CD8+ T-cells in atherosclerosis: recognizing their contribution

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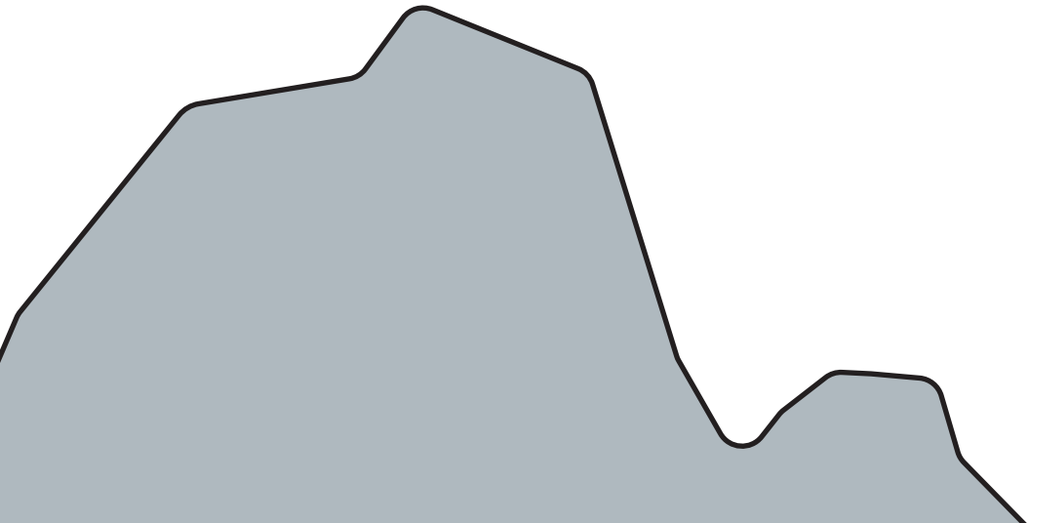

Chapter 1

General introduction

CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the leading cause of death worldwide, and comprises a number of disorders regarding the heart and the vascular system, including ischemic heart disease, myocardial infarction, angina pectoris, stroke, peripheral arterial disease, aortic aneurysm and pulmonary embolism[1]. The main underlying cause of CVD is the occlusion of blood vessels as a result of atherosclerosis. Atherosclerosis is characterized by the buildup of plaques in larger arteries, consisting of lipids, cholesterol and immune cells among others[2]. Gradual growth of these atherosclerotic lesions results in narrowing and hardening of arteries, potentially causing complete obstruction of the blood flow, called stenosis.

Disrupted blood flow may be accompanied by physical symptoms, depending on the location of the obstruction. Stenosis of the coronary artery, for example, causes chest pains and/or discomfort in the left arm and shoulder[3], while in cases of cerebral artery occlusion patients experience weakness of the muscles in the face, arms or legs, mostly on one side of the body[4]. More acute complications arise when the atherosclerotic plaques rupture and the formed thrombus causes direct and complete obstruction of larger arteries, resulting in a cardiovascular event or a stroke.

The main risk factors for atherosclerosis include hypertension, high cholesterol levels (particularly LDL cholesterol), an unhealthy diet high in saturated and trans-fats, smoking, obesity, coagulation, genetics, aging, diabetes, and chronic systemic inflammation caused by chronic inflammatory or autoimmune diseases (in example, rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus)[5].

Atherosclerosis-reducing therapies

First-line therapies for treating atherosclerosis typically focus on lifestyle modifications and pharmacologic interventions to manage risk factors and slow the progression of the disease. In case of an acute cardiovascular event, surgical techniques are required to restore blood flow. Surgical interventions, including coronary artery bypass grafting, carotid endarterectomy, angioplasty and stenting, are commonly used to attain revascularization. Despite their effectiveness, perioperative risks, including restenosis and recurrent cardiovascular events often occur. Vascular restenosis may be prevented or reduced by pharmacologic treatments. Current first-line pharmacologic therapies prescribed to control cardiovascular disease mainly focus on reducing blood pressure (beta-blockers, and angiotensin-converting enzyme inhibitors[6]), preventing clot formation (aspirin[7,8], antiplatelet therapies, and antithrombotic drugs[9]) and normalizing the lipid balance (statins, fibrates, and PCSK9 inhibitors[10]). These therapies have proven to be efficient in lowering the risk of cardiovascular events. Lowering systolic blood pressure by 5-mmHg, for instance, has been shown to reduce the risk of major cardiovascular events by approximately 10%, irrespective of previous diagnoses of cardiovascular disease, or initial blood pressure values[6]. Moreover, preventing coagulation with a low dose of aspirin reduced the risk of cardiovascular events by 21% and all-cause mortality by 13% in individuals with preexisting cardiovascular disease[8]. However, patients treated with anticoagulants suffer from an increased risk of severe bleeding, rendering this drug category unsuitable as a first-line treatment.

The most significant benefits have been observed with lipid-lowering therapies, such as statins. Statins reduce plasma cholesterol levels by inhibiting cholesterol synthesis and promoting the uptake of LDL by the liver[11]. Statin usage has been associated with a decreased relative risk of all-cause mortality of 8%, mostly explained by a reduced risk of stroke of 22%, and myocardial infarction of 33%[12]. Interestingly, statin usage did not significantly reduce the risk of cardiovascular mortality.

Despite the tremendous success of these treatments in lowering cardiovascular disease risk, a residual inflammatory risk for CVD remains in approximately 43-61% of patients with atherosclerosis[13]. Preclinical and clinical trials have demonstrated that dampening the inflammatory response can further curtail the risk of CVD[14–17]. Both broad-spectrum immunosuppressive drugs, as well as specific immunotherapies targeting one component of the immune system have demonstrated effective in reducing the residual risk for CVD. Treatment with a low dosage of colchicine, a broad-spectrum immunosuppressing agent[12], resulted in a cumulative reduction of 31% for the risk of cardiovascular death, spontaneous myocardial infarction, ischemic stroke, or ischemia-driven coronary revascularization[20]. Additionally, another treatment approach involved neutralizing the activity of an IL-1β using a monoclonal antibody called canakinumab. This treatment demonstrated a cumulative risk reduction of 15% for the same cardiovascular parameters[15]. These studies indicate that addressing inflammation poses a promising strategy for developing new therapies for cardiovascular diseases. Nevertheless, additional research is required to optimize and refine immunosuppressive treatments, as not all immunosuppressants appear effective[21,22], or are associated with severe side effects including fatal infection[15].

DISEASE PROGRESSION

Early atherosclerosis

Atherosclerosis is a slowly progressing disease, initiated by damage to endothelial cells[28]. Endothelial cell damage can be caused by noxious substances (e.g., elevated oxidized cholesterol levels, smoking-derived compounds, hyperglycemia, etc.) or altered hemodynamics (e.g., blood flow disturbance, or hypertension)[29]. Upon damage, endothelial cells become activated, resulting in the secretion of immune cell attracting chemokines and cytokines, the increased expression of adhesion molecules, and conformational changes that increase vascular permeability. This increased permeability allows for the accumulation of lipoproteins into the subendothelial layer of the arterial wall. Lipoproteins are particles that consist of apolipoproteins (e.g., ApoAI/II, ApoB48/100, ApoC, etc.), triglycerides, and water-insoluble lipids, including cholesterol[30]. They can be subdivided based on their density, size and composition into chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). VLDL and LDL are considered to be proatherogenic, while HDL, which is involved in cholesterol efflux, has atheroprotective properties[31]. In the arterial wall, these lipoproteins are exposed to various oxidative stressors, including reactive oxygen species (ROS), and enzymes resulting in oxidative, enzymatic and chemical modifications of the particles[31]. Modified lipoproteins hold immunogenic properties, enhancing endothelial cell and vascular smooth muscle cell (VSMC) activation, resulting in increased leukocyte attraction[2]. The attracted macrophages start to take up modified lipoproteins, leading to the formation of lipid-filled foam cells. The accumulation of foam cells in the vascular intima is the first hallmark of atherosclerotic plaque formation.[32]

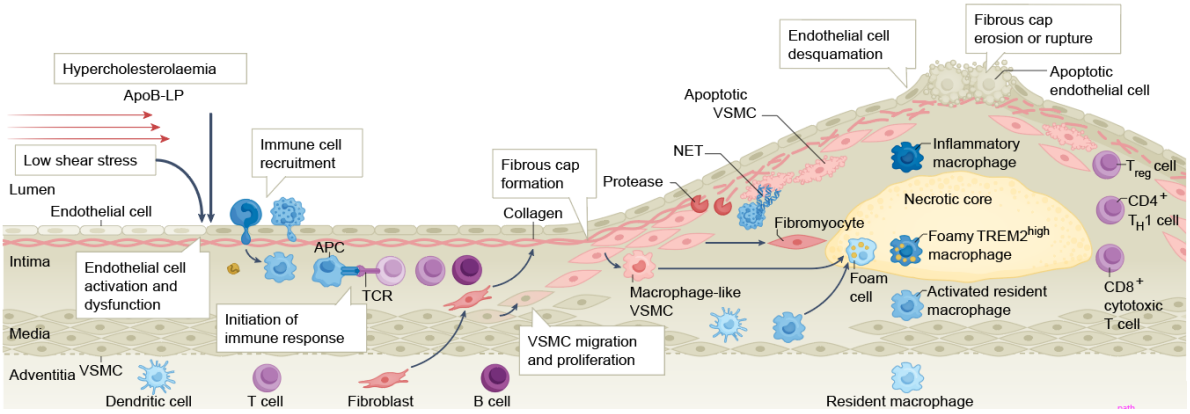

Figure 1. Initiation and progression of atherosclerosis. Atherosclerosis begins with endothelial cell damage from harmful substances or abnormal blood flow, attracting the immune system and increasing vascular permeability. This allows ApoB-containing lipoproteins (ApoB-LP) and immune cells to accumulate in the arterial wall's intimal layer. In the intima, these lipoproteins undergo oxidative changes, activating innate immune cells and promoting inflammation by inducing cytokine and chemokine release. Chemokines attract monocytes into the subendothelial space, where they become macrophages. These macrophages, together with macrophage-like vascular smooth muscle cells (VSMCs), ingest oxidized lipoproteins, and become lipid-laden foam cells forming a fatty streak. As the lesion progresses, antigen-presenting cells (APCs) activate T- and B-cells, enhancing inflammation. The buildup of lipids leads to foam cell apoptosis and secondary necrosis, resulting in necrotic core formation. VSMCs migrate and secrete collagen, creating a fibrous cap. Over time, advanced lesions become unstable due to necrotic core growth, fibrous cap thinning, endothelial cell loss, and calcification, eventually leading to rupture, thrombus formation, and cardiovascular events. *Adapted from Engelen et al. (2022) Nat. Rev. Cardiol 19(8):522-542*[32].

Advanced atherosclerosis

During the advancement of the atherosclerotic lesion, more complex structures arise. VSMCs migrate through the vessel intima and line the outer endothelial layer, where they deposit extracellular matrix molecules, like collagen, and promote the formation of a lesion-stabilizing fibrous cap[33,34]. Like the intralesional macrophages, these migratory VSMCs start to clear oxLDL from the surroundings, and obtain a foamy phenotype with macrophage-like characteristics[34–36]. At this stage, a larger variety of immune cells, including macrophages, dendritic cells, neutrophils, mast cells, NK-cells, B-cells and T-cells are recruited to the lesion[37]. The continuous influx of lipids, combined with nutrient deprivation, ongoing inflammation, and oxidative stress, leads to programmed cell death of foam cells. The excessive extent of cell death, accompanied by inadequate tissue clearance results in secondary necrosis of neighboring cells in the lesion[38]. The accumulation of necrotic debris, along with lipids and remnants from foam cells, forms necrotic cores within the lesion. Over time, atherosclerotic lesions become increasingly unstable, due to persistent inflammation, continuous necrotic core formation, and fibrous cap thinning.

EXPERIMENTAL MODELS TO STUDY ATHEROSCLEROSIS

To study the progression of atherosclerosis and discover potential treatments to combat cardiovascular disease, many pre-clinical *in vitro, ex vivo* and *in vivo* models have been developed. Despite tremendous advancement in comprehensive *in vitro* models, like culture systems closely resembling the human vasculature, they are not capable of capturing the full

complexity of the cellular interactions and inter-organ cross-talk that underlies atherogenesis. Therefore, *ex vivo* and *in vivo* studies remain a necessary tool for studying atherosclerosis.

Human endarterectomy samples

To gain direct insight into the pathological processes occurring in human disease, *ex vivo* samples of human atherosclerotic lesions are utilized. Human atherosclerosis is primarily studied using samples derived from the carotid or femoral arteries, obtained via endarterectomy surgery, or from the coronary arteries, which can be collected post-mortem following a fatal myocardial infarction. These lesions can then be analyzed to study the composition of the lesion, its cellular content, and the molecular characteristics. This allows researchers to correlate specific parameters, such as plaque characteristics to particular cell types, thereby identifying potential pathways for therapeutic interventions.

Pre-clinical mouse models

These identified pathways can subsequently be investigated more thoroughly using *in vivo* models. Atherogenesis may be studied *in vivo* in zebrafish, mice, rats, rabbits, pigs and non-human primates. Mice are predominantly used to study atherosclerosis, due to their rapid reproduction, relatively short life-span, and ease of genetic manipulation[23]. However, due to their inherently low cholesterol levels, and their relatively high HDL levels, mice are naturally resistant to atherosclerosis[24]. To circumvent this, genetic and dietary modifications have been used to change their cholesterol levels and lipoprotein profile to promote atherogenesis.

The LDL receptor knockout (*Ldlr*$^{-/-}$) mouse and the Apolipoprotein E knockout (*Apoe*$^{-/-}$) mouse are the most commonly used mouse models to study atherosclerosis. Both mouse strains exhibit elevated total cholesterol concentrations in plasma, which can be further enhanced by dietary changes. During a standard laboratory chow diet, containing 4-6% fat and <0.03% cholesterol, cholesterol levels are around 200-300 mg/dL in *Ldlr*$^{-/-}$[23], and 400-600 mg/dL in *Apoe*$^{-/-}$ mice[25]. By changing their diet to a cholesterol-rich Western-type diet, containing 21% fat and 0.15% cholesterol, cholesterol levels of *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice are substantially elevated to 1000-1500 mg/dL[23,26], accelerating atherosclerotic lesion formation.

The genetic background of these mice can be further modified to study specific components of the immune system. To investigate the function of the adaptive immune system, models such as the Nude mouse (lacking a thymus), SCID mouse (deficient in B and T cells), and *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ mice (lacking recombinase activating genes 1 or 2, resulting in the absence of B and T cells) have been developed. Additionally, mouse models like *Cd8a*$^{-/-}$ or *Cd4*$^{-/-}$ mice have been created to study specific subsets of the immune system. Furthermore, to comprehensively analyze the effect of a particular gene in a specific cell type, conditional knockout systems such as the Cre-Lox system can be utilized[27].

THE IMMUNE SYSTEM

The immune system protects the body against infections, pathogens, and malfunctioning or cancerous cells, and is comprised of an innate and adaptive arm. The innate immune system, comprised of macrophages, dendritic cells, neutrophils, NK-cells, and mast cells, forms the body's first line of defense and responds in a non-specific fashion against pathogens. In contrast, the adaptive immune system, consisting of B-cells, CD4^{+} T-cells, and CD8^{+} T-cells, is a highly specialized defense mechanism that requires more time to become activated but offers specific and long-lasting protection against pathogens. Both the innate- and adaptive immune system have been shown to contribute to the progression of atherosclerosis[37].

INNATE IMMUNE SYSTEM

The innate immune system is the body's first line of defense and is able to quickly respond to pathogens, upon stimulation of toll-like receptors (TLR) and NOD-like receptors (NLRs) on leukocytes. The innate immune system consists of monocytes, that develop into macrophages or dendritic cells, neutrophils, mast cells and NK-cells. These cells are activated upon stimulation by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[39]. PAMPs are molecular structures that are widely shared between pathogens, including lipopolysaccharides, flagellin, unmethylated cytosine-phosphate-guanine (CpG) DNA, and double-stranded RNA. DAMPs, also known as alarmins, are molecules associated with stressed, damaged, necrotic or dying cells, and include extracellular expression of heat shock proteins (HSPs), ATP, and S100 proteins. While PAMPs are more associated with responses against pathogens, DAMPs associate with tissue damage and are particularly relevant in the context of atherosclerosis. PAMPs and DAMPs, together with cholesterol crystals, reactive oxygen species (ROS), neutrophil extracellular traps (NETs), as well as exposure to TNFα or IL-1β can promote the activation of the NLRP3 inflammasome[40–44]. The NLRP3 inflammasome is a multiprotein complex involved in the production of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and interleukin-18 (IL-18), that further enhance inflammation.

Monocytes and macrophages

Monocytes are circulating immune cells that differentiate into macrophages or dendritic cells (DCs) upon entering tissues. Macrophages are specialized in phagocytosis of pathogens and cellular debris, efferocytosis, modulating the immune response by secreting cytokines, and although it is not their primary function, they are also involved in antigen presentation and priming of the adaptive immune response.

Originally, macrophages were considered to either exhibit a pro-inflammatory M1 phenotype, characterized by the production of proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6), or an anti-inflammatory M2 phenotype, that produce immune dampening cytokines (e.g., IL-10, TGF-β)[45]. However, in the atherosclerotic lesion, a broader range of macrophage subtypes has been identified[46–49]. Besides the foamy *TREM2*$^{+}$ macrophages, resident-like macrophages, interferon-inducible (IFNIC) macrophages, aortic-intima resident macrophages (MAC^{AIR}), and inflammatory macrophages reside in the lesion[46–49]. MAC^{AIR} and resident-like macrophages are present in both healthy and diseased aortas, and are the first to engulf oxLDL and obtain a foamy phenotype[50]. Although the overall consensus is that foam cells enhance inflammation by secreting cytokines and generating ROS, single-cell analysis of cells from human and mouse atherosclerotic lesions demonstrated that *Trem2*$^{+}$ foamy macrophages are characterized by the expression of genes involved in lipid metabolism, and only exhibit a limited expression of inflammation-associated genes[46]. This suggests that foam cells are more involved in homeostatic lipid processing, rather than contributing to inflammation. Inflammatory macrophages highly resemble the classical M1 macrophages, and are characterized by elevated expression of *NLRP3*, and production of proinflammatory mediators, such as IL-1β[46,51]. Of note, these proinflammatory macrophages constituted the predominant macrophage population and were exclusively expressed in atherosclerotic lesions, and were absent in healthy aortas[47,48,51].

Dendritic cells

Like macrophages, dendritic cells stem from circulating monocytes. Tissue specific microenvironmental cues, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, and PAMPs promote differentiation of monocytes into immature DCs. Immature DCs exhibit relatively high phagocytic capacity, and are specialized in capturing antigens from

their surroundings. Upon maturation through TLR stimulation or cytokine signaling, DCs upregulate the expression of major histocompatibility complex class-II (MHC-II) molecules, co-stimulatory molecules (e.g., CD80, CD86), and chemokine receptors, and develop increased antigen-processing and antigen-presenting capabilities[52]. This results in efficient presentation of foreign antigens on MHC-II, which can be recognized by $CD4^+$ T-cells. Moreover, exogenous antigens can also be cross-presented, resulting in their presentation on MHC-I, enabling the activation of $CD8^+$ T-cells. Upon maturation, DCs acquire the ability to migrate from the periphery towards secondary lymphoid organs via CCR7 signaling, to present antigens to T-cells and initiate adaptive immunity.

In the atherosclerotic lesion, DCs are considered to contribute to inflammation through the secretion of proinflammatory cytokines, including TNF-α and IL-12[53,54]. In early atherosclerosis, dendritic cells are considered to phagocytose oxLDL, resulting in a foamy phenotype. These early-stage foamy DCs maintain their capacity to migrate to secondary lymphoid organs and prime naïve T-cells. As the disease progresses, however, DC emigration from the plaque appears defective, resulting in the accumulation of stationary DCs in the atherosclerotic lesion[54]. Although the mechanism behind the impaired migration is not fully understood, it seems that the expression of CCR7 is inadequate under hyperlipidemic conditions. In the atherosclerotic lesion, T-cells have been shown to colocalize with DCs, and the retention of mature DCs in the plaque has been associated with enhanced local inflammation and local activation of effector/memory T-cells[55].

ADAPTIVE IMMUNE SYSTEM

The adaptive immune system consists of highly specialized lymphocytes, including B- and T-cells, that are capable of recognizing and responding to specific antigens with tailored immune responses. Single-cell analysis of human and mouse atherosclerotic lesions demonstrated that lymphocytes particularly concentrate in the lesions during advanced disease stages[48,56–58].

Although the adaptive immune system does not appear to be necessary for the development of advanced atherosclerotic lesions in mice, several studies in immunocompromised *scid/scid*, *Rag1*$^{-/-}$ or *Rag2*$^{-/-}$ mice, lacking an adaptive immune system, crossed with atherosclerosis prone *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice demonstrated that the adaptive immune system significantly accelerates lesion progression[59–63]. Song and colleagues demonstrated that the adaptive immune system substantially contributes to lesion development[62]. After 8 weeks on a Western-type diet (WTD), lesion development was reduced by 54% in double knockout mice, as compared with matched *Ldlr*$^{-/-}$ controls. Of note, this effect subsided over time. Moreover, fatty streak formation was reduced by 73% in *scid/scid Apoe*$^{-/-}$ mice that were fed a chow diet for 18 weeks[63], underlining the atherogenic capacity of the adaptive immune system.

B-cells

B-cells are specialized in the production of antibodies in response to antigen recognition via the B-cell receptor (BCR). During B-cell development in the bone marrow, sequential recombination involving heavy and light chain V(D)J genes leads to the formation of distinct B-cell receptors for each B-cell clone. These BCRs, and their soluble variants –also known as antibodies or immunoglobulins– recognize and bind to specific antigens, marking them for destruction by other immune cells or neutralizing their effects. Antibodies can be subdivided into five major subclasses, including IgM, IgD, IgG, IgE and IgA[64]. Upon antigen recognition, B cells become activated, undergo clonal expansion, and ultimately differentiate into plasma cells, which are capable of producing large quantities of antibodies. Moreover, B-cells serve as antigen presenting cells, facilitating antigen presentation to $CD4^+$ T-cells.

In the context of atherosclerosis, B-cells play a multifaceted role, contributing to both protective and pathogenic aspects of the disease. B-cells involved in atherosclerosis can be subdivided into B1 cells and marginal zone B cells, which are considered to produce atheroprotective IgM antibodies[65,66], follicular B2 cells, which promote atherosclerosis by secreting IgG antibodies and proinflammatory cytokines[67], and regulatory B-cells, that mitigate inflammation by secreting immunosuppressive cytokines (e.g., IL-10 and TGFβ)[68,69]. Auto-antibodies against plaque enriched proteins (oxLDL[70] and apoB100[71]) have been detected both healthy individuals, as well as cardiovascular disease patients. While anti-oxLDL IgM antibodies exert atheroprotective effects by reducing oxLDL-induced endothelial cell activation and foam cell formation[65,66], the function of other anti-oxLDL antibody remains debatable[67].

In mice, B-cells are generally considered to be atherogenic. Knockout of the immunoglobulin μ heavy chain (μMT) in apoE$^{-/-}$, resulting in a global loss of B-cells, significantly decreased lesion progression[72]. This atherogenic effect of B-cells was mainly attributed to their interaction with CD4^{+} T-cells, as a transfer B-cells lacking the expression of MHC-II, or co-stimulatory molecule CD40 into μMT$^{-/-}$apoE$^{-/-}$ mice failed to restore the atherogenic effect of B-cells. Moreover, transfer of wildtype B-cells into μMT$^{-/-}$*Apoe*$^{-/-}$ mice accelerated atherosclerosis and increased intralesional CD4^{+} T-cell numbers. Upon interaction, B cells can promote T cell activation, proliferation, and differentiation into effector subsets through antigen presentation and cytokine production.

T-cells

The second type of antigen-specific cell comprises T-cells, which can be subdivided into CD4^{+} and CD8^{+} T-cells. The former is classically known as T-helper cells, and is specialized in modulating the immune response by secreting cytokines. The latter, also known as cytotoxic T-cells, are renowned for killing virus infected or tumor cells, by releasing cytolytic molecules (e.g., granzymes, and perforin). T-cells originate from hematopoietic stem cells, which undergo maturation and selection in the thymus, hence earning the name “T-cells”. In the thymus, CD4^{+}CD8^{+} double positive precursors develop into two lineages, depending on the T-cell receptor (TCR) chains they obtain; the αβ or γδ lineage[73,74]. Each TCR has an antigen binding domain, constructed of a variable (V), diversity (D) and joining (J) segment. Through somatic DNA recombination, every T-cell obtains a unique TCR constructed of different V, D and J segments. This process is crucial for generating a diverse repertoire of T-cells, capable of recognizing a wide range of exogenous antigens. To avoid self-reactivity of T-cells against endogenous proteins T-cell precursors are selected. T-cells undergo negative selection to avoid autoimmunity. During negative selection, T-cells strongly engaging with self-peptides-MHC complexes are eliminated through apoptosis. Additionally, T-cells are positively selected for their capability to interact with MHC-peptide complexes; ensuring that only functional T-cells remain[75,76].

Upon selection, precursors will further develop into CD4^{+} or CD8^{+} T-cells, and naïve T-cells are released into circulation where they search for their cognate antigen presented on MHC-II (or human leukocyte antigen class-II [HLA-II] in humans) for CD4^{+} T-cells, or MHC-I (or HLA-I in humans) for CD8^{+} T-cells. These molecules present peptides typically ranging from 13-25 amino acids for MHC-II[77], and 8-11 amino acids for MHC-I[78]. Upon recognition of their cognate antigen, along with the proper molecular and chemical co-stimulation, T-cells undergo clonal expansion. Based on the co-stimulatory and chemical signals provided by the APCs and the microenvironment, T-cells can differentiate into different subtypes of effector cells, including Th1/Tc1, Th2/Tc2, Th17/Tc17 and ThReg/TcReg cells, whose functions are later discussed[79]. When inflammation is resolved, a subset of activated T-cells acquires a long-lived

memory phenotype, enabling for continuous surveillance and an immediate and robust response upon re-encountering the same antigens.

T-CELLS IN ATHEROSCLEROSIS

Recent single-cell advancements allowed for detailed analysis of the atherosclerotic microenvironment, demonstrating that T-cells constitute a variable but substantial proportion of the leukocyte population within the atherosclerotic lesions from both humans and mice[46–49,56,58,80,81]. The proportion of T-cells ranges between 20-65% of the immune cells infiltrate in human atherosclerotic lesions[48,58,82], and between 6-65% in mice[47,49,56,80]. T-cells accumulate mainly in the shoulder region, the fibrous cap, and the intima of human lesions[83]. Moreover, they are located adjacent to the atherosclerotic lesion in the adventitial tissue or artery tertiary lymphoid tissue.

As a whole, T-cells have been shown to exert a proatherogenic function. The absence of TCRαβ$^+$ CD4$^+$ and CD8$^+$ T-cells in *Tcrβ$^{-/-}$Apoe$^{-/-}$* mice protected against fatty streak formation, with a 49.4% reduction in lesion formation after 18 weeks and 38% reduction in lesion size after 1 year[84]. Despite the cumulative proatherogenic effect of T-cells, distinct CD4$^+$ and CD8$^+$ T-cell subsets may affect atherosclerosis differently.

CD4$^+$ T-cells in atherosclerosis

CD4$^+$ T-cells are generally considered to be pro-atherogenic mediators. Multiple studies have demonstrated that CD4$^+$ T-cell depletion, either by genetic manipulation, or via antibody-mediated depletion, results in a significantly reduced progression of atherosclerosis. In the late nineties, Emeson and colleagues demonstrated that CD4$^+$ T-cell depletion in wildtype mice fed an atherogenic diet resulted in an approximate atherosclerosis reduction of 70%[85]. A similar reduction in lesion development was observed in *Cd4$^{-/-}$Apoe$^{-/-}$* mice[86]. Likewise, transfer of CD4$^+$ T-cells into immunocompromised *scid/scid Apoe$^{-/-}$* mice resulted in a 164% increase in atherosclerotic lesions size, which was associated with significantly elevated circulating IFN-γ levels[63]. Conversely, there are also reports suggesting an atheroprotective function of CD4$^+$ T-cells, by ameliorating lesion formation in the aorta[84,87]. This might be due to the large discrepancy in T-cell function of different T-cell subsets.

CD4$^+$ T-cell (Th) subsets

The contradictory roles of T-cells in atherosclerosis may be attributed to their heterogeneous phenotypes and the diverse subsets that have been identified. Depending on co-stimulatory signals and cytokine exposure, T-cells can differentiate into various phenotypes, each playing a distinct role in regulating immune responses and maintaining tissue homeostasis. Additionally, T-cells can adapt their phenotypes based on microenvironmental cues, a phenomenon known as plasticity. Several T-cell subtypes have been implicated in the progression of atherosclerosis[88]. Single-cell analysis of human and mouse atherosclerotic lesions demonstrated that intralesional T-cells can obtain a wide variety of phenotypes, including a Th1, Th2, Th17, or a ThReg phenotype[48,57,58].

Th1

Exposure of T-cells to IL-7, IL-12, IL-27, and IFN-γ skews T-cells towards a Th1 phenotype, as well as co-stimulation via CD40-CD40L[89], or CD80-CD28[90]. Th1 cells are characterized by the expression of the transcription factor T-bet, chemokine receptors CXC-chemokine receptor 3 (CXCR3) and CC-chemokine receptor 5 (CCR5), and the secretion of pro-inflammatory cytokines, including IFN-γ and TNFα[91,92]. In the context of atherosclerosis, Th1 cells are the predominant CD4$^+$ T-cell subtype and are considered proatherogenic and associate with

increased inflammation and accelerated lesion formation[93,94]. Indeed, T-bet deficiency, or a knockout of IFN-γ or its receptor significantly suppresses atherosclerotic lesion formation[93,95,96], while administration of IFN-γ to *Apoe*$^{-/-}$ mice enhances lesion progression[97]. Besides IFN-γ, other Th1 produced cytokines have also been shown to contribute to lesion development. For instance, the inhibition of TNFα or suppression of its receptor, TNFR1, protects against atherosclerosis[98,99].

Th2

Exposure of T-cells to IL-4, IL-5, and IL-13, and co-stimulation via CD96-CD28 drives differentiation of T-cells towards a Th2 phenotype[90,100]. Th2 cells are characterized by the expression of transcription factor GATA3, and enhance Th2 differentiation by producing IL-4, IL-5, IL-13, and IL-33. The function of Th2 cells in the atherosclerotic lesion remains controversial. Higher circulating Th2 numbers have been associated with reduced subclinical disease burden, in terms of decreased carotid intimal media thickness[101] and their IL-4 production negatively correlated with the risk for myocardial infarction and stroke[102]. There is, however, no clear consensus on the function of IL-4 within the atherosclerotic lesion. IL-4 has been shown to antagonize Th1 responses and protects against atherosclerosis[103], while others report an atherogenic role of IL-4, as its depletion prevented lesion formation[104]. Additionally, multiple studies indicate that IL-4 levels do not affect lesion progression[105,106]. Other signature Th2 cytokines, including IL-5, IL-13, and IL-33 are considered to be atheroprotective, as increased plasma levels of these cytokines are associated with mitigated lesion development[107–109].

Th17

Th17 cells are induced by exposure of T-cells to IL-6, TGF-β, IL-1β, and IL-23, and are characterized by their expression of RORγT and secretion of IL-17, IL-21, IL-22, and potentially IL-10. The signature cytokine produced by Th17 cells, IL-17, is considered pro-atherogenic. Knockout or inhibition of IL-17, or its associated receptor, protected against atherosclerosis after Western type diet in *Ldlr*$^{-/-}$ mice[110–113]. Moreover, the IL-17-producing Th17 have been associated with autoimmunity and affect the progression of atherosclerosis via activation of endothelial cells, macrophage recruitment, and cytokine production[111–113].

Th17 cells have been shown to be particularly plastic within the atherosclerotic lesion, with subtypes simultaneously expressing IL-17 and IFN-γ or IL-17 and IL-10[114,115]. These divergent phenotypes may be induced by environmental cues. For instance, exposure of T-cells to IL-6 and TGF-β induces a less pathogenic Th17 subtype that produces IL-10 concomitantly with IL-17, which suppresses inflammation and ameliorates atherosclerosis[116,117]. Interestingly, antigen-specific multilineage-committed T-cells have been detected in circulation of cardiovascular disease patients. ApoB100 specific CD4^{+} T-cells have been shown to exhibit a plastic phenotype during disease progression, switching from a regulatory phenotype during early lesion development, towards a Th17/Th1 phenotype as the disease progresses[118,119].

ThReg

These regulatory T-cells are crucial for maintaining self-tolerance and are notorious for their immunosuppressive capacity, which is essential for resolving tissue damage in inflammation. Approximately 5-10% of circulating CD4^{+} T-cells exhibit a regulatory phenotype during normal physiology. T-cells can differentiate into Tregs upon exposure to cytokines IL-2, IL-10 and TGF-β, co-inhibition by APCs via PD-1L and CTLA-4, or chemicals including retinoic acid and rapamycin[120]. Signature molecules expressed by regulatory T-cells include transcription factor FOXP3, CTLA-4, LAG-3 and CD25 (IL-2RA), along with the secretion IL-

10, IL-35, and TGF-β[121]. IL-10 and TGF-β mitigate inflammation by inhibiting B- and T-cell activation, promoting immune tolerance against self-peptides, and driving immune cell differentiation towards a regulatory phenotype. Both IL-10 and TGF-β protect against atherosclerosis development[122,123].

Besides modulating immunity with immunosuppressive cytokines, Tregs can inhibit co-stimulation by APCs by binding to CD80/CD86 or MHC-II with CTLA-4 or LAG-3, respectively, reducing their ability to activate effector T-cells[121,124]. Additionally, Tregs can also induce the expression of indoleamine 2,3-dioxygenase (IDO) in dendritic cells, resulting in tryptophan catabolism and suppressed T-cell proliferation. Tregs can also directly dampen effector T-cell responses. They inhibit effector T-cell expansion by producing adenosine via CD39 and CD73, which binds to the adenosine receptor and inhibits T-cell expansion. Besides secreting adenosine, they depriving effector T-cells of nutrients, by consuming high levels of IL-2, which is essential for the survival of effector T-cells.

The protective function of $CD4^+$ ThRegs has been widely acknowledged in the context of atherosclerosis. Removal of $CD25^+CD4^+$ T-cells, including activated $CD4^+$ T-cells and regulatory $CD4^+$ T-cells, enhanced atherosclerotic lesion formation, suggesting that naturally occurring $CD4^+$ T-cells control lesion development[125]. Moreover, complete ablation of $Foxp3^+$ ThRegs in *Ldlr*$^{-/-}$ mice during the progression of atherosclerosis significantly enhances progression of atherosclerosis[126,127]. Authors suggest that besides mitigating inflammation, ThRegs may also affect the lipid profile and reducing circulating cholesterol levels[126]. Moreover, clinical data indicates a strong inverse correlation between Threg numbers and IL-10 levels, and myocardial infarction[128,129].

Increasing ThReg numbers using IL-2-complexes[130,131], anti-CD3 therapies[132] or adoptive transfer of ThRegs[133] offer promising strategies to combat atherosclerosis. These therapies have proven efficient in shifting the ThReg versus T-cell ratio, and protecting against atherosclerosis. However, these therapies do not necessarily expand naturally occurring, or antigen-specific ThRegs. Antigen-specificity of ThRegs has been shown to boost ThReg function and might be essential for controlling atherosclerosis. Absence of antigen recognition by $CD4^+$ T-cells aggravates atherosclerosis, potentially due to the loss of antigen-specific $CD4^+$ T-cells[134,135].

Autoreactive $CD4^+$ T-cells in atherosclerosis

The presence of activated T-cells in the plaque suggests there is an autoimmune component to atherosclerosis, including accumulation of T-cells that exhibit a strong memory phenotype with hallmarks of chronic stimulation and T-cell exhaustion, apparent from a large proportion of $CD4^+CD45RA^{low}CCR7^{low}$ TEM and high expression of activation markers CD69 and CD38[58,136], and the presence of circulating autoantibodies in patients with atherosclerosis[71]. These hallmarks indicate the potential of self-reactive $CD4^+$ and $CD8^+$ T-cells within the atherosclerotic lesion. Recently, single-cell TCR sequencing of human endarterectomy samples revealed clonally expanded TCR repertoires within the atherosclerotic lesion[81,136,137].

Interestingly, the clonal expansion level of $CD4^+$ T-cells within the atherosclerotic lesion appeared to be much more pronounced in comparison to their circulating counterparts[136], supporting the hypothesis that $CD4^+$ T-cells are activated and clonally expand in response to atherosclerosis related auto-antigens. Moreover, $CD4^+$ T-cell clones enriched in the atherosclerotic lesion exhibited substantially more activated phenotype[136]. These potentially antigen-specific $CD4^+$ T-cells have been suggested to hold atheroprotective properties, as the absence of the antigen presentation on MHC-II aggravates atherosclerosis[134]. Of note, in this study, the $CD4^+$ T-cells, including ThRegs, were virtually abolished, potentially explaining the observed phenotype.

Indeed, auto-reactive $CD4^+$ T-cells recognizing oxLDL-[138] or ApoB100-derived[118,119] antigens have been detected in circulation of patients with cardiovascular disease. For instance, approximately 0.17% of the circulating $CD4^+$ T-cells population has been shown to be reactive against ApoB100 peptide P18 (SLFFSAQPFEITAST)[118]. The majority of these ApoB100 specific $CD4^+$ T-cells exhibit a regulatory phenotype in healthy individuals and healthy mice, but differentiate towards a more pro-atherogenic multi-lineage exThReg phenotype (co-expressing FoxP3 and Tbet, or Foxp3 and Rorγt) during disease progression[118,119]. Based on the phenotype switching, it seems plausible that ApoB100 reactive $CD4^+$ T-cells hold atheroprotective properties in health and during initial disease stages, but become atherogenic in advanced atherosclerosis.

The function of antigen-specific T-cells in atherosclerosis may be studied in more detail by therapeutically inducing a T-cell response against a specific target within the atherosclerotic lesion, using vaccination strategies or engineered T-cells (e.g. TCR-engineered T-cells, exhibiting genetically engineered TCRs; or CAR-T-cells, expressing a chimeric antigen receptor (CAR)). Since the 1960s numerous vaccination studies demonstrated the atheroprotective effects of T-cells directed against LDL, oxLDL or ApoB[118,139–143]. The protective effects of immunization with whole ApoB100, or ApoB100 derived peptides, have been mainly attributed to the significant induction of IL-10 producing ThRegs[118,144].

While vaccination against LDL, oxLDL, or ApoB has shown an atheroprotective effect, immunization targeting other antigens within the atherosclerotic lesion may impact plaque formation differently. For instance, a dual effect has been observed upon immunization against heat shock protein 60. Heat shock proteins are produced by any cell type in response to stressful conditions. Vaccination against HSP60 has demonstrated heterogeneous effects, with some studies reporting enhanced atherogenesis following vaccination[145,146], while others observed an immunosuppressive effect and reduced lesion progression[139,147].

$CD8^+$ T-cells in atherosclerosis

Although there is a general consensus about the function of $CD4^+$ T-cells in the atherosclerotic lesion, the role of $CD8^+$ T-cells remains more debatable. Depletion of $CD8^+$ T-cells resulted, on the one hand, in an atheroprotective effect by reducing intralesional macrophage content and enhancing lesion stability[149]. On the other hand, $CD8^+$ T-cell depletion has been shown to accelerate lesion progression and was associated with increased cellular infiltrate into the atherosclerotic lesions, including an increased number of macrophages, and decreased lesion stability[150,151]. The atherogenic effect of $CD8^+$ T-cells seems primarily attributable to those producing cytolytic mediators such as perforin, granzyme B (GZMB), or TNFα[150]. Additionally, there is a substantial amount of data concluding that the overall impact of $CD8^+$ T-cells does not significantly contribute to lesion development[84,85,152].

$CD8^+$ T-cell (Tc) subsets

Similar to $CD4^+$ T-cells, $CD8^+$ T-cells can be divided into various subsets based on their transcription factor profiles and cytokine production, including Tc1, Tc2, Tc17 and TcReg $CD8^+$ T-cells. While the contribution of different $CD4^+$ T-cell subsets to atherosclerosis has been well characterized, the function of the different $CD8^+$ T-cell subsets is less understood.

IFN-γ-producing Tc1 $CD8^+$ T-cells are generally considered to be atherogenic, as IFN-γ production by $CD8^+$ T cells has been shown to regulate monopoiesis during early lesion development in *Ldlr*$^{-/-}$ mice[151]. However, some studies have reported controversial results, suggesting that IFN-γ production by CD8+ T cells does not affect atherosclerosis or may even reduce vascular stenosis[153,154].

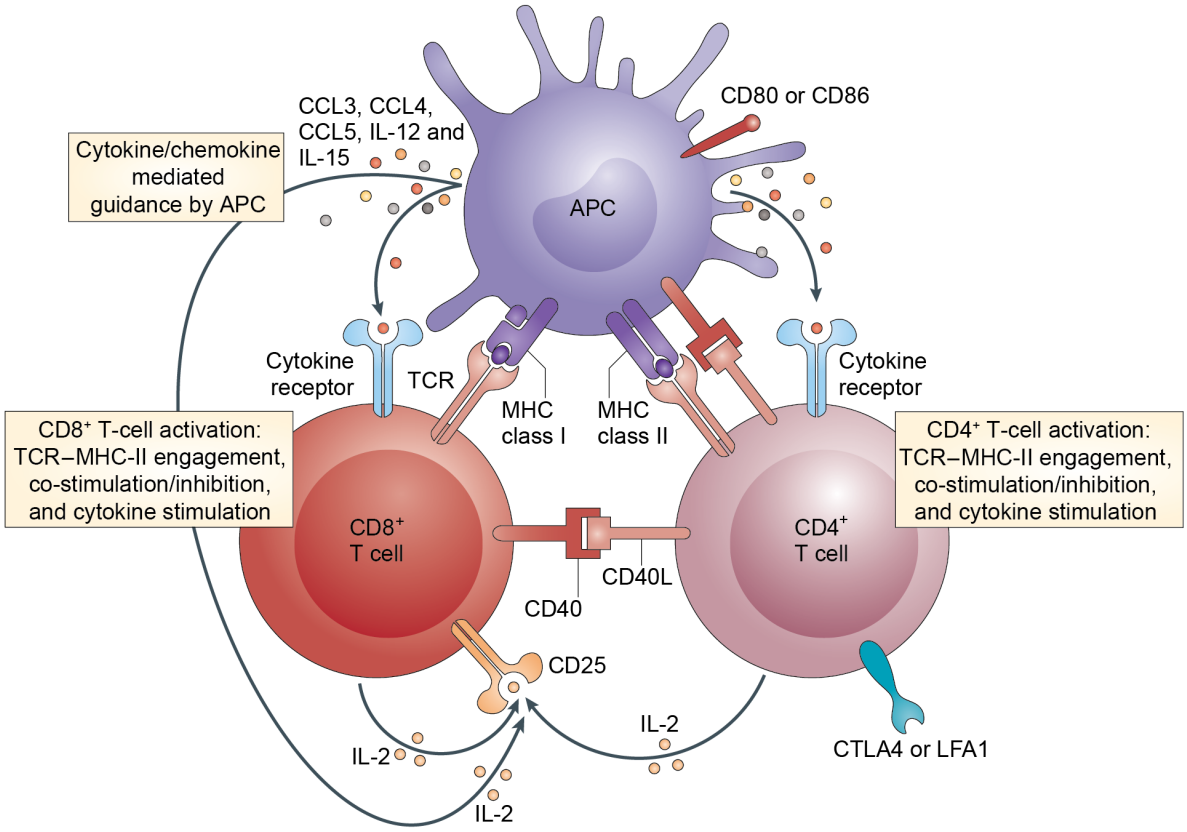

Figure 2. CD4⁺ and CD8⁺ T-cell activation. Activated antigen-presenting cells (APCs) recruit lymphocytes by secreting cytokines and chemokines. T-cells are activated through interaction with their cognate antigen presented on MHC-I/HLA-I for CD8⁺ T-cells and MHC-II/HLA-II for CD4⁺ T-cells by APCs, eliciting a highly specific immune response. This antigen binding, along with co-stimulatory or co-inhibitory signals (e.g. CD80/CD86-CD28/CTLA-4, CD40-CD40L) and cytokine secretion from APCs, determines the extent of T-cell activation and initiates a cascade of intracellular signaling events that lead to T-cell proliferation, differentiation, and cytokine production. CD4⁺ T-cells provide essential help to CD8⁺ T-cells during activation through the secretion of cytokines and additional co-stimulatory signals. CCL3-5, CC-chemokine ligand 3-5; IL-12/15, interleukin 12/15; TCR, T-cell receptor; CTLA4, cytotoxic T lymphocyte antigen 4; LFA1, lymphocyte function-associated antigen 1. *Adapted from Laidlaw et al. (2016) Nat. Rev. Immunol 16(2):102-11*[148].

In addition to Tc1, the Tc17 subset also exhibits a dual function. Elevated levels of circulating IL-17+ CD8+ T-cells associate with increased myocardial infarction risk, indicating a pathogenic role in cardiovascular disease. Conversely, our research, elaborately discussed in **chapter 2**, demonstrates a non-atherogenic function for Tc17 cells.[156]. In contrast to the Tc1 and Tc17 subsets, TcReg CD8⁺ T-cells hold a strong immunosuppressive capacity and have been associated with an atheroprotective function. Depletion of CD25⁺CD8⁺ T-cells, which includes both activated and regulatory CD8⁺ T-cells, aggravated atherosclerosis, while adoptive transfer with CD25⁺CD8⁺ T-cells ameliorated lesion growth[157]. Moreover, disrupted interaction between CD8⁺ T-cells and Qa-1, a nonclassical MHC-I molecule that induces a regulatory phenotype in CD8⁺ T-cells, results in accelerated atherosclerosis progression, expansion of follicular helper T-cells and germinal center B-cells, increased plasma antibody concentrations, and formation of adventitial ectopic germinal centers in atherothrombotic arteries[158–160]. In

other chronic inflammatory and autoimmune diseases Qa-1-restricted $CD8^+$ T-cells have been associated with maintaining self-tolerance and preventing auto-immunity[160].

Autoreactive $CD8^+$ T-cells in atherosclerosis

In contrast to $CD4^+$ T-cells, understanding the implications of auto-reactive $CD8^+$ T-cells in atherosclerosis has proven to be more challenging. Single-cell TCR sequencing of human endarterectomy samples demonstrated that although half of the plaque-derived $CD8^+$ T-cells exhibit a clonally expanded TCR, their level of clonal expansion was lower compared to those in circulation[136]. Despite clonal $CD8^+$ T-cell expansion not being exclusive to atherosclerotic lesions, these clonally expanded $CD8^+$ T-cells in plaques exhibited a significantly more cytotoxic expression profile compared to singular $CD8^+$ T-cell clones. Additionally, $CD8^+$ T-cell clones enriched in plaques displayed a highly activated phenotype, suggesting interactions with antigens presented within the atherosclerotic lesion[136]. These antigen-specific $CD8^+$ T-cells may protect against the progression of atherosclerosis, as the absence of MHC-I molecules significantly exacerbated fatty streak formation[161]. Interestingly, lesion development was not affected in a similar study setup where antigen recognition by $CD8^+$ T-cells was prevented using *Tap1*$^{-/-}$ mice, which lack the machinery for antigen processing[162]. These studies underscore the inconsistent findings regarding antigen-specific $CD8^+$ T-cells in the context of atherosclerosis, highlighting the need for elaborate research to elucidate their function.

The effect of antigen-specific $CD8^+$ T-cells in the atherosclerotic lesion may highly depend on the cell type that they are targeting. $CD8^+$ T-cells directed against smooth muscle cells, for example, have been shown to enhance vascular inflammation and lesion progression[163], while vaccination against endothelial cell markers, including CD99[164] and VEGFR2[165], attenuated atherosclerosis by inhibiting leukocyte infiltration and angiogenesis, respectively. $CD8^+$ T-cell responses directed against macrophages and foam cells within the atherosclerotic lesion may have dual effects. $CD8^+$ T-cells against the P210 peptide of ApoB100 induced through immunization, attenuate atherosclerosis by reducing plaque size and intralesional macrophage infiltrate[166–168]. Interestingly, other studies attributed the protective effect of P210 to either its associated regulatory T-cell responses[169,170], or the production of atheroprotective antibodies by B-cells[171–173]. Attempts to induce a $CD8^+$ T-cell response against other fragments of the ApoB100 protein, including vaccinating humanized mice with ApoB100 peptides predicted to bind HLA-A2 *in silico*, remained unsuccessful[174].

Controversially, macrophage killing by $CD8^+$ T-cells has also been associated with increased atherosclerosis in *Apoe*$^{-/-}$ mice that harbor a deficiency for E3-ligase CBL-B[175]. The increased production of IFN-γ and GZMB by $CD8^+$ T-cells has been associated with enhanced macrophage killing and accelerated atherosclerosis progression[153]. It has been hypothesized that increased macrophage apoptosis during early disease stages has an protective effect, while it seems enhances lesion development during later disease stages[176]. This might be explained by the increased expression of CD47, the "don't eat me" signal, that is increasingly expressed during disease progression, and results in apoptotic cell accumulation followed by secondary necrosis[177].

In addition to spontaneously occurring autoimmune responses against the atherosclerotic lesion, various studies suggest that pathogenic infections may elicit a mimicry response, potentially resulting in autoimmunity. Antibodies against bacterial HSPs, targeting mycobacterium tuberculosis HSP65, Escherichia coli HSP60, and 60-kD chlamydial HSP, for instance, appeared to be recognize specific human HSP60 epitopes as well[145,178]. Chowdhury and colleagues suggested the presence of cross-reactive virus- and self-specific $CD8^+$ T-cells in the atherosclerotic lesion[179]. They demonstrated that $CD8^+$ T-cells expressing a virus associated

$TCR_{\alpha\beta}$ (CASSIGLYGYTF-CAMSGGAGGTSYGKLTF), responsive to immunodominant influenza peptide GILGFVFTL, are also reactive against cells expressing TSPAN17 or ZIP9, two proteins highly expressed by endothelial cells, smooth muscle cells and fibroblasts. These cross-reactive $CD8^+$ T-cells may modulate the progression of atherosclerosis.

Bystander T-cells

Besides activation through TCR engagement, T-cells can also become activated upon exposure to a pro-inflammatory environment. This antigen non-specific form of T-cell activation is also known as bystander activation. T-cells can obtain a bystander phenotype upon exposure to type-1 IFN, IL-12, IL-15, IL-18, IL-1, and TNF-α[180]. These bystander $CD8^+$ T-cells undergo homeostatic expansion[181], and produce pro-inflammatory mediators including cytokines (e.g., IFN-γ[182]), and cytolytic molecules (e.g., GZMB and NKG2D[183]). The activity of bystander $CD8^+$ T-cells is significantly influenced by the type and level of cytokine exposure. For example, engagement of IL-18R leads to increased IFN-γ production by $CD8^+$ T-cells, while exposure to IL-15 boosts their cytotoxic abilities[180]. T-cells are exposed to many different cytokines within the atherosclerotic lesion, including type-1 IFNs, IL-12, IL-15, IL-18, IL-1, and TNF-α, potentiating a bystander $CD8^+$ T-cell response.

TOOLS FOR IMMUNO-PROFILING OF T-CELLS

Single-cell analysis of the atherosclerotic lesion

To gain detailed insights into the cellular and molecular heterogeneity within atherosclerotic lesions, various techniques are employed to analyze cellular composition at single-cell resolution. In the 1980s, the usage of immunostainings was proposed, allowing for the detection of two markers simultaneously[184]. Currently, this technique has evolved allowing for the staining of up to 16 markers[185]. Immunostainings are used for protein-level analysis, and can be used to visualize markers using microscopy, flow cytometry or Cytometry by Time-of-Flight (CyTOF). Flow cytometry analysis allows for the detection and quantification of particle size, complexity and the presence of 1-20 fluorescently labeled markers, as cells or particles flow in a fluid stream through a laser beam. CyTOF is based on a similar principle as flow cytometry, but uses a different detection method that is based on heavy metal isotopes conjugated to antibodies for cell labeling, enabling the detection of about 50 markers simultaneously[49,58].

Additionally to protein-level analysis, RNA-based analysis have tremendously advanced in recent years. Although bulk RNA sequencing, which measures the average gene expression of certain genes across a sample, was already introduced in 1995 by Schena et al.[186], in recent years, this technique has advanced to a single-cell resolution. Single-cell RNA sequencing (scRNAseq) enables high resolution quantification of the mRNA transcriptome for each cell. Moreover, multiple single-cell technologies can be integrated, allowing for combined profiling of the genome, epigenome, transcriptome, proteome, metabolome, and the immune repertoire to identify cellular characteristics[187]. These single-cell methodologies have uncovered novel cellular targets and provided new perspectives on signaling pathways and molecular mechanisms driving the progression of atherosclerosis. They represent a valuable tool for comprehending the pathophysiology underlying the progression of atherosclerosis.

T-cell receptor sequencing

An example of a combined multimodal sequencing technique is single-cell TCR sequencing (scTCRseq), combining scRNAseq and TCR sequencing. This technique allows for functional correlation between T-cell phenotype and TCR clonality, providing insights into the phenotypic characteristics and activation status of each clone. ScTCRseq forms a versatile and powerful approach for enhancing our understanding of T-cell biology in the atherosclerotic lesion[136].

While scTCRseq offers valuable insights into T-cell clonality and phenotype, it does not provide information on the specific antigens recognized by these T-cells. Despite numerous computational algorithms proposed to predict T-cell targets based on structural similarities in TCR composition or peptide sequence, they have not yet achieved satisfactory accuracy[188]. Other, more labor intensive tools are still required for uncovering the cognate antigens of T-cells, including 1) *in silico* screening for auto-peptides by identifying peptides with a high affinity for HLA-I or HLA-II, followed by a re-stimulation assay with the identified peptides, 2) identification of antigen-specific T-cells using peptide presenting tetramers, 3) or immunization of TCR-transgenic mice with certain peptides, followed by sequencing of the clonally expanded TCRs[71]. Although these tools have shown to be efficient in identifying TCR-antigen combinations, they rely on prior knowledge of potential targets.

To screen for potential T-cell targets in a more unbiased way, immunopeptidomics can be used. Immunopeptidomics is a branch of proteomics that focuses on the identification and characterization of peptides presented by MHC or HLA molecules[189]. To analyze which peptides are presented on MHC/HLA, cells are lysed and MHC/HLA molecules are isolated through immunoprecipitation. Peptides are eluted and purified from the precipitate, and analyzed by mass spectrometry. The mass spectrometry results are matched with a reference library of for example the human proteome, resulting in a list of presented peptides and their original proteins. Thus, this method poses an powerful tool for the identification of peptides that potentially contribute to autoimmunity.

THESIS OUTLINE

Atherosclerosis is the most prominent underlying pathology of cardiovascular disease and an important cause of major adverse cardiovascular events. Chronic inflammation drives growth and destabilization of atherosclerotic plaques. Recent single-cell technologies demonstrated that T-cells, including $CD4^+$ and $CD8^+$ T-cells, form a major immune population within human and mouse atherosclerotic lesions. These T-cells show signs of recent TCR activation, and clonal expansion, suggesting an autoimmune component in the progression of atherosclerosis. Although $CD4^+$ T-cells have been widely studied in the context of atherosclerosis, understanding the implications of $CD8^+$ T-cells in disease progression has proven to be more challenging, due to inconsistent findings. This thesis focuses on elucidating the effects of different $CD8^+$ T-cell subsets, and their potential targets, within the atherosclerotic lesion.

In **chapter 2**, we investigated the impact of Tc17 $CD8^+$ T-cells on the progression of atherosclerosis. In this study, *ex vivo* cultured Tc0 and Tc17 $CD8^+$ T-cells were adoptively transferred into *Cd8*$^{-/-}$*Ldlr*$^{-/-}$ mice. Atherosclerosis development of Tc17 or Tc0 transferred mice was compared to that of untreated *Cd8*$^{-/-}$*Ldlr*$^{-/-}$ mice. Our findings demonstrate that even though Tc17 cells accumulated in the atherosclerotic lesion, they appear to be non-atherogenic. In **chapter 3**, we studied the contribution of resident memory T-cells to the progression of atherosclerosis. By interrogating a single-cell RNA sequencing dataset of human atherosclerotic lesions, and integrating predefined T_{RM} cells, we identified a small proportion of T-cells that strongly exhibit a T_{RM} -associated transcription profile. The presence of T_{RM} cells in atherosclerotic lesions was corroborated using a mouse model that enabled tracking of Hobit. These lesion-derived T_{RM} constituted a minor T-cell population, and were characterized by the expression of CD69 and CD49α, and associated with a reduced amount of intralesional macrophages and increased collagen content.

Chapter 4 focuses on elucidating the effect of antigen recognition by T-cells in the atherosclerotic lesion. By employing various mouse models lacking antigen-specific $CD8^+$ T-cell interactions, we demonstrate that TCR engagement is not required for $CD8^+$ T-cells to

affect lesion development. Manipulation of antigen-specific $CD4^+$ T-cell responses on the other hand, was associated with reduced lesion stability and elevated intralesional $CD8^+$ T-cells numbers. To comprehend the discrepancy between $CD4^+$ and $CD8^+$ antigen recognition within the atherosclerotic lesion, antigen presentation on MHC-I/HLA-I and MHC-II/HLA-II was compared. Using $ApoB^{OVA\text{-}TG}$ mice, which are designed to present MHC-I and MHC-II ovalbumin peptides in atherosclerotic lesions, we demonstrate that $CD4^+$ T-cells become primed by ApoB-derived OVA peptides, whereas $CD8^+$ T-cells were not. Analyzing the immunopeptidome of human atherosclerotic lesions revealed a wide array of atherosclerosis-associated antigens presented on HLA-II, while only a limited number was presented on HLA-I. In **chapter 5**, we confirmed the presence of antigen non-specific $CD8^+$ T-cells in human atherosclerotic lesions. We identified virus-associated $CD8^+$ T-cells within human atherosclerotic lesions by matching TCRs from a human atherosclerosis scTCRseq dataset with databases containing TCRs of known specificity. These virus-associated $CD8^+$ T-cells exhibited a virtually identical phenotype compared to other $CD8^+$ T-cells within the atherosclerotic lesion, even though they appeared to be antigen non-specific due to the absence of pathogen-derived antigen presentation on HLA-I in human atherosclerotic lesions. In **chapter 6**, we aimed to obtain a deeper understanding of antigen-specific $CD8^+$ T-cell responses against ApoB100 derived antigens in the atherosclerotic lesion. As ApoB100 derived peptides do not prime naïve $CD8^+$ T-cells (**chapter 5**), $Ldlr^{-/-}$ or $ApoB100^{OVA\text{-}TG}$ mice received an adoptive transfer of αCD3 and αCD28 activated ovalbumin-specific OTI $CD8^+$ T-cells. This study demonstrates that previously activated OTI $CD8^+$ T-cells are capable of recognizing ApoB100-derived OVA antigens, and that targeting of ApoB100 presenting cells enhanced lesion stability by reducing necrotic core formation. Findings of these studies are summarized and discussed in **chapter 7**.

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