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

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General Introduction

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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].

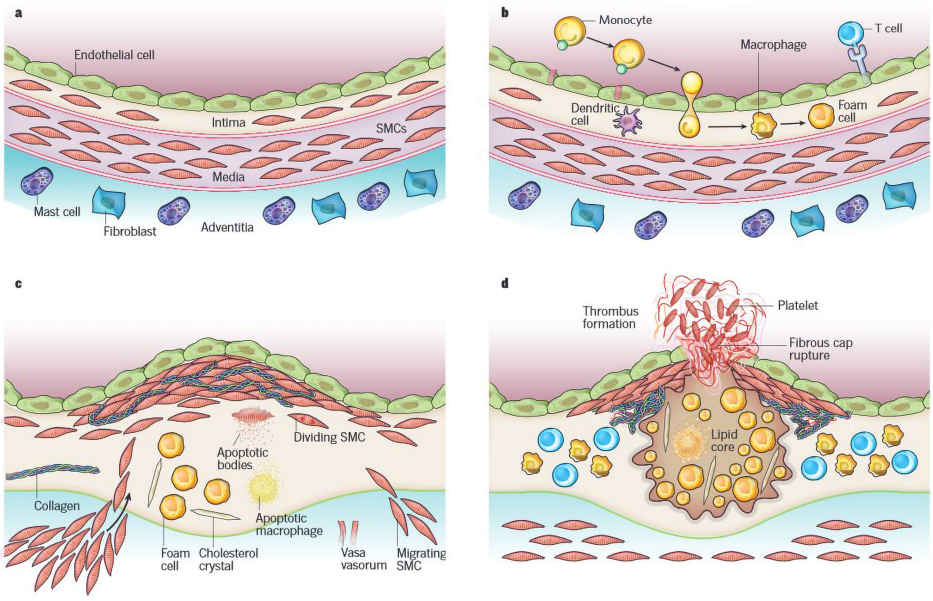

Figure 1: Stages in the development of atherosclerotic lesions. The healthy artery and the changes that occur during disease progression to thrombosis are shown. (A) The normal artery is lined by ECs that are in contact with blood. (B) Atherosclerosis is initiated by adhesion of blood leukocytes to the 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].

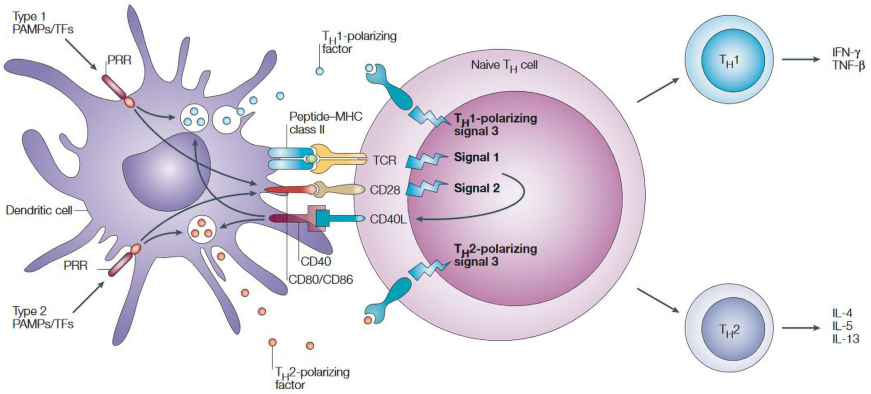

Figure 2: T-cell stimulation and polarization require three dendritic cell-derived signals. Signal 1 is the antigen-specific signal that is mediated through T-cell receptor (TCR) triggering by MHC-associated peptides processed from pathogens or self-molecules after internalization through Pattern Recognition 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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