereas the necrotic areas were increased. When investigating the T-cell compartment within the lesions, we observed a striking increase in the percentage of inflammatory $CD4^+$ T helper 1 (Th1) cells associated with a decrease in percentage of Th2 cells upon CD8 depletion. Of note, this increase was only observed locally within the lesions and not at other sites in the body. Together, these results indicate that $CD8^+$ T-cells exert a protective effect in advanced atherosclerosis by reducing macrophage and Th1 cell content (Fig. 1). This is in agreement with previously published studies, demonstrating that antigen-specific $CD8^+$ cells can reduce atherosclerosis by mounting a cytolytic response against APCs [37].

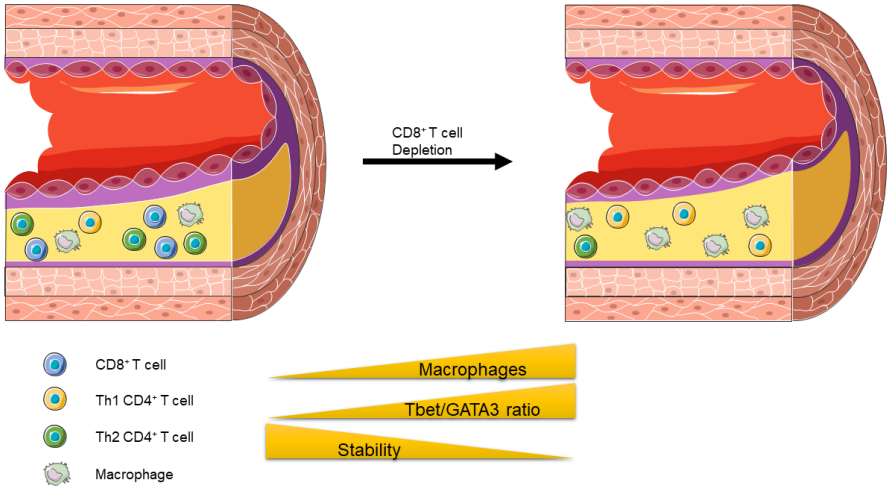

Figure 1: Schematic overview of the effects of $CD8^+$ T-cell depletion on the advanced stages of atherosclerosis. $CD8^+$ T-cell depletion increases plaque macrophage and Th1 cell content and reduces plaque stability. *From van Duijn et al. Cardiovasc Res. 2019 Mar 15;115(4):729-738.*

Indeed, we suggest a role for Fas-Fas ligand (FasL)-mediated cell death in establishing these atheroprotective effects. We found an increased expression of FasL on $CD8^+$ T-cells in the lesion microenvironment, which is known to induce apoptosis of activated $CD4^+$ T-cells [38], and Th1 cells specifically are known to have a high susceptibility to FasL-induced cell death [39, 40]. Moreover, lipid-loaded macrophages are vulnerable to Fas-FasL-mediated cell death as well [41]. Neutralizing the function of FasL in vivo using a monoclonal antibody resulted in a similar skewing of $CD4^+$ T-cell subsets in favor of Th1 cells, as well as an increased lesional macrophage content. Thus, the protective and lesion-stabilizing effect of $CD8^+$ T-cells in advanced atherosclerosis is at least in part mediated via FasL-induced apoptosis of Th1 cells and macrophages. This

protective effect of $CD8^+$ T-cells may be exploited therapeutically, by boosting $CD8^+$ T-cell function in the advanced stages of atherosclerosis, which should result in lesion stabilization.

3. $CD8^+$ T-cells modulate the immune environment locally within the lesion via several distinct mechanisms

In **Chapter 3** we observed that the function of $CD8^+$ T-cells within the lesion microenvironment differs from that in the secondary lymphoid organs. As the atherosclerotic microenvironment contains many lipid-derived and inflammatory stimuli, this may affect the $CD8^+$ T-cell phenotype specifically at this site. In **Chapter 4** we set out to investigate how the lesion microenvironment affects $CD8^+$ T-cell function. We demonstrated that $CD8^+$ T-cells derived from aortic lesions of $apoE^{-/-}$ mice show a dysfunctional phenotype, characterized by impaired cytokine production, when compared to their counterparts in the spleen. This phenotype was associated with an increased expression of the ectonucleotidase CD39. This enzyme is involved in the conversion of extracellular ATP, a danger-associated molecular pattern that has been linked to atherosclerosis development [42], into the immunomodulatory compound adenosine [43]. Adenosine produced by $CD8^+CD39^+$ T-cells has previously been shown to reduce cytokine production by other T-cells [44]. Indeed, we showed that pharmacological inhibition of CD39 is able to partly reverse the dysfunctional $CD8^+$ T-cell phenotype that was observed in the lesions of the $apoE^{-/-}$ mice. Mechanistically, we showed that TCR signaling induces expression of CD39 in the lesions. Transplantation of OT.1 bone marrow (which gives rise to $CD8^+$ T-cells unable to recognize any atherosclerosis-specific antigen) into $LDLr^{-/-}$ recipient mice resulted in a marked reduction in CD39-expression on lesion-derived $CD8^+$ T-cells compared to transfer of wild-type bone marrow. These results indicate that it is the antigen-specific stimulation of $CD8^+$ T-cells in the lesion environment that drives the unique phenotype observed at this location (Fig. 2). In agreement with this, recent work by others has shown that TCR activation induces reactive oxygen species that activate signaling cascades resulting in increased CD39 expression [44], and that TCR signaling specifically drives CD39 expression on $CD8^+$ T-cells in the tumor microenvironment as well [45, 46]. Importantly, we confirmed that in human atherosclerosis there is also a strong microenvironment-specific upregulation of CD39 on $CD8^+$ T-cells in the plaques compared to matched blood samples. Thus, the results we observed in our mouse models may be translated to a clinical setting, and boosting $CD39^+CD8^+$ T-cell function could be an interesting approach for the treatment of atherosclerosis.

In **Chapter 5**, we revisited lesion-localized $CD8^+$ T-cell responses, focusing on determining which $CD8^+$ T-cell subsets are present within the plaque microenvironment. Upon activation of $CD8^+$ T-cells, cytokines released by APCs can drive the differentiation of the $CD8^+$ T-cells into various subsets, which are characterized by distinct cytokine release profiles [47]. We observed that in the lesion microenvironment, there is a reduction in the content of IFN-γ-producing Tc1 cells, but a large increase in the percentage of Tc17 cells. To investigate how these cells affect atherosclerosis develop-

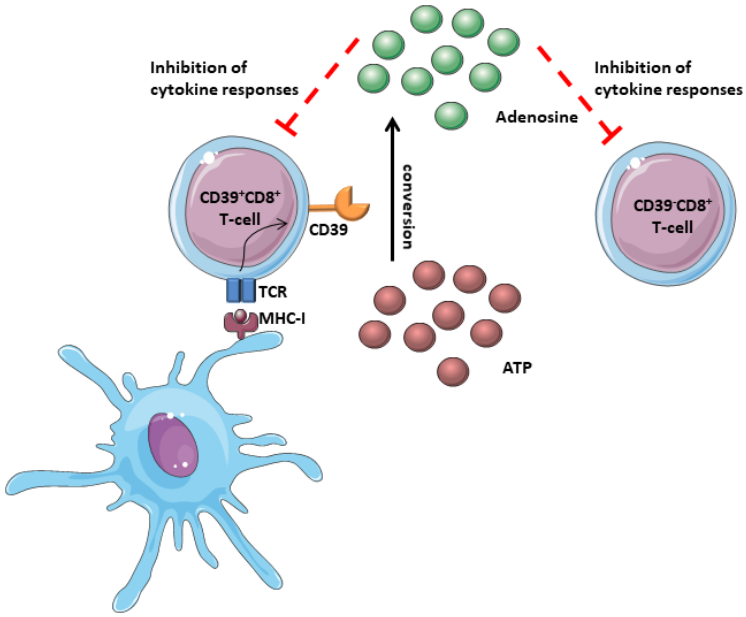

Figure 2: Schematic overview of the lesion-localized induction of CD39 on CD8$^+$ T-cells, and how this in turn affects the inflammatory status. TCR signaling induces CD39 expression on CD8$^+$ T-cells. The CD39$^+$CD8$^+$ T-cells convert pro-inflammatory ATP into anti-inflammatory adenosine, which in turn is able to reduce cytokine production by different CD8$^+$ T-cell subsets. *From van Duijn et al. Atherosclerosis. 2019 Jun;285:71-78*

ment, we performed an adoptive transfer of Tc17 cells or undifferentiated Tc0 cells into CD8-deficient LDLr$^{-/-}$ mice. Whereas Tc0 cells differentiated into a Tc1 phenotype upon injection, as evident from their increased IFN-γ production and T-bet expression, Tc17 cells demonstrated lower levels of IFN-γ production compared to the Tc0 cells and retained their ability to produce IL-17A. This shows that both of these subsets show plasticity in vivo in response to the inflammatory stimuli present in atherosclerotic mice [37, 48]. Adoptive transfer of Tc17 cells resulted in smaller atherosclerotic lesion size compared to the transfer of Tc0 cells. Moreover, there was a decrease in plaque macrophage content as well as a reduction in inflammatory CD4$^+$ Th1 cells in the aortas of the Tc17-treated mice (Fig. 3). As the Tc17 cells produced more IL-17 but fewer IFN-γ compared to the Tc0 cells, the atheroprotective function of Tc17 cells can be ascribed to either or both of these phenotypical differences. Nonetheless, these findings show that different Tc subsets are present in atherosclerotic lesions, and suggest that skewing the CD8$^+$ T-cell phenotype towards a less atherogenic subset, such as the Tc17 subset, may provide a novel therapeutic avenue for the treatment of atherosclerosis.

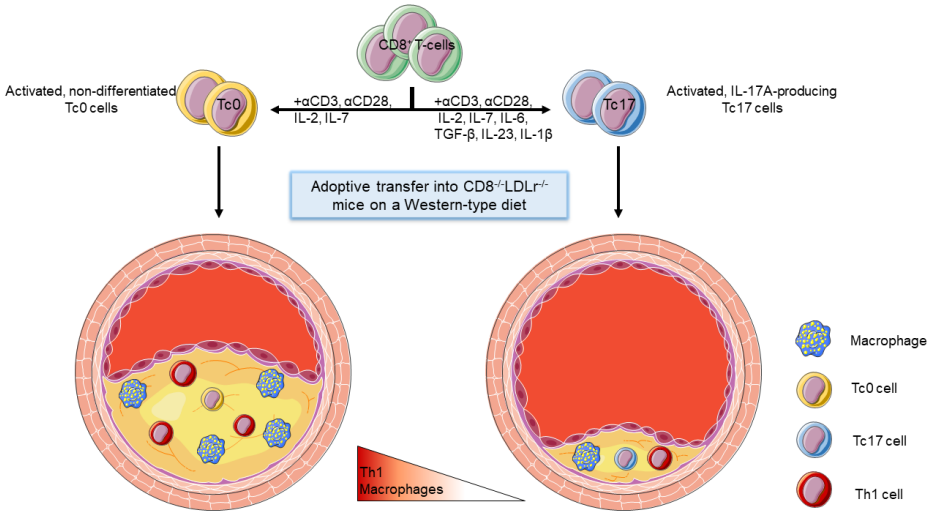

Figure 3: Schematic overview of how the adoptive transfer of Tc0 and Tc17 cells affects atherosclerosis development in CD8$^{-/-}$LDLr$^{-/-}$ mice. CD8^{+} T-cells were polarized towards a Tc0 or Tc17 phenotype and injected once weekly into CD8$^{-/-}$LDLr$^{-/-}$ mice fed a WTD for 6 weeks. Transfer of Tc17 cells resulted in smaller lesions associated with a reduced Th1 cell and macrophage content. *From van Duijn et al. Tc17 CD8^{+} T-cells accumulate in murine atherosclerotic lesions and modulate local inflammatory responses (submitted manuscript).*

4. CD8^{+} T-cell directed vaccination in atherosclerosis

As we determined that CD8^{+} T-cells can exert anti-atherogenic functions under certain conditions, we looked into ways in which we could harness the atheroprotective functions of CD8^{+} T-cells, while inhibiting their pro-inflammatory functions, as a potential treatment strategy for atherosclerosis. An interesting strategy to induce antigen-specific CD8^{+} T-cell responses is via vaccination. In **Chapter 6**, we reviewed how to design a particle-based vaccine in order to skew the immune response towards the desired direction. As pathogens have different dimensions and shapes, they stimulate the immune system in a specific way, inducing different immunological effects. Similarly, in this review, we focused on how the physicochemical characteristics of size, shape, and rigidity of the particulate antigen delivery system affects the biodistribution, cellular uptake, antigen presentation and the resulting immune response in murine models. Regarding the induction of CD8^{+} T-cell responses, we reviewed studies reporting that small particles in the nano-size range are easily transported from the site of injection to the lymphatic system [49]. Moreover, we discussed that small nanoparticles are taken up efficiently by APCs and localize rapidly into late endosomes [50], which subsequently fuse with the lysosomes from where the nanoparticles escape via the phagosome-to-cytosol pathway, resulting in higher MHC class I presentation [51]. Furthermore, we reviewed studies revealing that smaller sized nanoparticles are more effective than larger particles in inducing CD8^{+} T-cell responses, and this effect is most pronounced when these small particles are (nearly) spherical, probably due to

enhanced uptake of spherical particles [52, 53].

Using the knowledge we obtained regarding particulate vaccine design, we set out to induce atheroprotective $CD8^+$ T-cell responses via liposomal vaccination in **Chapter 7**. Liposomes are spherical vesicles composed of one or multiple bilayers of phospholipids which enclose an aquatic core. Liposomes are an efficient delivery system for antigens, as they enhance antigen uptake by APCs and initiate immune activation within these cells. Positively charged (cationic) liposomes below 200 nm in size were previously shown to induce strong antigen-specific $CD8^+$ T-cell responses in a cancer mouse model [54]. However, we were unable to induce antigen-specific $CD8^+$ T-cell responses in $LDLr^{-/-}$ mice by vaccinating against the ApoB100-derived peptide QSFDLSVK using cationic DOPC:DOTAP liposomes. Consequently, no effects of this treatment on atherosclerosis development were observed. This failure to induce immunogenicity was not due to the formulation, as we showed that vaccination using the exact same liposomes encapsulating the model peptide SIINFEKL was able to induce antigen-specific $CD8^+$ T-cell responses in $LDLr^{-/-}$ mice. Moreover, using dendritic cell vaccination in which the QSFDLSVK peptide was loaded directly onto the DCs, we were able to induce antigen-specific $CD8^+$ T-cell responses. Thus, immune responses can be directed against this peptide sequence in $LDLr^{-/-}$ mice. Most likely, there were issues with the stability of the peptide during the cross-presentation process in vivo, by which the antigen comes into contact with the acidic environment and proteases in the lysosomes before it gains access to the cytosol. As we have shown compelling data in this thesis supporting a protective role for $CD8^+$ T-cells via antigen-specific mechanisms, we believe further exploration into activating these cells could be of great value for atherosclerosis research.

5. Perspectives

In recent years, great progress has been made with regard to the research into immunotherapy of atherosclerosis. The aforementioned CANTOS trial [14] has already shown that targeting the inflammatory component of atherosclerosis can reduce the number of cardiovascular events, although adverse effects occur due to the general immune suppression. As $CD8^+$ T-cells are a part of the adaptive immune response, and therefore function in an antigen-specific manner, they could prove to be interesting tools for targeting the immune system in atherosclerosis in a more specific manner, reducing the occurrence of adverse effects such as the ones observed in the CANTOS trial. In this thesis, the contribution of $CD8^+$ T-cells to the pathogenesis of atherosclerosis has been studied, and several mechanisms through which $CD8^+$ T-cells exert protective functions have been identified. We hope these findings will prove to be useful starting points for developing therapeutic interventions in atherosclerosis.

In **Chapter 3**, we have shown a protective function for $CD8^+$ T-cells that was at least in part mediated via the increased expression of FasL on these cells. Activation-induced cell death, such as that induced by Fas/FasL signaling, is one of the main mechanisms used by the immune system to prevent autoimmunity, through controlling T-cell re-

sponses [55]. In mouse models, immunotherapies using genetic modulation of FasL were shown to reduce autoimmune diseases [56, 57], providing proof-of-concept for targeting the Fas/FasL-axis in atherosclerosis. Unfortunately, the administration of agonistic Fas antibodies or recombinant FasL results in severe hepatotoxicity and even death of animal models [58], eliminating the use of this strategy for the treatment of atherosclerosis. Alternatively, bispecific antibodies consisting of an agonistic Fas antibody hybridized to a second antibody directed against a different target antigen on the same cell were shown to be effective in inducing apoptosis of the target cells, while reducing non-specific adverse effects [59]. Potentially, this strategy could be employed to eliminate the atherogenic Th1 cells from the lesions. Alternatively, FasL-expressing $CD8^+$ T-cells could be employed as a therapy. Adoptive transfer of T-cells retrovirally transduced to overexpress FasL resulted in effective killing of prostate cancer cells in mice [60], suggesting it could be interesting to transduce patient-derived $CD8^+$ T-cells in a similar fashion ex vivo and subsequently transfer them back. It must be noted that none of the methods discussed above target the immune system in an antigen-specific manner, and thus a risk of generalized immune suppression remains a concern with these methods. In order to avoid this, one could consider the use of chimeric antigen receptor (CAR) T-cell therapy. These cells are constructed from T-cells isolated from the patient, which are genetically modified to express a CAR construct, which allows a cell to bind a certain target cell surface antigen via a single-chain variable fragment domain. This domain is linked to intracellular signaling molecules that are usually associated with T-cell receptor signaling, which induce T-cell activation upon binding of the antigen. Thus, a CAR T-cell can recognize antigens irrespective of MHC-I presentation but instead needs to bind a structure expressed on the target cell membrane. Upon recognition of this structure, CAR T-cells mediate their cytotoxic effect via the release of the cytotoxic perforin and granzymes, the release of cytokines, and via the Fas-FasL axis [61]. Thus, employing these T-cells in atherosclerosis would enable FasL-mediated killing of target cells in an antigen-specific fashion. For instance, once could design CAR T-cells that target scavenger receptor A-I, which is overexpressed on plaque macrophages, in order to kill these pro-atherogenic cells [62].

In **Chapter 4**, we describe that lesion-localized $CD8^+$ T-cells express high levels of CD39, which results in local modulation of inflammatory responses. As we and others have shown that TCR signaling is required to induce CD39 expression [44], it could be of interest to increase the number of CD39-expressing immunomodulatory $CD8^+$ T-cells via TCR signaling. Vaccination would provide an excellent method to upregulate CD39 expression, as this specifically activates $CD8^+$ T-cells in an antigen-specific manner. In **Chapter 7** we set out to test this hypothesis in an atherosclerotic mouse model. However, our liposomal vaccination method did not induce any antigen-specific $CD8^+$ T-cell responses toward the ApoB100-derived peptide. Therefore, we will have to be improve our strategy, using a more effective combination of peptide antigen and delivery system that is able to induce strong immune responses. It could be of interest to look into what antigens are naturally presented upon MHC-I molecules within the atherosclerotic lesions in murine models and human patients by the use of mass spectrometry [63]. This will provide an overview of what peptide fragments of which proteins are presented to $CD8^+$ T-cells in an atherosclerotic context and may thus lead

to the identification of a relevant antigen to boost atheroprotective $CD8^+$ T-cell functions in both preclinical and clinical studies. Once a suitable antigen is identified, it is important to assess if vaccination can indeed boost CD39- and FasL-expression levels on $CD8^+$ T-cells, reduce inflammatory $CD4^+$ T-cell responses, and induce cytolytic responses towards APCs. If this is found to be the case, there remains a need for studies exploring the potential of vaccination to treat atherosclerosis in humans, as most studies on vaccination in this disease have been performed in murine models [64]. Humans express different variants of the MHC-I molecules than mice, termed human leukocyte antigens (HLA), which have different binding affinities compared to their murine counterparts. Transgenic mouse lines that express HLA class I molecules have been developed [65], which may prove useful for investigating the translational value of a vaccination strategy. Finally, as we observed the increase in CD39 only in the lesional microenvironment, the effect of inducing CD39-expressing cells systemically will have to be studied. Possibly, most of the antigen-specific cells home towards the atherosclerotic sites, as this is where their cognate antigen is expressed, but one must be cautious of potential adverse effects.

As shown in **Chapter 5**, the skewing of $CD8^+$ T-cells towards different Tc subsets can affect the development of atherosclerotic lesions in a murine model. We report a protective effect of IL-17A-producing Tc17 cells compared to IFN-γ-producing Tc1 cells. Of note, treatment with a blocking antibody against IL-17A in psoriatic arthritis patients resulted in the occurrence of six major adverse cardiovascular events in the treated group, whereas no such events occurred in placebo-controlled patients [66]. Similarly, trials testing anti-IL-17A antibodies in ankylosing spondylitis resulted in three major adverse cardiovascular events in the treated group versus none in the controls [67]. Although these outcomes were not statistically significant, they could indicate that blocking the function of IL-17 may prove detrimental for the prevention of cardiovascular events, agreeing with the protective role we described for Tc17 cells. Conversely, administration of IL-17A may be beneficial against atherosclerosis development, although systemic administration may result in unwanted adverse effects. Therefore, adoptive transfer of IL-17A-producing $CD8^+$ T-cells, specifically targeted to the atherosclerotic lesions, may be preferable. Specific accumulation in the lesion may be enhanced by using $CD8^+$ T-cells that recognize antigens presented in the plaque. For instance, in the field of cancer research, adoptive transfer of ex vivo activated tumor-reactive $CD8^+$ T-cells skewed towards a Tc1 or Tc17 phenotype reduced melanoma growth [68]. Furthermore, adoptive transfer of antigen-specific OT.1 $CD8^+$ T-cells skewed towards the Tc17 phenotype controlled tumor-growth in early and late-stage melanoma in OVA tumor-bearing mice [69]. These experimental studies demonstrate the potential of combining adoptive T-cell transfer therapy with the skewing of those T-cells towards the desired phenotype ex vivo. Interestingly, ex vivo stimulation of cells under Tc17-polarizing conditions with a small-molecule RORγ-agonist was shown to potentiate the anti-tumor activity of both CAR-expressing human T-cells and tumor-specific $CD8^+$ T-cells [70], demonstrating that this approach could work in a clinical setting as well. Therefore, we suggest it is of interest to look into adoptive transfer of $CD8^+$ T-cells skewed towards the Tc17 phenotype ex vivo for the treatment of atherosclerosis, which allows for specific delivery of IL-17 to the atherosclerotic lesions.

Collectively, this research suggests there is potential to target $CD8^+$ T-cells in order to modulate the immune response in atherosclerosis. However, in doing so, one must be very careful to induce only the atheroprotective functions of the $CD8^+$ T-cells and monitor if no adverse effects are induced simultaneously. Moreover, much of the data demonstrating a protective role for $CD8^+$ T-cells has been collected in murine models, although we show that at least some of this can be translated to human atherosclerosis as well. Before an effective $CD8^+$ T-cell-based therapy can be developed, more research is required to confirm the atheroprotective function of this subset in human atherosclerotic lesions.

6. Conclusion

In summary, the research described in this thesis has shed new light on the phenotype and function of $CD8^+$ T-cells in atherosclerosis, demonstrating lesion-localized atheroprotective functions. This implies that antigen-specific stimulation of $CD8^+$ T-cell responses may have therapeutic potential. Great progress is currently being made towards developing antigen-specific T-cell therapies in numerous diseases. Moreover, immune-based therapies in general currently receive increasing attention for the treatment of atherosclerosis. Therefore, it is not a question of *if*, but rather of *when* effective immune-based treatment of cardiovascular diseases will be within our reach.

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9

Nederlandse Samenvatting

1. Inleiding

1.1. Atherosclerose wordt gekenmerkt door de ophoping van vetten en een chronische ontstekingsreactie in de vaatwand

"Hart- en vaatziektenïs een brede term die alle aandoeningen van het hart en het vaatsysteem omvat, zoals hartinfarcten en beroertes. Hart- en vaatziekten vormen een belangrijk probleem voor de samenleving, aangezien 31% van alle sterftegevallen wereldwijd aan deze aandoeningen te wijten valt [1]. De belangrijkste risicofactoren voor het ontwikkelen van hart- en vaatziekten zijn een hoog cholesterolgehalte in het bloed, een hoge bloeddruk, roken, diabetes, obesitas en een weinig actieve levensstijl [2]. Daarnaast verhogen erfelijke aandoeningen zoals familiaire hypercholesterolemie en onderliggende auto-immuunziekten zoals reuma het risico [3].

Het belangrijkste onderliggende ziekteproces dat hart- en vaatziekten veroorzaakt is atherosclerose, ook wel slagaderverkalking genoemd. Atherosclerose is een chronische ontstekingsziekte die vooral de middelgrote en grote arteriën aantast. Het ziekteproces begint al tijdens de kinderjaren en langzaam maar zeker bouwen zich over tientallen jaren vettige plaques op in de vaatwand [4]. Dit proces begint op plaatsen in de bloedvaten waar het endotheel, de binnenste laag cellen van de bloedvaten, disfunctioneel wordt door een verstoring van de bloedstroming. Hierdoor wordt het endotheel meer permeabel, en kunnen de "slechte"lage-dichtheid lipoproteïnen (LDL), die cholesterol in het bloed vervoeren, zich gaan ophopen in de vaatwand [5]. Deze ophoping initieert een ontstekingsreactie, waarbij er moleculen op het endotheel tot expressie komen die immuuncellen rekruteren naar de plek van de vetophoping [6]. Onder de gerekruteerde immuuncellen zijn macrofagen, een celtype dat is gespecialiseerd in het opruimen van dode celresten, en ook LDL-deeltjes kan opnemen. In het geval van atherosclerose raken de macrofagen overladen met LDL-deeltjes, wat resulteert in de vorming van schuimschellen. Deze cellen hebben zoveel vetten opgenomen dat ze er ‘schuimachtig’ uitzien als ze met de microscoop worden bekeken. Schuimcellen kunnen niet meer weggeraken uit de plaques en hierdoor ontstaat een lokale ophoping van vetten en immuuncellen [7]. De schuimcellen produceren ook inflammatoire cytokines, signaalstoffen die het immuunsysteem verder stimuleren [8]. Nadat een bepaalde toxische drempel aan vetopname is overschreden, wordt celdood geïnduceerd via apoptose (een gereguleerd, niet-inflammatoir proces) of via necrose (een verstorend, pro-inflammatoir proces). Dit kan leiden tot het vrijgeven van gevaarsignalen die het ontstekingsproces nog verder versterken [9].

Naast macrofagen worden ook dendritische cellen (DCs) gerekruteerd naar de plaques. Dit celtype is normaliter betrokken bij het opnemen van bacteriën of virussen in het geval van een infectie. Vervolgens worden deze ziekteverwekkers afgebroken en kleine stukjes van de eiwitten die daaruit vrijkomen worden gepresenteerd op zogenaamde ‘major histocompatibility complex’ (MHC) moleculen. Dit proces wordt antigeenpresentatie genoemd, en resulteert in de activatie van het adaptieve immuunsysteem. Het adaptieve immuunsysteem wordt gestimuleerd door de DCs om specifiek de ziekteverwekkers aan te vallen via de herkenning van deze antigenen. In het geval van

atherosclerose worden de DCs geactiveerd door de gevaarsignalen die aanwezig zijn in de plaque en kunnen deze cellen dode celresten of LDL-deeltjes opnemen. Vervolgens migreren de DCs door het lymfesysteem naar de lymfeknopen, waar ze antigenen uit het opgenomen materiaal kunnen presenteren aan de cellen van het adaptieve immuunsysteem, de T- en B-cellen [10].

Bovenop de activatie van het adaptieve immuunsysteem, worden ook de gladde spiercellen in de vaatwand geactiveerd door de ontstekingsreactie. Deze cellen vormen vervolgens een fibreuze laag bovenop de plaque, die zorgt voor stabiliteit [11]. Atherosclerotische plaques kunnen voor lange tijd asymptomatisch blijven, maar uiteindelijk kan de progressieve groei van deze plaques leiden tot complicaties. Doordat de plaques een gedeelte van het bloedvat afsluiten, kan de bloedstroming afnemen, wat kan leiden tot een tekort aan zuurstoftoevoer naar bepaalde weefsels (hypoxie). Verminderde zuurstoftoevoer naar het hart kan leiden tot pijn op de borst (angina pectoris) [12]. Daarnaast kan de fibreuze laag van gladde spiercellen op de plaque dunner worden door de voortgaande ontstekingsreacties, en uiteindelijk scheuren. Dit leidt tot de vorming van een bloedprop, ook wel trombus genoemd, die ofwel lokaal de bloedstroming kan verstoren, ofwel zich met de bloedstroom kan verplaatsen en op andere locaties kan leiden tot problemen. Als een trombus de kransslagaders die het hart van zuurstof voorzien blokkeert, is er sprake van een hartinfarct [13]. Een blokkering van de bloedvaten in de hersenen kan leiden tot een herseninfarct [14].

1.2. Behandeling van atherosclerose

Bij patiënten met acute klinische symptomen zijn chirurgische ingrepen nodig. De vaatwand kan verwijd worden door middel van ballon-angioplastie, waarbij een ballonnetje wordt opgeblazen binnen het verstopte bloedvat, om de bloedstroming te herstellen. Deze ingreep wordt vaak gecombineerd met het plaatsten van een metalen buisje (stent) in het bloedvat, die het teruggroeien van de plaque verhindert [15]. Een alternatief is een bypassoperatie, waarbij een omleiding voor de verstopte slagader wordt gevormd door de transplantatie van een gezonde ader ter hoogte van de verstopte kransslagader van het hart of slagader in het been [16]. Tot slot is endarterectomie ook een optie, waarbij het aangetaste bloedvat wordt geopend en de plaque chirurgisch wordt verwijderd [17].

Naast deze acute ingrepen, ligt de nadruk van de behandeling van atherosclerose vooral op het aanpakken van de risicofactoren, zoals het advies geven over een gezonder dieet en een actievere levensstijl. Daarnaast richten de beschikbare medicijnen zich op het verlagen van het cholesterolgehalte in het bloed. De meest voorgeschreven medicijnen zijn statines, die de aanmaak van cholesterol remmen en de opname van cholesterol uit het bloed verhogen, en op die manier het aantal LDL-deeltjes in het bloed verminderen [18]. Hoewel het behandelen met statines resulteert in een 30% verlaging van het risico op een cardiovasculaire gebeurtenis (zoals een hartaanval) [19], is er een grote behoefte aan nieuwe medicijnen om dit risico verder te verlagen. Zo wordt onder andere onderzoek gedaan naar medicijnen die aangrijpen op het immuunsysteem. Een grote klinische studie (de CANTOS trial) heeft het effect van een antilichaam bestudeerd

dat de functie van het pro-inflammatoire cytokine IL-1β blokkeert. De resultaten laten zien dat remmen van het immuunsysteem, in combinatie met het gebruik van cholesterolverlagende middelen, het risico op hart- en vaatziekten verder kan verlagen [20]. Helaas had de algemene remming van het immuunsysteem in deze studie ook tot bijwerking dat meer patiënten overleden aan opportunistische infecties. Het remmen van het immuunsysteem voor de behandeling van atherosclerose is dus zeker veelbelovend, maar het is van belang om zulke ernstige bijwerkingen te voorkomen door specifiekere therapieën te ontwikkelen die uitsluitend het ziekteproces aanpakken.

1.3. De rol van T-cellen in atherosclerose

T-cellen zijn een interessant celtype om te bestuderen als een mogelijk aangrijpingspunt voor een nieuwe behandeling van atherosclerose, aangezien deze cellen in een doelgerichte en antigeen-specifieke manier hun effecten uitoefenen. Zoals hierboven beschreven, worden T-cellen geactiveerd door DCs, die antigenen presenteren in de lymfe klieren. DCs presenteren deze antigenen op hun celoppervlak, ingekapseld in MHC-moleculen, die binden aan de T-celreceptoren (TCR) op het oppervlak van T-cellen. Dit induceert een activerend signaal. Daarnaast geven de DCs ook costimulatoire signalen door aan de T-cellen en scheiden ze cytokines uit, die de T-cellen specifiek instrueren hoe ze zij zich vervolgens moeten ontwikkelen. Vervolgens ondergaan de T-cellen klonale expansie, wat resulteert in een grote verzameling cellen die hetzelfde antigeen kunnen herkennen. Zowel helper T-cellen, die gekenmerkt worden door expressie van CD4, als cytotoxische T-cellen, die gekenmerkt worden door expressie van CD8, zijn aanwezig in humane atherosclerotische plaques [21]. Er is al veel onderzoek gedaan naar de rol van $CD4^+$ T-cellen in het ziekteproces. Zo is bekend dat de zogenaamde T-helper 1 (Th1)-cellen bijdragen aan het onstekingsproces door de productie van inflammatoire cytokines zoals interferon γ (IFN-γ) en tumor necrose factor α (TNF-α) en daardoor de ontwikkeling van atherosclerose versterken [22, 23]. Er is veel minder bekend over de rol van $CD8^+$ T-cellen in deze ziekte. $CD8^+$ T-cellen zijn ook wel bekend als "killer"T-cellen, omdat ze celdood kunnen induceren in andere, voornamelijk virus-geïnfecteerde cellen. Dit gebeurt door middel van het binden van Fas-ligand (FasL), dat tot expressie komt op de $CD8^+$ T-cellen, aan de Fas-receptor op de doelcellen, wat apoptose initieert. Ook kunnen ze de toxische moleculen perforine en granzyme uitscheiden, die andere cellen kunnen lyseren. Daarnaast produceren $CD8^+$ T-cellen ook pro-inflammatoire cytokines zoals IFN-γ en TNF-α [24].

1.4. Doel van het onderzoek

Het doel van het in dit proefschrift beschreven onderzoek was ten eerste om de functie van $CD8^+$ T-cellen in atherosclerose op te helderen, met name binnen de omgeving van de plaque. Ten tweede wilden wij deze kennis gebruiken om te testen of een therapie die zich richt op $CD8^+$ T-cellen gebruikt kan worden om de inflammatoire respons in atherosclerose te remmen.

2. $CD8^+$ T-cellen zijn beschermend in de geavanceerde stadia van atherosclerose

In **Hoofdstuk 2** presenteren wij een overzicht van wat er tot nu toe bekend is over $CD8^+$ T-cellen in atherosclerose. $CD8^+$ T-cellen zijn aanwezig in zowel beginnende als meer gevorderde plaques in patiënten met atherosclerose [21, 25]. Er zijn echter nog veel onduidelijkheden over de functie van deze cellen in het ziekteproces. Recente experimentele studies in muizen hebben laten zien dat cytokine-producerende $CD8^+$ T-cellen de vorming van beginnende atherosclerotische plaques stimuleren door het rekruteren van monocyten, die zich ontwikkelen tot inflammatoire macrofagen in de lesies [26]. Ander onderzoek laat juist zien dat het induceren van $CD8^+$ T-cellen door middel van vaccinatie met een peptide afkomstig van het eiwitgedeelte van LDL een beschermende functie heeft op de ontwikkeling van atherosclerose. Dit beschermende effect wordt gemedieerd door de cytotoxische functies van $CD8^+$ T-cellen op antigeen-presenterende cellen [27, 28]. Daarnaast laten verschillende studies het gunstige effect zien van regulatoire $CD8^+$ T-cellen, die de immuunrespons onderdrukken en daardoor atherosclerose remmen [29, 30]. In patiënten met hart- en vaatziekten zijn hogere aantallen van (geactiveerde) $CD8^+$ T-cellen in het bloed geassocieerd met een toename in cardiovasculaire complicaties [31–33]. Echter, ook in mensen geldt dat regulatoire $CD8^+$ T-cellen een anti-atherogene functie uitoefenen [34]. Samengevat laten deze studies zien dat de rol van $CD8^+$ T-cellen afhankelijk is van zowel de subset van deze cellen als het stadium van atherosclerose.

Het meeste onderzoek dat tot nu toe verricht is naar de rol van $CD8^+$ T-cellen in atherosclerose, richt zich op de initiële stadia van de ontwikkeling van plaques. Vanuit een klinisch oogpunt is het echter relevanter om de meer geavanceerde stadia van atherosclerose te bestuderen, aangezien patiënten dan pas symptomen beginnen te ervaren, zoals kortademigheid en pijn op de borst, en zich melden bij een dokter. Therapeutische interventie wordt daarom vaak ook pas vanaf dit stadium mogelijk, en dientengevolge is het interessant om dit stadium te bestuderen vanuit een oogpunt van geneesmiddelontwikkeling. Daarom hebben wij in **Hoofdstuk 3** bekeken of er enige correlatie bestaat tussen $CD8^+$ T-cellen en de aanwezigheid van andere celtypen in humane plaques. Dit hebben we bestudeerd in materiaal dat verwijderd wordt na endarterectomie-operaties, via een samenwerking met het Westeinde ziekenhuis in Den Haag. We vonden dat als er percentueel meer $CD8^+$ T-cellen aanwezig zijn in de atherosclerotische plaques, er percentueel minder macrofagen in de plaques teruggevonden worden. Dit kan erop duiden dat de aanwezigheid van $CD8^+$ T-cellen het aantal macrofagen in de plaques kan reguleren, en dat deze cellen dus een beschermende functie uitoefenen. Om deze hypothese verder te testen, hebben we dit onderzocht in een muismodel. We hebben gebruik gemaakt van de LDL-receptor deficiënte ($LDLr^{-/-}$) muis, die genetisch gemodificeerd is om minder LDL op te nemen uit de bloedstroom. Dit leidt tot hoge cholesterolwaarden in het bloed en daarom ontwikkelt deze muis atherosclerose zodra deze een cholesterolrijk dieet gevoerd wordt [35]. Om specifiek het effect van $CD8^+$ T-cellen in de geavanceerde stadia te bestuderen, zijn de muizen eerst 10 weken op een cholesterolrijk dieet gezet, zodat de plaques zich konden ontwikkelen. Vervolgens kregen de muizen nogmaals 6 weken een cholesterolrijk dieet, waarbij ze ook geïnjecteerd

werden met ofwel een antilichaam dat alle $CD8^+$ T-cellen uit het lichaam van de muizen verwijderde, ofwel een controle-antilichaam dat geen effect uitoefende. Aan het einde van het experiment zijn de atherosclerotische plaquegrootte alsmede belangrijke parameters van plaquestabiliteit gemeten in het driekleppengebied in het hart van de muizen, een locatie waar vanwege turbulente bloedstroming vaak atherosclerose ontstaat.

Hoewel de afwezigheid van $CD8^+$ T-cellen de plaquegrootte niet beïnvloedde, had het verwijderen van deze cellen wèl effect op de samenstelling van de plaques. In de behandelde groep was er een toename in het aantal macrofagen in de plaques. Dit is in overeenstemming met de correlatie die we beschreven tussen $CD8^+$ T-cellen en macrofagen in het humane endarterectomiemateriaal. Daarnaast beschreven we dat de behandelde groep minder stabiele plaques had, aangezien er minder collageen aanwezig was in de fibreuze laag van gladde spiercellen boven op de plaque, waardoor deze gemakkelijker kan scheuren. Bovendien leidde het verwijderen van de $CD8^+$ T-cellen ook nog eens tot een toename van de pro-inflammatoire Th1-cellen. Interessant genoeg zagen we deze toename alleen in de plaques van de behandelde muizen, maar niet op andere locaties in het lichaam. Deze resultaten geven aan dat $CD8^+$ T-cellen bijdragen aan de stabiliteit van de plaque, door het remmen van verschillende pro-inflammatoire immuuncellen. Deze beschermende rol van $CD8^+$ T-cellen in een geavanceerd stadium van atherosclerose komt overeen met een eerder gepubliceerde studie, waarin $CD8^+$ T-cellen een beschermende rol uitoefenden via het doden van antigeen-presenterende cellen (waar macrofagen ook onder vallen) [36]. Aangezien wij hebben gevonden dat het beschermende effect van $CD8^+$ T-cellen specifiek in de plaques plaatsvond, hebben wij ook gekeken naar verschillen tussen $CD8^+$ T-cellen in de plaque en die in de milt, als representatief orgaan voor de systemische immuunrespons. We vonden dat $CD8^+$ T-cellen in de plaques een hogere expressie hadden van Fas-ligand, een molecuul dat celdood kan induceren in andere cellen. Vooral Th1-cellen en macrofagen die veel vetten hebben opgenomen zijn hier erg gevoelig voor [37–39]. Het neutraliseren van de functie van Fas-ligand leidde inderdaad tot de aanwezigheid van meer Th1-cellen en macrofagen in de plaques. Daarom kunnen we uit deze studie concluderen dat $CD8^+$ T-cellen een beschermende functie uitoefenen in de geavanceerde stadia van atherosclerose, via het reduceren van de aanwezigheid van Th1-cellen en macrofagen. Dit beschermende effect wordt (gedeeltelijk) gemedieerd via het induceren van celdood door de hoge expressie van Fas-ligand op de $CD8^+$ T-cellen specifiek in de plaques.

3. $CD8^+$ T-cellen moduleren de lokale immuunrespons in de plaque

Zoals we hebben laten zien in **Hoofdstuk 3**, kunnen lokaal in de plaques aanwezige $CD8^+$ T-cellen een andere functie uitoefenen dan elders in het lichaam. Aangezien de lokale omgeving in de atherosclerotische plaque veel inflammatoire signalen en lipiden bevat, vroegen wij ons af of dit de functie van $CD8^+$ T-cellen beïnvloedt. In **Hoofdstuk 4** beschrijven we de verschillen in eigenschappen van $CD8^+$ T-cellen uit de plaque en de milt van apolipoproteine E deficiënte ($apoE^{-/-}$) muizen. Deze muizen heb-

ben een verminderde opname van zeer-lage dichtheid lipoproteïne (VLDL)- en LDL-deeltjes en ontwikkelen daardoor spontaan atherosclerose, zelfs als ze een normaal dieet gevoerd krijgen. De $CD8^+$ T-cellen uit de plaques van deze muizen leken disfunctioneel, aangezien ze minder pro-inflammatoire cytokines produceerden vergeleken met hun tegenhangers in de milt. Deze verminderde productie van cytokines was geassocieerd met een hogere expressie van het enzym CD39 op het celmembraan van de $CD8^+$ T-cellen. Dit enzym is betrokken bij het omzetten van adenosine trifosfaat (ATP), een pro-inflammatoir "gevaar"signaal, naar het anti-inflammatoire molecuul adenosine [40, 41]. Het farmacologisch remmen van CD39 in deze muizen leidde zoals verwacht tot een hogere inflammatoire cytokineproductie door $CD8^+$ T-cellen in de plaque. Daarnaast laten we zien dat antigeen-specifieke stimulatie van de TCR nodig is om CD39-expressie te induceren. Om dit aan te tonen hebben we een beenmergtransplantatie uitgevoerd, waarbij de aanwezige immuuncellen van de muizen werden verwijderd door bestraling, waarna er nieuw beenmerg werd geïnjecteerd waaruit nieuwe immuuncellen voortkwamen. Als we beenmerg in $LDLr^{-/-}$ muizen transplanteerden waaruit $CD8^+$ T-cellen voortkwamen die via de TCR geen atherosclerotisch antigeen konden herkennen, zagen we een flinke reductie in de expressie van CD39 op $CD8^+$ T-cellen in de plaques, vergeleken met de transplantatie van normaal beenmerg. Dit is in overeenstemming met data uit kankeronderzoek, waar TCR-signalen ook specifiek CD39-expressie induceren op $CD8^+$ T-cellen in de tumoromgeving [42, 43]. Bovenal laten we in dit onderzoek zien dat in humane atherosclerose er ook een sterk verhoogde CD39-expressie was op $CD8^+$ T-cellen in de plaques verkregen via endarterectomie vergeleken met bloedmonsters van dezelfde patiënten. Dit toont aan dat de resultaten die wij hebben verkregen in de muizenstudies ook klinische relevantie hebben.

In **Hoofdstuk 5** hebben wij onderzocht of de lokale ontstekingsreactie in de atherosclerotische plaque ook de daar aanwezige subsets van $CD8^+$ T-cellen beïnvloedt. Op het moment van $CD8^+$ T-celactivatie door de DCs, kunnen de door DCs uitgescheiden cytokines de $CD8^+$ T-cellen beïnvloeden om te differentiëren naar verschillende subsets. Deze subsets worden gekarakteriseerd door de productie van verschillende inflammatoire cytokines. Zo produceert de Tc1-subset vooral de inflammatoire cytokines IFN-γ en TNF-α, de Tc2-subset voornamelijk IL-4 en IL-5, en de Tc17-subset vooral IL-17A. Daarnaast wordt elke subset geassocieerd met de expressie van een andere transcriptiefactor, een eiwit dat de genexpressie in de cel reguleert en op die manier de verschillende effectorfuncties van elke subset teweegbrengt [44]. In $apoE^{-/-}$ muizen hebben wij bepaald dat er procentueel minder Tc1-cellen in de plaque waren vergeleken met de milt, maar dat er juist een toename was in het percentage Tc17-cellen. Om te bestuderen hoe Tc17-cellen de ontwikkeling van atherosclerose beïnvloeden, hebben we deze cellen ingespoten in CD8-deficiënte $LDLr^{-/-}$ muizen. Deze muizen hebben van zichzelf geen $CD8^+$ T-cellen, waardoor het effect van de geïnjecteerde cellen goed bestudeerd kan worden. Ter controle werden ongedifferentieerde Tc0-cellen ingespoten. Tot onze verassing zagen we dat de Tc0-cellen zich meer gingen gedragen als Tc1-cellen zodra ze werden geïnjecteerd, aangezien ze IFN-γ en T-bet, de karakteristieke transcriptiefactor van Tc1 cellen, verhoogd tot expressie brachten. Tc17-cellen produceerden ook meer IFN-γ na injectie, maar minder dan de Tc0-cellen, en meer IL-17A, zoals verwacht. Het is aannemelijk dat de inflammatoire omgeving in de atherosclerotische muizen deze

veranderingen induceert [36, 45]. De muizen in de Tc17-behandelde groep hadden kleinere atherosclerotische plaques vergeleken met de Tc0-behandelde groep. Daarnaast waren er minder macrofagen in de plaques van de Tc17-behandelde muizen en was er een vermindering van het percentage inflammatoire Th1-cellen. Aangezien er zowel een toename in IL-17A productie als een vermindering van IFN-γ productie was in de Tc17-groep ten opzichte van de Tc0-groep, kunnen de verschillen in atherosclerose aan beide veranderingen ten grondslag liggen. Desalniettemin laten deze bevindingen zien dat er verschillende subsets van $CD8^+$ T-cellen aanwezig zijn in de atherosclerotische plaques, en dat het mogelijk interessant kan zijn vanuit een therapeutisch oogpunt om de $CD8^+$ T-cellen te differentiëren naar een minder atherogene subset, zoals de Tc17-subset.

4. Inductie van $CD8^+$ T-cellen door middel van vaccinatie voor de behandeling van atherosclerose

Aangezien we in de voorgaande hoofdstukken hebben aangetoond dat $CD8^+$ T-cellen een anti-atherogene functie kunnen uitoefenen onder bepaalde omstandigheden, was het een logische volgende stap om een therapie te ontwikkelen die deze beschermende rol benut. Een interessante strategie om antigeen-specifieke $CD8^+$ T-cellen te induceren is via vaccinatie. In **Hoofdstuk 6** hebben wij een literatuuronderzoek uitgevoerd naar hoe de immuunrespons wordt beïnvloed door de eigenschappen van de deeltjes waarin het antigeen aan de antigeen-presenterende cellen wordt afgeleverd. Ziekteverwekkers zoals bacteriën en virussen verschillen van vorm en andere eigenschappen, en stimuleren op die manier het immuunsysteem elk in een specifieke manier. Op eenzelfde manier kan het ontwerp van een deeltjesvormig vaccin de immuunrespons beïnvloeden. In dit hoofdstuk bespreken we hoe de fysisch-chemische eigenschappen van grootte, vorm, en rigiditeit van een deeltjesvormig vaccin de distributie van dit deeltje door het lichaam, de opname door cellen, de antigeen-presentatie en de resulterende immuunrespons kunnen beïnvloeden. Met betrekking tot $CD8^+$ T-cellen bespreken we hier verschillende studies die demonsteren dat deeltjes in de grootte van nanometers gemakkelijk getransporteerd worden van de injectieplaats naar het lymfestelsel [46]. Daarnaast worden kleine nanodeeltjes efficiënt opgenomen door DCs [47]. Tot slot zijn kleine nanodeeltjes meer effectief dan grote deeltjes in het induceren van $CD8^+$ T-celimmuunreacties, vooral als deze deeltjes bolvormig zijn, aangezien dit de opname van de deeltjes vergemakkelijkt [48, 49].

Met de vergaarde kennis over hoe de eigenschappen van deeltjesvaccins immuunreacties kunnen beïnvloeden, hebben we in **Hoofdstuk 7** vervolgens een vaccinatiestrategie gekozen om beschermende $CD8^+$ T-cellen te induceren in atherosclerose. Het doel was om een cytotoxische $CD8^+$ T-celrespons op te wekken tegen antigeen-presenterende cellen die LDL-deeltjes hebben opgenomen, wat zou moeten resulteren in een vermindering van het aantal macrofagen in de plaque alsmede een verminderde stimulatie van het adaptieve immuunsysteem. Daarnaast zou vaccinatie ook de expressie van CD39 kunnen verhogen, wat de immuunrespons verder zou kunnen remmen. Door middel van de Immune Epitope Database Analysis Resource (IEDB), een online pro-

gramma voor het voorspellen van T-celepitopen [50], hebben we een voorspelling gemaakt welke peptide-sequentie in het ApoB100 eiwit, het belangrijkste eiwit op het LDL-deeltje, zeer waarschijnlijk goed in het MHC-I molecuul kan binden. Vervolgens hebben we dit peptide in een liposomaal deeltje ingekapseld. Liposomen zijn doorgaans bolvormige deeltjes die bestaan uit een of meerdere bi-lagen van fosfolipiden die een vettige membraan vormen welke een waterhoudende holte omsluiten. Antigenen verpakt in liposomen worden beter opgenomen door antigeen-presenterende cellen vergeleken met opgeloste antigenen. Daarnaast induceren de liposomen immuunactivatie in de antigeen-presenterende cellen. Eerder werk heeft laten zien dat positief geladen liposomen met een diameter kleiner dan 200 nanometer sterke antigeen-specifieke $CD8^+$ T-celresponsen kunnen induceren in een muismodel van kanker [51]. Daarom hebben wij gekozen om dit type liposomen te gebruiken in onze vaccinatiestudie. $LDLr^{-/-}$ muizen werden gevaccineerd met liposomen die het peptide bevatten, met alleen lege liposomen, of met alleen het vrije peptide. Na 10 weken op een cholesterolrijk dieet en twee boost-vaccinaties hebben we het effect op atherosclerose-ontwikkeling in deze muizen geëvalueerd. Helaas waren we niet in staat om antigeen-specifieke $CD8^+$ T-cellen te detecteren na de vaccinaties, waardoor er ook geen effect van de vaccinatie op atherosclerose waarneembaar was. Echter waren we wel in staat om antigeen-specifieke $CD8^+$ T-cellen te induceren als we een ander peptide in de liposomen inkapselden, wat aantoont dat de problemen met de immunogeniciteit in onze vaccinatiestudie niet door de liposomen veroorzaakt werden. Daarnaast waren we ook in staat om antigeen-specifieke $CD8^+$ T-cellen te induceren als we ons ApoB100-afgeleide peptide direct op DCs laadden en vervolgens met deze cellen vaccineerden, wat aantoont dat dit peptide wel degelijk immunogeen is. Het is aannemelijk dat er problemen waren met de stabiliteit van het peptide tijdens het kruispresentatieproces na vaccinatie, waarbij het peptide in contact komt met een zure omgeving en verschillende enzymen die het peptide kunnen afbreken. Het gebruik van een meer stabiel peptide zou deze problemen wellicht kunnen verhelpen. Ondanks dat de uitkomsten van deze studie geen afdoend bewijs hebben geleverd dat onze vaccinatiemethode beschermend werkt, hebben we in dit proefschrift overtuigende data gepresenteerd die een beschermde rol van $CD8^+$ T-cellen laat zien. Daarom zijn we van mening dat verder onderzoek naar vaccinatie gericht op $CD8^+$ T-cellen van grote waarde kan zijn in het onderzoeksveld van hart- en vaatziekten.

5. Perspectieven

In de laatste jaren is er veel vooruitgang geboekt in het onderzoek naar immuuntherapieën voor de behandeling van atherosclerose. In dit proefschrift hebben wij de rol van $CD8^+$ T-cellen in deze ziekte onderzocht, en meerdere mechanismen geïdentificeerd waardoor $CD8^+$ T-cellen een beschermende functie uitoefenen. We hopen dat deze bevindingen nuttige uitgangspunten zullen blijken te zijn voor de ontwikkeling van nieuwe therapeutische interventies voor de behandeling van atherosclerose.

In **Hoofdstuk 3** lieten we zien dat FasL-expressie op de $CD8^+$ T-cellen bijdraagt aan het verwijderen van de pro-inflammatoire Th1-cellen en macrofagen in de plaques.

Daarom kan het mogelijk interessant zijn om een hogere expressie van FasL te induceren op T-cellen, en deze T-cellen vervolgens toe te dienen als celtherapieproduct, een methode die al effectief is gebleken in de behandeling van prostaatkanker in een muismodel [52]. Een ander alternatief is om T-cellen uit patiënten te isoleren, en in deze cellen een zogenaamd "chimeric antigen receptor"(CAR) construct tot expressie te laten brengen, waarmee deze CAR-T-cellen specifiek een gericht celtype kunnen aanvallen en celdood kunnen induceren via onder andere FasL-signalering [53]. Door deze therapie op macrofagen te richten, kan de plaquevorming wellicht worden tegengegaan.

In **Hoofdstuk 4** beschrijven we dat activatie van $CD8^+$ T-cellen leidt tot een verhoogde expressie van CD39, wat de pro-inflammatoire cytokineproductie in de plaque vermindert. Daarom hebben we in **Hoofdstuk 7** getest of we beschermende $CD8^+$ T-cellen konden induceren door middel van vaccinatie. Helaas was onze vaccinatiemethode niet in staat antigeen-specifieke $CD8^+$ T-cellen te induceren tegen het ApoB100-afgeleide peptide. Om in de vorige sectie genoemde redenen, zou verder onderzoek naar een verbeterde vaccinatiestrategie desalniettemin kunnen leiden tot de ontwikkeling van een effectieve therapie voor de behandeling van atherosclerose.

Tot slot hebben wij met het in **Hoofdstuk 5** beschreven onderzoek aangetoond dat IL-17A-producerende Tc17-cellen de vorming van atherosclerotische lesies verminderen vergeleken met IFN-γ-producerende Tc1-cellen. Dit laat zien dat de verschillende subsets van $CD8^+$ T-cellen ieder hun eigen effect uitoefenen op het ziekteproces. Daarom kan het interessant zijn om T-cellen te isoleren uit het bloed, buiten het lichaam naar het Tc17-subtype te differentiëren en het resulterende celtherapieproduct vervolgens terug te geven aan de patiënt, zodat deze aanpaste T-cellen een beschermende functie kunnen uitoefenen. Deze methode is al succesvol gebleken in het versterken van de anti-tumor activiteit van T-cellen in muismodellen en humane cellen [54, 55], wat suggereert dat deze aanpak ook in een klinische setting van toepassing kan zijn.

Samenvattend is er zeker potentie voor het ontwikkelen van een $CD8^+$ T-cel-gerichte therapie voor de behandeling van hart- en vaatziekten. Men moet echter zorgvuldig zijn in het sturen van de $CD8^+$ T-celfunctie naar een beschermende functie, en het induceren van pro-inflammatoire bijwerkingen zien te voorkomen. Daarnaast zijn veel van de onderzoeksgegevens die een beschermende rol voor $CD8^+$ T-cellen laten zien verkregen in muismodellen, alhoewel wij hebben aangetoond dat dit ten minste gedeeltelijk vertaald kan worden naar een klinische setting. Voordat er een effectieve $CD8^+$ T-cel-gerichte therapie kan worden ontwikkeld, is echter meer onderzoek nodig om de beschermende functie van $CD8^+$ T-cellen in humane atherosclerose te bevestigen. Daarnaast is er meer onderzoek nodig om de bruikbaarheid, veiligheid en effectiviteit van mogelijke therapieën te onderzoeken.

6. Conclusie

Het in dit proefschrift beschreven onderzoek werpt een nieuw licht op de functie van $CD8^+$ T-cellen in atherosclerose en laat zien dat deze cellen in de plaque beschermende effecten uitoefenen. Dit onderzoek biedt nieuwe aanknopingspunten

voor de ontwikkeling van immuunmodulerende therapieën voor de behandeling van atherosclerose, zoals antigeen-specifieke stimulatie van $CD8^+$ T-cellen door middel van vaccinatie. Er wordt momenteel grote vooruitgang geboekt in het ontwikkelen van antigeen-specifieke T-celtherapieën voor de behandeling van verschillende ziektes. Daarnaast is er toenemende aandacht binnen het veld van atherosclerose onderzoek naar de ontwikkeling van immuunmodulerende therapieën, die gecombineerd kunnen worden met het gunstige effect van het verlagen van de cholesterolwaarden in het bloed. Daarom is de vraag niet *of*, maar *wanneer* er effectieve immuun-gerichte therapieën voor de behandeling van hart- en vaatziekten binnen ons handbereik zullen zijn.

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Curriculum Vitae

Janine van Duijn werd op 2 juni 1992 geboren in Leiden. In 2010 behaalde ze haar VWO-diploma aan het Pieter Groen College te Katwijk. In datzelfde jaar begon ze aan de opleiding Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden. Ze behaalde haar Bachelor of Science-titel *cum laude* in juli 2013. Haar bachelor onderzoeksproject is uitgevoerd aan de afdeling Biofarmacie van het Leiden Academic Centre for Drug Research, onder begeleiding van Dr. V. Frodermann en Prof. Dr. J. Kuiper. In dit project heeft Janine onderzoek gedaan naar de immuun-modulerende rol van de *Staphylococcus aureus* bacterie, wat heeft geresulteerd in een wetenschappelijke publicatie in het tijdschrift *The Journal of Internal Medicine*.

In september 2013 is ze begonnen aan de Master studie Bio-Pharmaceutical Sciences aan de Universiteit Leiden. Haar eerste masterstage werd uitgevoerd tussen september 2013 en juli 2014 aan het Leiden Academic Centre for Drug Research, onder begeleiding van Dr. V. Frodermann en Prof. Dr. J. Kuiper. Deze stage werd afgesloten met een verslag getiteld "Specific β5 Proteasomal Subunit Inhibition in Atherosclerosis". Voor deze stage ontving Janine de Suzanne Hovinga Award voor de beste masterthesis, alsmede de KNMP (Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie) studentenprijs. Van januari 2015 tot juli 2015 voerde zij een tweede wetenschappelijke stage uit aan het Kennedy Institute of Rheumatology, onderdeel van Oxford University. Ze deed daar onderzoek binnen de afdeling Cardiovascular Inflammation onder de begeleiding van Prof. Dr. C. Monaco. De titel van haar onderzoeksproject was "Interferon regulatory factor 5 regulates B cell responses in atherosclerosis". Ze behaalde in september 2015 haar Master of Science-graad *summa cum laude*.

Van augustus 2015 tot december 2019 was Janine als promovendus werkzaam bij de afdeling BioTherapeutics van het Leiden Academic Centre for Drug Research onder begeleiding van Dr. B. Slütter, Prof. Dr. W. Jiskoot en Prof. Dr. J. Kuiper. Voor het presenteren van haar onderzoek ontving ze in 2016 de Trainee Poster Award op de American Association of Immunologists Immunology meeting en de posterprijs op het LACDR Spring symposium. In 2019 ontving ze de Young Investigator Fellowship op de European Atherosclerosis Society Conference.

Sinds Januari 2020 is zij werkzaam als Science & Business Trainee bij Janssen Vaccines, Leiden, onderdeel van de *Johnson & Johnson group*.

List of Publications

J. van Duijn, M. van Elsas, N. Benne, M. Depuydt, A. Wezel, H. Smeets, I. Bot, W. Jiskoot, J. Kuiper, B. Slütter. CD39 identifies a microenvironment-specific anti-inflammatory $CD8^+$ T-cell population in atherosclerotic lesions. Atherosclerosis. 2019 Jun;285:71-78.

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. 2019 Mar 15;115(4):729-738.

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J. van Duijn[1], N. Benne[1], J. Kuiper, W. Jiskoot, B. Slütter. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. J Control Release. 2016 Jul 28;234:124-34.

E. Kritikou, M.A.C. Depuydt, M.R. de Vries, K.E. Mulder, A.M. Govaert, M.D. Smit, J. van Duijn, A.C. Foks, A. Wezel, H.J. Smeets, B. Slütter, P.H.A. Quax, J. Kuiper, I. Bot. Flow Cytometry-Based Characterization of Mast Cells in Human Atherosclerosis. Cells. 2019 Apr 9;8(4): E334.

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. 2019 Jan;280:132-139.

E. Kritikou, T. van der Heijden, M. Swart, J. van Duijn, B. Slütter, A. Wezel, H.J. Smeets, P. Maffia, J. Kuiper, I. Bot. Hypercholesterolemia Induces a Mast Cell-$CD4^+$ T Cell Interaction in Atherosclerosis. J Immunol. 2019 Mar 1;202(5):1531-1539.

N. Benne, J. van Duijn, F. Lozano Vigario, R.J.T. Leboux, P. van Veelen, J. Kuiper, W. Jiskoot, B. Slütter. Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. J Control Release. 2018 Dec 10;291:135-146.

T. van der Heijden, E. Kritikou, W. Venema, J. van Duijn, P.J. van Santbrink, B. Slütter, A.C. Foks, I. Bot, J. Kuiper. NLRP3 Inflammasome Inhibition by MCC950 Reduces Atherosclerotic Lesion Development in Apolipoprotein E-Deficient Mice-Brief Report. Arterioscler Thromb Vasc Biol. 2017 Aug;37(8):1457-1461.

V. Frodermann, J. van Duijn, G.H. van Puijvelde, P.J. van Santbrink, H.M. Lagraauw, M.R. de Vries, P.H. Quax, I. Bot, A.C. Foks, S.C. de Jager, J. Kuiper. Heat-killed Staphylococcus aureus reduces atherosclerosis by inducing anti-inflammatory macrophages. J Intern Med. 2016 Jun;279(6):592-605.

V. Frodermann, J. van Duijn, M. van Pel, P.J. van Santbrink, I. Bot, J. Kuiper, S.C. de Jager. Mesenchymal Stem Cells Reduce Murine Atherosclerosis Development. Sci Rep. 2015 Oct 22;5:15559.

PhD Portfolio

Cursussen

2018	R & Python for Data science
2017	Cytoflex Flow Cytometry course
2017	Effective Communication
2017	Introduction to Teaching and Supervision
2016	Time- & Self-Management
2016	Communication in Science
2016	Data Management
2016	Advanced Immunology
2015	Advanced Drug Delivery & Drug Targeting
2015	Scientific Conduct
2015	PhD Introductory Course on Drug Research

Presentaties

2019 Rembrandt symposium, Noordwijkerhout, Nederland

2019 Scandinavian Society for Atherosclerosis Research, Humbleæk, Denemarken

2019 LACDR Spring symposium, Leiden, Nederland

2019 European Atherosclerosis Society Conference, Maastricht, Nederland

2018 LACDR Spring symposium, Leiden, Nederland

2018 Rembrandt symposium, Noordwijkerhout, Nederland

2017 Figon Dutch Medicine Days, Ede, Nederland

2017 Scandinavian Society for Atherosclerosis Research, Humbleæk, Denemarken

2017 LACDR Spring symposium, Leiden, Nederland

2017 Rembrandt symposium, Noordwijkerhout, Nederland

2016 American Association of Immunologists Immunology meeting, Seattle, Verenigde Staten van Amerika

2016 LACDR Spring symposium, Leiden, Nederland