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Characterization of age-associated immunity in atherosclerosis

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Chapter 1

GENERAL INTRODUCTION

CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is an umbrella term for all diseases related to the heart and blood vessels, including ischemic heart disease, stroke, angina pectoris, and aortic aneurysm. Atherosclerosis is the main pathology underlying most CVDs and is characterized by the gradual buildup of atherosclerotic plaques, or lesions, in the arterial wall.¹ Continuous growing of plaques lining the arteries can result in narrowing of the artery, also called stenosis, which can obstruct normal blood flow and, depending on the location, cause symptoms. For example, patients can suffer from chest pain upon significant coronary artery stenosis.² When atherosclerosis affects the peripheral arteries, patients can experience intermittent claudication with concomitant major discomfort in the leg or even gangrene that endangers limb viability.³ More problematic and acute complications occur when destabilized atherosclerotic lesions erode or rupture, resulting in thrombus formation. These blood clots can completely occlude the blood vessel and lead to cardiovascular events, such as a stroke or a heart attack, depending on the tissue or organ affected.²

Risk factors such as smoking, high cholesterol levels due to unhealthy diet, and sedentary lifestyle are modifiable, while unmodifiable risk factors include age, sex, and genetic predisposition, such as familial hypercholesterolemia.^{3,4} Furthermore, people suffering from chronic inflammatory or autoimmune diseases (e.g., systemic lupus erythematosus and rheumatoid arthritis) are at increased risk to develop CVD.^{5,6} Sex is also an important risk factor, as CVD develops about 10 years later in women than in men⁷, but women have a poorer prognosis and are more likely to die following an acute cardiovascular event.⁸ Aging is considered the dominant risk factor for CVD which is illustrated by the globally increasing numbers of death caused by cardiovascular disease with increasing age (Figure 1).^{9,10} In fact, the estimated CVD-caused death rate in 70+ year old individuals (2,624 CVD deaths per 100,000 individuals) is 7-fold higher than in 50-69 year old individuals, and even 84-fold higher than in 15-49 year old individuals in 2019.¹⁰ Although we know that coronary atherosclerotic plaques are already present in a third of young individuals aged 20-29 years, about 85% of >50-year old asymptomatic individuals show evidence of coronary atherosclerosis.¹¹ As the average lifespan of humans is increasing, more people belong to the age group of 65 years and older. With CVD being the leading cause of death in this age group, CVD forms a global burden on healthcare and economy.

ATHEROSCLEROSIS

Healthy blood vessels are constructed of three layers: the intima, media and adventitia. The intima lines the lumen, the space where the blood circulates. The intimal layer is protected by a monolayer

of endothelial cells that in homeostatic conditions acts as a selective barrier for molecules to travel between the blood and tissue. The media mainly consists of vascular smooth muscle cells (VSMCs), while fibrous connective tissue construct the adventitial layer. In atherosclerosis, the protective layer is breached, allowing lipids and immune cells to enter the intimal layer that lead to the formation of atherosclerotic plaques. The pathophysiology of atherosclerosis can be categorized into an initial and advanced stage, which will be discussed below.

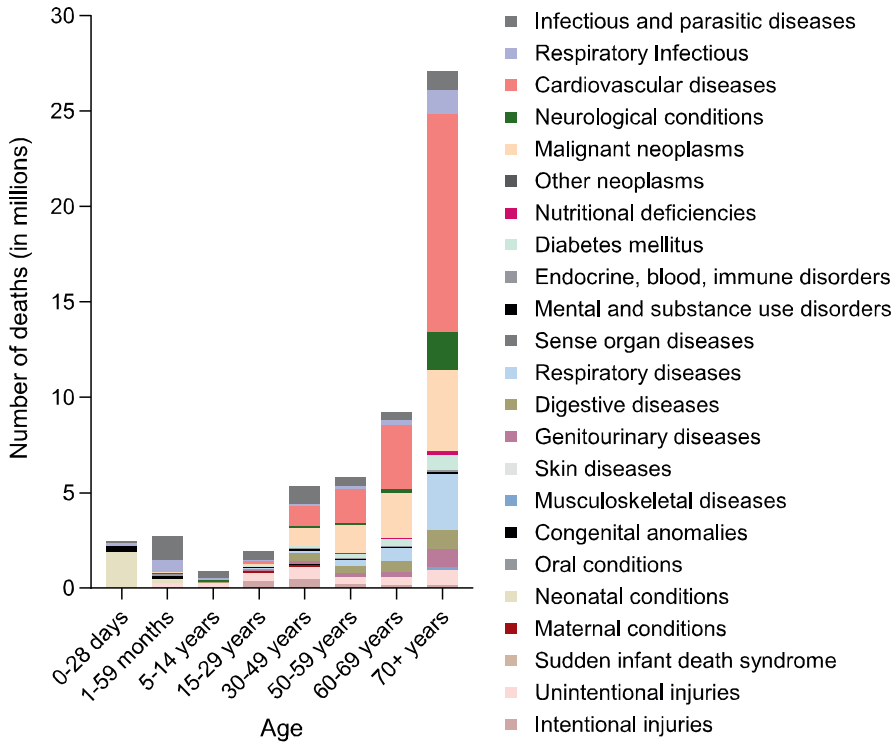


Figure 1. Age-specific causes of global mortality in 2019.

Number of total deaths (in millions) due to a specific cause is indicated by height of bar, with cardiovascular disease (red bar) being the leading cause of death in the age-group of 70+ years according to the Global Burden of Disease Study 2019. *Data from Global Burden of Disease Collaborative Network. Global Burden of Disease Study 2019 (GBD 2019) Reference Life Table (2021) Institute for Healthy Metrics and Evaluations (IHME).*¹⁰

Initial atherosclerosis

Atherogenesis is initiated by the entry of cholesterol-rich plasma lipoproteins in the sub-endothelial layer of the arterial wall, usually at sites of disturbed blood flow and endothelial dysfunction (Figure 2). Chylomicrons, very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) are apolipoprotein B (apoB)-containing carriers of water insoluble cholesterol, triglycerides, and other lipids. LDL is the most documented lipoprotein and is colloquially stigmatized as “bad cholesterol”, while high-density lipoprotein (HDL) is involved in

cholesterol efflux.^{1,12} Similar to LDL is lipoprotein(a), or Lp(a), which is an LDL particle with an apolipoprotein(a) attached to the apoB molecule. Lp(a) has gained attention since the last decade as an independent risk factor for CVD, because there is strong evidence for causality between Lp(a) levels and cardiovascular outcomes, even at low levels of LDL.¹³ When apoB-containing lipoproteins enter the intima, they are trapped by interactions with interstitial extracellular matrix components (e.g. collagen, elastin and proteoglycans) and are subjected to oxidative, enzymatic, and chemical modifications. These modifications form pro-inflammatory and immunogenic properties, which can activate endothelial cells and VSMCs. Subsequently, chemokine signaling, and expression of adhesion molecules, including VCAM-1, ICAM-1, P- and E-selectin, induce the migration of circulating monocytes across the endothelium into the intima where they differentiate into macrophages. Macrophages express scavenger receptors, such as CD36 and class A scavenger receptors (SR-A), which initiate the uptake and clearance of trapped lipoproteins. Excessive ingestion of lipoproteins, including intimal oxidized LDL (oxLDL), causes macrophages to exhibit a foamy appearance, hence they are named lipid-laden foam cells. The accumulation of foam cells, and initial infiltration of monocytes and macrophages lead to thickening of the intimal layer, or fatty streak formation. At this stage, the process is reversible, as a study showed that complete regression of fatty streaks can be achieved by sufficiently lowering plasma cholesterol levels.¹⁴ However, fatty streaks are generated in the subclinical phase of atherosclerosis disease and usually progress into advanced lesions that eventually can induce clinical symptoms.

Advanced atherosclerosis

Uptake of lipid particles by antigen-presenting cells, including macrophages and dendritic cells, also results in presentation of antigen to specific T cells that are then recruited to the plaque. These T cells secrete inflammatory cytokines and contribute to the ongoing inflammation. Chemokine signaling also leads to recruitment of other immune cells including neutrophils, mast cells and B cells. The continuous lipid accumulation and immune cell influx cause the development of advanced atherosclerotic lesions. Characteristic of advanced plaques are formation of a fibrous cap that surrounds an environment containing activated immune cells, cholesterol crystals and lipids. VSMCs migrate to the intima and accumulate below the endothelial layer, where they produce extracellular matrix molecules including collagen, and promote fibrous cap formation.¹⁵ Like macrophages, migrated VSMCs can take up lipid particles, and adopt a foam cell phenotype with macrophage-like features, thereby contributing to further lesion development.¹⁵⁻¹⁷ As the plaque progresses, continuous lipid uptake by macrophages and VSMCs cannot be maintained, due to excessive deposition of lipid particles in the arterial wall, leading to programmed cell death of foam cells. Phagocyte-mediated clearance of these apoptotic cells through efferocytosis is crucial for tissue homeostasis. However, excessive death of cells and insufficient removal by efferocytosis in advanced stages result in secondary necrosis.¹⁸ The consequent release of cellular contents (e.g. cellular debris

and lipids) and concomitant inflammation elicit the formation of a necrotic core in the plaque and recruitment of more immune cells. A thick fibrous cap and presence of extracellular matrix molecules are initially adequate to stabilize the plaque, with which patients can be asymptomatic for years.¹⁹ Over time, however, plaques become less stable due to increased inflammation, growing of the necrotic core, thinning of the fibrous cap, and degradation of extracellular matrix components mediated by decreased collagen production and increased production of matrix metalloproteases. Furthermore, calcified areas put mechanical stress on the plaque, which can trigger erosion or rupture of the plaque that eventually lead to thrombus formation and blocking of blood flow.²⁰ As a result, clinical manifestations such as a myocardial infarction or ischemic stroke occur.

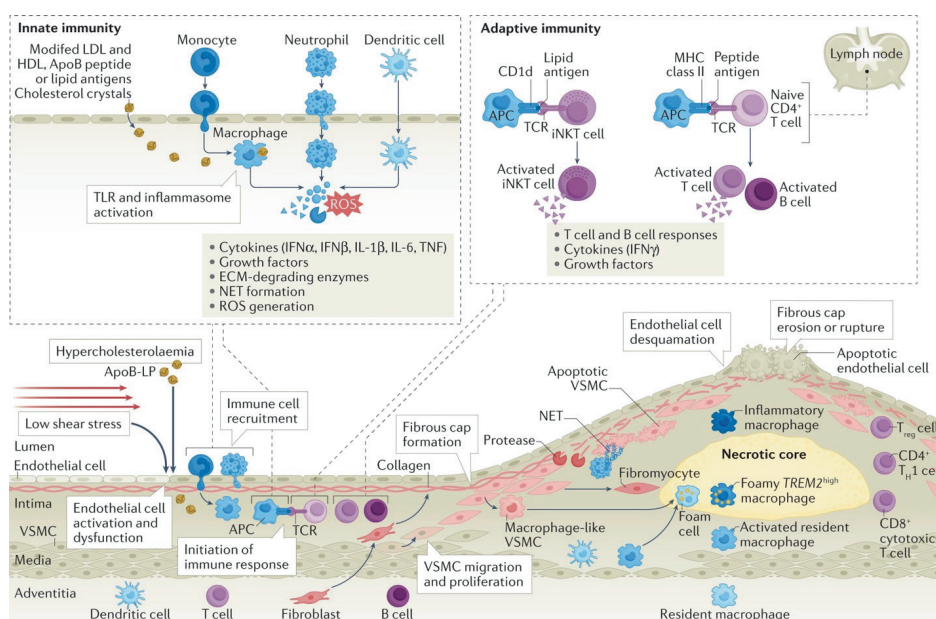


Figure 2. Atherosclerotic plaque development.

Atherosclerosis is initiated at sites of low shear stress where endothelial cells become activated and dysfunctional. Endothelial dysfunction allows entry of circulating ApoB-containing lipoproteins (ApoB-LP) in the intimal layer, where ApoB-LPs can undergo oxidative modifications. Modified ApoB-LPs are recognized by innate immune cells, which triggers responses promoting inflammation, including the secretion of cytokines and growth-factors, upregulation of co-stimulatory molecules and stimulation of monocyte recruitment to the subendothelial space, which subsequently differentiate into macrophages. Macrophages and migrated vascular smooth muscle cells (VSMCs) can take up lipoproteins in the subendothelial space and become lipid-laden foam cells that form a fatty streak. As the plaque progresses, excessive lipid-uptake by foam cells cannot be maintained and leads to apoptosis. Apoptotic cells are initially cleared by phagocytes, but excessive cell death and insufficient clearance result in secondary necrosis that leads to necrotic core formation. Additionally, antigen-presenting cells present antigens to T cells, which elicits T and B cell activation and recruitment to the atherosclerotic plaque where they further contribute to ongoing inflammation. Migration, activation and proliferation of VSMCs below the endothelial layer promote fibrous cap formation that initially stabilizes the plaque. Over time, however, plaques become less stable due to fibrous cap thinning, increased necrotic core formation and calcification. Subsequent erosion or rupture of the plaque leads to thrombosis and concomitant cardiovascular events. *Adapted from Engelen et al. (2022) Nat. Rev. Cardiol 19(8):522-542.*²¹

Widely-used preclinical models of atherosclerosis

Despite the utility of refined *in vitro* microvascular systems in mechanistic studies, animal models of atherosclerosis remain a necessary tool to understand the complex molecular mechanisms and inter-organ crosstalk underlying atherosclerotic plaque formation, progression and associated cardiovascular events. Advantages of animal models include inducibility of disease, reproducibility, control of environmental factors, and access to appropriate controls.²² Various vertebrate species have been used to model atherosclerosis, such as zebrafish, rats, rabbits, pigs, and non-human primates. The mouse remains the predominantly used animal model for atherosclerosis, because of its rapid reproduction, ease of genetic modification, cost-effective housing, and relatively short life span.²³ Notably, mice have a different lipoprotein profile compared to humans and are naturally resistant to atherogenesis. While most of the cholesterol in humans is transported in atherogenic LDL particles, HDL particles are the main cholesterol transporters in wildtype mice.²⁴ To bypass this, genetic and dietary manipulations affecting lipid metabolism were applied to develop appropriate mouse models of atherosclerosis. The two most commonly used genetically modified strains are the LDL receptor knockout (*Ldlr*^{-/-}) and the apolipoprotein E knockout (*ApoE*^{-/-}) mouse. Both strains exhibit increased concentrations of total plasma cholesterol that induce atherosclerotic plaque formation.

ApoE^{-/-} mice have increased VLDL and chylomicron fractions that elevate plasma cholesterol levels to 400–600 mg/dL, allowing spontaneous atherosclerotic lesion development when fed a regular chow diet (containing 4–6% fat and <0.03% cholesterol).²⁵ A Western diet (containing 21% fat and 0.15% cholesterol, similar to the everyday diet in Western countries) quadruples plasma cholesterol levels, thereby accelerating and exacerbating plaque development in *ApoE*^{-/-} mice.²⁶ Besides its role in lipid metabolism, apoE is also involved in immune regulation. Importantly, apoE deficiency can lead to altered cholesterol efflux, enhanced proliferation, leukocytosis and spontaneous development of ectopic lymphoid structures that may promote inflammation in this mouse model.^{27–29} In contrast, lipid metabolism and the immune system in *Ldlr*^{-/-} mice are less perturbed. *Ldlr*^{-/-} mice have mild hypercholesterolemia (total plasma cholesterol levels of 200–300 mg/dL), owing to an increase of mainly LDL particles, which shows a higher resemblance to the human lipoprotein profile. A western diet that increases plasma cholesterol levels to ~1000 mg/dL, is needed to induce plaque development in young *Ldlr*^{-/-} mice²³, which diverges from the actual plaque development in humans that occurs more gradually. Additionally, the use of an adeno-associated virus with a gain-of-function for pro-protein convertase subtilisin/kexin type 9 (PCSK9) can induce atherosclerosis in mice without germline genetic engineering.³⁰ PCSK9 is an enzyme that binds hepatic LDL receptors and marks them for degradation, resulting in reduced expression of LDL receptors and concomitant increased levels of circulating LDL.³¹ Overexpression of PCSK9 is established by a single intravenous injection of the virus, which generates a similar atherosclerosis phenotype as the *Ldlr*^{-/-} mouse.³²

THE IMMUNE SYSTEM IN ATHEROSCLEROSIS

The immune system is a physiological defense instrument that protects the body from infection and tissue damage, and it can be categorized into two arms: the innate and adaptive immune system. Most immune cells, or leukocytes, originate from hematopoietic stem cells in the bone marrow. These pluripotent stem cells give rise to two main categories of leukocytes: the myeloid and lymphoid lineage. Innate immunity comprised by myeloid cells include neutrophils, monocytes, macrophages, mast cells and dendritic cells. These cells are the first and immediate responders to danger signals and disease-causing pathogens, including bacteria and viruses. Pathogens express regular patterns of molecular structure known as pathogen-associated molecular patterns (PAMPs) that are not found in the host and can be recognized by pattern recognition receptors (PRR) present on innate immune cells. Besides PAMPs, PRR-bearing immune cells also recognize damage-associated molecular pattern molecules (DAMPs), which are endogenous cell-derived molecular structures that are a product of tissue damage or trauma. Recognition of such molecule patterns, or antigens, through PRRs activates the immune cells and induces an immune response. Some innate immune cell types, such as macrophages and dendritic cells can internalize and present antigens on their cell surface to lymphoid cells of the adaptive immune system, specifically T cells. Upon encountering an antigen for the first time, the adaptive immune response is relatively slow. Unique to the adaptive immune system, however, is their capability of acquiring immunological memory that mediates the generation of an immediate and stronger immune response against the antigen upon any subsequent exposure, resulting in life-long protection against encountered antigens. Both innate and adaptive immune cells are important players in atherosclerosis. The functionality of immune cell populations that are central to this thesis will be described in the section below.

INNATE IMMUNITY

Monocytes and macrophages

During homeostasis, monocytes circulate in the blood and can either re-enter the bone marrow or migrate into tissues. The two main monocyte populations are classical monocytes (CCR2⁺Ly6C^{high} in mice and CD14⁺CD16⁻ in humans) and non-classical monocytes (CCR2⁺Ly6C^{low} in mice and CD14^{int}CD16⁺ in humans).^{21,33} Classical monocytes produce high levels of pro-inflammatory cytokines and chemokines, while non-classical monocytes secrete lower quantities of pro-inflammatory agents and are considered less inflammatory than classical monocytes.^{34,35} Hypercholesterolemia increases circulating classical monocyte levels and their activation status.^{36,37} In atherosclerosis, classical monocytes play a pivotal role in the initiation of atherosclerosis, as they are recruited to the vessel wall in response to an activated endothelium and accumulation of intimal lipid particles, where they enter the intimal layer

and can differentiate into macrophages that promote atherogenic processes.³³ Alternatively, classical monocytes can transform into non-classical monocytes that patrol the endothelium on the vascular luminal side to remove debris and maintain endothelial cell function.³⁸ Although non-classical monocytes are generally considered atheroprotective, a prospective study showed that higher levels of non-classical monocytes are associated with carotid intima-media thickening in men.³⁹

Macrophages are well-known contributors to atherosclerosis disease pathology. Many functions have been described for macrophages, including phagocytosis, efferocytosis, antigen-presentation and lipid-processing. Classically, macrophage phenotypes were divided into two extremes on the inflammation spectrum: pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages that are experimentally polarized macrophage phenotypes.^{40–42} However, a broader range of macrophage subtypes in atherosclerotic lesions have been defined by recent single-cell studies in human and mouse^{43–53}, which subdivided the macrophage pool into four common populations: resident-like macrophages, foamy TREM2-expressing macrophages, interferon-inducible (IFNIC) macrophages, and inflammatory macrophages.⁵⁴ Tissue resident-like macrophages are present in both healthy and diseased aortas.^{44,45,55} LYVE1-expressing tissue-resident macrophages originate from embryonic precursors and inhibit collagen production by VSMCs.⁵⁶ In mice, aortic-intima resident macrophages (MAC^{AIR}) are seeded by circulating monocytes at birth but are sustained by local proliferation during homeostasis.⁵⁵ MAC^{AIR} macrophages are one of the earliest cholesterol-engulfing foam cells during atherosclerosis initiation, but as the plaque progresses, they are outnumbered by recruited monocyte-derived foamy macrophages. Foam cells are notorious players in atherosclerosis and owe their name to the foamy-like appearance that is a result of intraplaque lipid engulfment. Multiple single-cell studies identified *Trem2*⁺ foamy macrophages, which express genes associated with lipid processing, but low levels of inflammatory-associated genes, suggesting a homeostatic lipid-processing function.^{54,57} A recent study showed that *Trem2* expression is important for macrophages to differentiate into foam cells.⁵⁸ Interestingly, a meta-analysis showed that a subset of *Trem2*⁺ macrophages were enriched for *Tnf* and *Il1b* transcripts compared to MAC^{AIR} macrophages, although at lower levels than bona fide pro-inflammatory macrophages, while antigen-processing and presenting related genes were higher expressed in MAC^{AIR} macrophages.⁵⁴ Furthermore, a recent scRNA-seq study identified a subset of peripilin 2^{hi} (PLIN2^{hi})/TREM1^{hi} macrophages in human carotid plaques, displaying a transcriptomic signature associated with lipid accumulation and inflammation.⁵¹ Functional experiments showed that homeostatic TREM2^{hi} macrophages can transition into these inflammatory (PLIN2^{hi})/TREM1^{hi} macrophages through TLR2 signaling. Bona fide inflammatory macrophages express high levels of IL-1 β , a well-known cytokine in atherosclerosis as targeted in the CANTOS trial, as well as other pro-inflammatory genes including *Cxcl2*, *Tnf*, *Tlr2*, *S001a9*, and *Nlrp3*, which is part of the inflammasome that mediates secretion of IL-1 β .

This signature highly resembles the phenotype of the classical M1 macrophage. Notably, these pro-inflammatory macrophage populations were only found in atherosclerotic plaques, but not in healthy aortas, and they represent the largest macrophage subset in both mouse and human plaques and are considered major drivers of plaque inflammation.^{44,45,53,57,59,60}

Dendritic cells

Classically, dendritic cells (DCs) are described as professional antigen-presenters and hence play a main role in bridging the innate and adaptive immune system. DCs can also perform other immune functions such as phagocytosis and share some phenotypic similarities with macrophages.⁶¹ A common DC progenitor gives rise to plasmacytoid dendritic cells (pDCs), and conventional dendritic cells (cDCs), which are subdivided in type 1 (cDC1) and type II (cDC2) dependent on BATF3 and IRF4 expression, respectively.⁶² pDCs are normally located in the circulation and lymphoid organs and can produce high quantities of type I interferon (IFN) and pro-inflammatory cytokines in response to pathogens.⁶³ In atherosclerotic lesions, pDCs are found to co-localize with T cells and contribute to atherogenesis by promoting vascular inflammation through the secretion of IFN, pro-inflammatory cytokines and immune cell-attracting chemokines.⁶⁴ On the other hand, cDCs internalize antigens in the vessel wall, migrate to secondary lymphoid organs via CCR7 signaling, and present the processed antigen on major histocompatibility complex (MHC) I or II, to naïve T cells in lymphoid tissues (e.g. draining lymph nodes) that recognize the presented antigen via their antigen-specific T cell receptor (TCR). Depending on the received co-stimulatory or co-inhibitory signal (e.g. CD80/CD86, CD40) and cytokine secretion, the naïve T cell acquires an effector or immunosuppressive phenotype. cDC1s are generally involved in cross-presentation of antigens and drive cytotoxic CD8⁺ T cell responses, while cDC2s mainly promote CD4⁺ T cell priming.⁶⁵ Tolerogenic DCs have immunosuppressive properties and are able to induce tolerogenic responses, including the expansion of regulatory T cells⁶⁶. In atherosclerosis, vaccination with oxLDL-pulsed DCs and adoptive transfer of ApoB100-loaded DCs in mice dampened inflammatory T cell responses and reduced atherosclerotic plaque size.^{67,68}

Mast cells

Mast cells are part of the myeloid lineage and innate immune system, and seed vascularized tissues that are exposed to the exogenous milieu, such as skin, airways peritoneal cavity.⁶⁹ Stem cell-derived mast cell progenitors are released from the bone marrow into the circulation and mature into mast cells upon entering peripheral tissues. Mast cells are characterized by the expression of tyrosine kinase receptor for stem cell factor (c-Kit or CD117) and the high-affinity receptor for immunoglobulin E (FcεRI).^{70,71} Activation of mast cells mainly occurs through binding of antigen-sensitized IgE to FcεRI, but also via IgG binding, toll-like receptor and complement receptor signaling. When activated, mast cells release the contents of their cytoplasmic granules, which contain a plethora of agents, including mast cell-specific

proteases, histamine, growth factors, and cytokines and thereby induce a specific immune response. Furthermore, mast cells can function as non-classical antigen-presenters in cross-talk with the adaptive immune system.⁷²⁻⁷⁴ Although they are highly known for their role in allergies and immune responses against infections and toxins, mast cells also contribute to atherosclerosis pathology. The number of mast cells in atherosclerotic plaques increases as the plaque progresses and is also associated with the occurrence of future cardiovascular events.^{75,76} Functionally, mast cells promote atherosclerosis by releasing pro-inflammatory cytokines and matrix-degrading proteases that lead to fibrous cap thinning and intraplaque neovascularization, resulting in plaque destabilization.⁷⁷⁻⁷⁹

ADAPTIVE IMMUNITY

T cell development

T cells derive from common lymphoid progenitors (CLP), which travel from the bone marrow to the thymus and give rise to T cell precursor cells (i.e. thymocytes), while losing the potential to become B cells or natural killer T cells.⁸⁰ Interaction with thymic stromal cells triggers the differentiation and the expression of T cell-specific markers such as CD2 and Thy.1, but does not induce the expression of CD3, CD4 nor CD8 yet. These immature CD4⁻CD8⁻ double-negative (DN) thymocytes give rise to two T cell lineages that either express $\gamma\delta$ (which will lack CD4 and CD8 when matured) or $\alpha\beta$ genes that encode the T cell receptor (TCR). Each TCR has a unique antigen-binding site, which is variable due to V(D)J recombination. DN thymocytes go through different phases of differentiation and, simultaneously, somatic DNA rearrangements of the TCR β chain locus. At the end phase of DN thymocyte differentiation, the expressed β chains pair with the TCR α chain, and together with CD3 molecules allows for the assembly of a pre-TCR complex, thereby committing to the $\alpha\beta$ lineage. Further differentiation results in the expression of CD8 and CD4, and thus the formation of CD4⁺CD8⁺ double-positive (DP) thymocytes. In the DP stage, the α chain locus rearranges and a functional $\alpha\beta$ TCR complex is produced. Besides a place for T cell maturation, the thymus is a crucial organ where important screening occurs of (self-)reactivity for each thymocyte. The majority of DP thymocytes fail to sufficiently interact with peptide-MHC complexes and are subjected to cell death by neglect. In contrast, negative selection occurs when thymocytes react too strongly to self-peptide-MHC complexes and go into apoptosis to prevent autoimmunity if released from the thymus. A fraction of thymocytes that show low self-reactivity but sufficient TCR signaling, is positively selected. Depending on the affinity for MHCII or MHCI and signaling strength through the CD4 or CD8 co-receptor, a thymocyte will either commit to the CD4⁺ or CD8⁺ T cell lineage, respectively, and differentiate into mature naïve single-positive T cells with a unique, functional TCR that is specific for an antigen. After maturation, naïve T cells are released from the thymus and migrate to peripheral lymphoid tissues, such as the spleen

or lymph nodes, where they can be activated upon encountering an antigen-MHC molecule complex that is presented by an antigen-presenting cell.⁸¹

CD4⁺ T cells

Antigen presentation to naïve CD4⁺ T cells in lymphoid tissues elicits the clonal expansion and polarization into a distinct CD4⁺ T cell subset. Co-stimulatory or co-inhibitory signals and cytokines provided by the antigen-presenting cell determine the CD4⁺ T cell phenotype that the primed T cell differentiates into. Via homing receptors, T cells migrate to atherosclerotic lesions, where they exert their pro-atherogenic or atheroprotective function through the secretion of corresponding cytokines. In atherosclerotic lesions various CD4⁺ T cell subsets have been identified, including T helper subtypes (Th) Th1, Th2, Th9, Th17, Th22, follicular T helper cells (Tfh), and regulatory T cells (Treg).⁸²

Th1

Single-cell studies showed that the majority of CD4⁺ T cells are Th1 cells in human atherosclerotic plaques.^{52,53} Interferons, including IFN alpha and gamma (IFN- α and IFN- γ), and interleukins IL-12 and IL-18 drive the polarization towards the Th1 phenotype.⁸³ Th1 cells are defined by their expression of the T-box transcription factor TBX21 (T-bet), chemokine receptors CXCR3 and CCR5 that mediate plaque-homing, and the secretion of pro-inflammatory cytokines, such as IFN- γ and tumor necrosis factor (TNF).^{84,85} Experimental studies have demonstrated a pro-atherogenic role for Th1 cells in atherosclerosis. Disruption of IFN- γ signaling or T-bet deficiency protected mice from atherosclerosis, while administration of IFN- γ to *ApoE*^{-/-} mice promoted atherosclerotic lesion development.^{84,86–88} Mechanistically, IFN- γ can mediate plaque destabilization by influencing macrophage polarization, and inhibiting VSMC proliferation^{89,90}, although some studies have also shown the ability of IFN- γ to induce VSMC proliferation and promote plaque stability.^{91,92}

Th2

Th2 differentiation is induced by IL-4 and IL-33 that leads to the upregulation of GATA3, the key transcription factor of Th2 cells. GATA3 promotes the production of IL-4, IL-5, and IL-13.⁹³ Although the pro-atherogenic character of Th1 is well-established, the role of Th2 cells in atherosclerosis is controversial. High Th2 cell numbers and IL-4 secretion in CVD patients were associated with reduced risk of CVD, suggesting an atheroprotective role for Th2 cells.⁹⁴ However, a study that induced Th2 responses in mice immunized with ApoB100 had no effect on atherosclerosis.⁹⁵ Conflicting results have been reported on Th2-specific IL-4 secretion. In *ApoE*^{-/-} mice, IL-4 inhibited Th1 responses and reduced atherosclerotic plaque formation, while depletion of IL-4 in high-fat diet-fed *Ldlr*^{-/-} mice also led to decreased lesion formation.^{96,97} IL-5 has been shown to promote atheroprotective humoral immune responses⁹⁸ and IL-5 deficiency slightly reduced plaque formation in *ApoE*^{-/-} mice, which suggests an atheroprotective role.⁹⁹

The latter study also showed that plasma IL-5 levels were inversely associated with presence of carotid plaque but could not predict cardiovascular events. IL-13 also has an atheroprotective effect on lesion development.¹⁰⁰ It should be noted that the effect of T cell-produced IL-5 and IL-13 as well as Th2-specific depletion in atherosclerosis is yet to be investigated.

Th17

Th17 cells are induced by IL-23, express the transcription factor ROR γ t and mainly secrete IL-17.¹⁰¹ In endothelial cells and immune cells, IL-17 promotes the secretion of IL-6, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor and chemokine, which can all have pro-atherogenic effects.¹⁰¹ Interestingly, IL-6 and transforming growth factor beta (TGF- β) can induce a Th17 subtype that apart from IL-17 also produces anti-atherogenic IL-10.^{102,103} Experimental studies investigating the role of IL-17 in atherosclerosis yielded conflicting results, where pro-atherogenic, atheroprotective, but also no effects were reported.¹⁰⁴⁻¹¹¹ In some clinical studies higher plasma IL-17 levels and increased circulating Th17 cell numbers were associated with cardiovascular events^{112,113}, while larger clinical studies showed that plasma IL-17 levels did not differ between individuals with or without cardiovascular disease¹¹⁴, but also that low levels of IL-17 were associated with a higher risk of cardiovascular events.¹¹⁵ Notably, IL-17 is also produced by $\gamma\delta$ T cells and innate lymphoid type 3 cells (ILC3).¹¹⁶ Studies examining isolated Th17-specific mechanisms in atherosclerosis are needed to fully comprehend their function.

Tfh

A subset of activated T cells migrates towards B cell follicles in lymphoid organs; hence they are named follicular T helper cells (Tfh). Here, Tfh cells directly interact with B cells in germinal centers to induce B cell proliferation, differentiation and antibody isotype switching by secreting IL-21 and IL-4.¹¹⁷ Tfh cells are characterized by the expression of transcription factor BCL-6, chemokine receptor CXCR5, and co-stimulatory and co-inhibitory molecules ICOS and PD-1¹¹⁷. In *ApoE*^{-/-} and *Ldlr*^{-/-} mice, hypercholesterolemia increased the number of CXCR3⁺ Tfh cells, and Tfh cells from *ApoE*^{-/-} mice showed higher expression of genes associated with inflammation and more potential to induce IgG2 immunoglobulins.¹¹⁸ Interestingly, middle-aged (40 weeks old) *ApoE*^{-/-} mice showed increased Tfh numbers compared to young (6 weeks old mice).¹¹⁹ Marginal zone B cells and regulatory CD8⁺ T cells were shown to control hypercholesterolemia-induced Tfh cells and the germinal center B cell response.^{119,120} Blocking of the ICOS-ICOSL axis in *ApoE*^{-/-} mice resulted in decreased Tfh cell numbers in lymphoid organs and reduced atherosclerosis development.¹²¹ Furthermore, stimulating B cells with IFN- γ strongly upregulated PD-L1, which inhibited PD-1-mediated Tfh cell responses, resulting in reduced atherosclerosis burden.¹²² These data suggest that Tfh cells promote atherosclerosis development.

Treg

Regulatory T cells (Tregs) are crucial for maintaining self-tolerance and resolving tissue damage in inflammation. To achieve this, Tregs regulate the immune system by dampening proliferative and inflammatory responses of immune cells. Natural Tregs develop in the thymus where they are trained to tolerate self-antigens.¹²³ In secondary lymphoid organs, naïve CD4⁺ T cells differentiate into inducible or peripheral Tregs through stimulation of tolerogenic DCs, exposure to TGF- β and IL-2, weak TCR interaction, weak co-stimulatory signaling and strong co-inhibitory signaling.^{124–127} A hallmark of Tregs is the expression of the transcription factor FOXP3, which induces upregulation of IL-2RA (CD25) and GITR, that are critical for their immunosuppressive function.¹²⁸ Tregs exert their immunosuppressive function by producing the anti-inflammatory cytokines IL-10, TGF- β , and IL-35, and also via co-inhibitory molecules CTLA-4 and LAG-3.¹²⁹ In *ApoE*^{-/-} mice, a high-fat diet reduced the number of Tregs and increased plaque development.¹³⁰ Clinical studies associated lower percentages of Tregs of total T cells with plaque vulnerability and severity of coronary artery disease^{131,132}, although it should be noted that total T cell numbers increased with increased plaque vulnerability. Experimental studies that specifically targeted functional Tregs show a protective role for these cells in atherosclerosis. Both vaccination against FOXP3 and depletion of FOXP3⁺ Tregs promoted atherosclerotic lesion development^{127,133}, while IL-2-complex induced expansion of Tregs and inhibited atherosclerosis development.^{125,134} FOXP3 is crucial for Treg function, as a study in Western diet-fed *ApoE*^{-/-} mice showed that Tregs lose their FOXP3 expression as atherosclerosis progresses, resulting in the loss of their immunosuppressive function. Furthermore, a fraction of these Tregs converted into pro-atherogenic BCL-6⁺ Tfh cells.¹²¹ Other studies also revealed Treg plasticity, as Tregs in advanced atherosclerosis in mice exhibited co-expression of FOXP3 and T-bet and lost their regulatory and atheroprotective phenotype when converted into these so-called exTregs.^{135–138} These studies indicate that during atherosclerosis progression, Tregs lose their immunosuppressive functions. Maladaptive Treg conversion into exTregs co-expressing FOXP3 and RORC has also been suggested^{139,140}, although another study showed that human plaques showed no indications of Treg-Th17 plasticity, as there was no co-expression of FOXP3 and RORC, nor overlapping clones between Treg and Th17 clusters.¹⁴¹ Hence, more research into the function of Tregs and their potential pathologic conversion in the atherosclerotic environment is required.

Nonetheless, the protective potential of Tregs in atherosclerosis led to the set-up of the LILACS trial, which is studying the potential therapeutic effects of low-dose IL-2 therapy in stable CVD patients, with preliminary data showing an effective expansion of regulatory T cells.^{142,143} Similarly, the IVORY trial is currently assessing the effects of low-dose IL-2 on vascular inflammation in patients with acute CVD.¹⁴⁴

CD8⁺ T cells

While CD4⁺ T cell specificity is restricted to antigens presented on MHCII molecules by antigen-presenting cells, CD8⁺ T cells recognize antigen-MHCI complexes presented by all nucleated cells. Upon recognition of an antigen, CD8⁺ T cells can mature into cytotoxic T cells that can kill the antigen-bearing target cell via three main cytotoxic pathways. First, the secretion of inflammatory cytokines, such as TNF- α and IFN- γ , can induce apoptosis and inflammation. Apoptosis of a target cell can also be initiated by ligating Fas ligand (FasL) present on the surface of CD8⁺ T cells, to the Fas receptor on target cells. Alternatively, CD8⁺ cells can release perforin and granzymes that perforate the target cell and trigger apoptotic signaling cascades.¹⁴⁵ After clearance of the antigen-bearing cells, effector T cells go into apoptosis, or differentiate into memory T cells that can rapidly proliferate and respond more effectively upon any subsequent exposure to the antigen.¹⁴⁶ However, chronic antigen stimulation or inflammation, and terminal differentiation can push T cells into an exhausted state.¹⁴⁷ Exhausted CD8⁺ T cells exhibit a weakened effector cytokine response, and upregulated expression of co-inhibitory receptors, such as PD-1.^{148,149}

Both protective and atherogenic effects of CD8⁺ T cells in atherosclerosis have been reported. Its conflicting role is probably due to CD8⁺ T cell heterogeneity and stage-dependent differential effects during disease progression. Genetic knockout of CD8⁺ T cells in chow diet-fed *ApoE*^{-/-} mice did not affect initial nor advanced atherosclerotic lesion development, but mechanisms that may have compensated for the loss of CD8⁺ T cells were not evaluated in this study.¹⁵⁰ In contrast, antibody-mediated depletion of total CD8⁺ T cells in *ApoE*^{-/-} and *Ldlr*^{-/-} mice led to decreased atherosclerosis burden after 6 or 8 weeks of atherogenic diet feeding, while leaving CD4⁺ T cell unaffected.^{151,152} Adoptive transfer of CD8⁺ T cells in lymphocyte-deficient *ApoE*^{-/-} mice led to CD8⁺ T cell infiltration in plaques and increased atherosclerosis burden. Moreover, reconstitution with CD8⁺ T cells lacking perforin, granzyme B and TNF- α but not IFN- γ , failed to aggravate atherosclerotic plaques.¹⁵¹ However, antibody-depleted mediation of CD8⁺ T cells in advanced atherosclerosis destabilized established plaques, which was accompanied by increased Th1 CD4⁺ T cell numbers in the plaque.¹⁵³ Protective effects of CD8⁺ T cells were also demonstrated by a study in which immunization with ApoB100-derived peptide induced the expansion of CD8⁺ T cells and the reduction of atherosclerotic plaque development.¹⁵⁴ Different CD8⁺ T cell subtypes may explain the mixed effects reported in CD8⁺ T cell depletion studies. Numbers of ROR γ t⁺IL-17⁺ CD8⁺ T cells (Tc17) that resemble Th17 CD4⁺ T cells, were increased in advanced atherosclerotic plaques of *ApoE*^{-/-} mice compared to the spleen, but adoptive transfer of these cells into CD8-deficient *ApoE*^{-/-} mice did not affect atherosclerosis.¹⁵⁵ The protective role of CD8⁺ T cells is probably caused by a subset called regulatory CD25⁺CD8⁺ T cells, which were able to reduce lesion size, decrease macrophage content and inhibit proliferation of CD4⁺ T cells in *ApoE*^{-/-} mice, compared to CD25⁻CD8⁺ T cells.¹⁵⁶

In clinical studies, coronary artery disease patients showed enhanced levels of circulating cytotoxic CD8⁺ T cells.^{145,157,158} Furthermore, single-cell studies of human atherosclerotic plaques found different subpopulations of CD8⁺ T cells with an effector-memory phenotype, terminally differentiated cytotoxic phenotype, exhausted phenotype, and central-memory phenotype.^{52,53,141,159}

$\gamma\delta$ T cells

Unlike $\alpha\beta$ CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells do not react to conventional antigens presented on MHC molecules, but respond quite rapidly to non-peptide antigens, such as lipids and phosphorylated nucleotides. Hence, $\gamma\delta$ T cells lie in between the innate and adaptive immune system. Although presence of $\gamma\delta$ T cells in human atherosclerotic plaques has been reported^{141,160}, $\gamma\delta$ T cells are seldomly studied in atherosclerosis. In mice, the percentage of splenic $\gamma\delta$ T cells increases when fed a high-fat diet.¹⁶¹ Flow cytometric analysis showed that aortic $\gamma\delta$ T cells produced IL-17. An experimental study investigating genetic deficiency of $\gamma\delta$ T cells in *ApoE*^{-/-} mice, suggested that $\gamma\delta$ T cells promote early-stage atherosclerotic lesion development¹⁶², while a similar study showed no effect on atherosclerosis.¹⁶¹ The latter result was also confirmed by a recent study in *Ldlr*^{-/-} *Tcr δ* ^{-/-} mice that lacked the global pool of $\gamma\delta$ T cells.¹⁶³ However, the same study showed that IL-23 receptor (IL-23R) expressing $\gamma\delta$ T cells, which are the main cell type expressing *Il17a* in the aorta of atherosclerotic *Ldlr*^{-/-} mice, are able to drive early atherosclerotic plaque formation with increased necrotic cores.

B cells

Another branch of the adaptive immune system consists of B cells that are renowned for their ability to produce antibodies, also known as immunoglobulins. Similar to the T cell's TCR, B cells express B cell receptors (BCR), which are formed by membrane-bound immunoglobulins coupled to a signaling component, consisting of CD79a and CD79b. Immunoglobulins are composed of two heavy chains and two light chains that are linked together by disulfide bonds. There are five major heavy-chain classes or isotypes, specifically IgM, IgD, IgG, IgE and IgA, of which IgG also holds subclasses (e.g. IgG1, IgG2, IgG3 and IgG4 in humans). The specific isotype determines the function of the immunoglobulin when secreted as an antibody, as the tail region of the corresponding isotype can interact with Fc-receptor-expressing immune cells or complement molecules that drive a particular immune response. The heavy and light chains can be categorized into constant and variable domains. Variability in sequences of the heavy and light chain variable domains make up the unique epitope-binding site of the immunoglobulin, which can bind to specific antigens. In the developmental stages of the B cell in the bone marrow, sequential heavy chain and light chain V(D)J gene recombination result in the expression of a mature BCR which is unique for every B cell clone. Aside from the bone marrow, other tissues including the fetal liver, are a source for B lymphopoiesis.¹⁶⁴ Further development takes place in secondary lymphoid organs, such as the spleen and lymph nodes,

where immature B cells differentiate into mature or naïve B cells. The two main B cell lineages are innate-like B1 cells and conventional B2 cells, which differ in their ontogeny, location, function and expression of characteristic markers.

B1 cells originate from the fetal liver and are mainly located at mucosal surfaces, particularly the pleural and peritoneal cavity, although B1 cells that secrete high levels of antibodies are also found in the spleen and bone marrow.¹⁶⁵ In mice, B1 cells are further divided into CD5⁺ B1a and CD5⁻ B1b subtypes. B1a spontaneously produce naturally occurring IgM antibodies in the absence of infection or immunization¹⁶⁶, while B1b cells seem to develop parallelly to B2 cells to drive T cell-dependent or -independent, long-lasting IgM responses to pathogens.^{167,168}

The majority of the B cell compartment is taken up by B2 cells derived from splenic immature B cells, originating from bone marrow progenitors. B2 cells can be split into CD21⁺CD23⁻ follicular (FO) and CD21^{high}CD23⁻ marginal zone (MZ) B cells. Similar to B1 cells, MZ B cells can exert innate-like functions, such as generating a rapid humoral response to circulating antigens in the blood that pass through the red pulp. MZ B cells mainly produce IgM antibodies. Although MZ B cells do not display circulating properties in mice, IgM⁺ MZ-like B cells have been found in the circulation of humans^{169,170}. In contrast to MZ B cells, FO B cells can leave the spleen and migrate to distant locations.¹⁷¹ When encountering an antigen that is recognized by their BCR, FO B cells travel towards the T cell border within lymphoid organs to interact with cognate CD4⁺ T helper cells and become fully activated. While a part of these activated B cells travels to extrafollicular foci and turn into low-affinity antibody-secreting short-lived plasmablasts, some activated FO B cells return to B cell follicles and together with Tfh cells form germinal centers (GC), where further differentiation occurs. The GC reaction involves rapid proliferation, isotype switching and affinity maturation through somatic hypermutation and subsequent clonal selection. This results in the generation of memory B cells and long-lived plasma cells that secrete antibodies with high affinity for the specific antigen. While affinity maturation only occurs in GCs, isotype switching or class-switching is not restricted to GCs and can also occur in extrafollicular sites.¹⁷² Related to this is the formation of tertiary lymphoid structures in tissues such as adipose tissue (fat-associated lymphoid clusters; FALCs) and aortic adventitia (artery tertiary lymphoid organs; ATLOs), which harbor immune cells, particularly adaptive immune cells.^{173,174} ATLOs have been identified in predominantly in the lower abdominal part of aortas of aged *ApoE*^{-/-} mice, and in human atherosclerotic tissues.¹⁷⁴ Interestingly, FALC-residing B cells were described to increase with age.¹⁷⁵ FALCs and ATLOs contain many B cells that are suggested to orchestrate their immune functions in atherosclerosis from within these lymphoid structures.¹⁷³

Investigation of the total B cell pool in atherosclerotic mouse models has produced mixed results. Initially, B cells were suggested to be protective, as adoptive transfer of splenic B

cells rescued splenectomy-driven acceleration of atherosclerosis in *ApoE*^{-/-} mice. Similarly, transplantation of bone marrow from B cell-deficient μ MT mice into lethally irradiated *Ldlr*^{-/-} mice led to increased atherosclerotic plaque development compared to controls.¹⁷⁶ However, μ MT-dependent B cell deficiency in *ApoE*^{-/-} mice reduced atherosclerosis compared to control *ApoE*^{-/-} mice, indicating a pro-atherogenic role for B cells.¹⁷⁷

Distinct B cell subsets display different effector mechanisms in atherosclerosis. B1 cells are atheroprotective, owing to their production of IgM antibodies that recognize oxidation-specific epitopes (OSE) present on oxidized LDL particles (oxLDL), apoptotic cells and microvesicles.¹⁷⁸ IgM can reduce oxLDL-induced activation of endothelial cells and foam cell formation.¹⁷⁹ Experimental studies demonstrated that decreased levels of B1a cells increased atherosclerotic plaques, whereas adoptive transfer of B1a or B1b cells both attenuated atherosclerosis.^{180,181} Adoptive transfer of B1a versus B1b cells into *ApoE*^{-/-}*Rag1*^{-/-} mice showed enhanced CCR6-mediated splenic migration, superior IgM production and IgM repertoire diversification by B1b cells compared to B1a cells.¹⁸² Similarly, MZ B2 cells protect against atherosclerosis by producing protective-IgM antibodies, of which human circulating CD24^{hi} MZ B cells were found to be the major producers of atheroprotective IgMs and their frequency in CVD patients inversely correlated with coronary artery disease severity.¹⁸³ Additionally, MZ B cells can suppress Tfh cells and atherogenic Thf-mediated FO B responses¹⁸⁴ Tay et al. showed that FO B2 cells promote atherosclerosis development by transitioning into plasma cells that produce pathogenic IgG antibodies and pro-inflammatory cytokines.¹⁸⁵ *Ldlr*^{-/-} mice treated with an agonistic antibody specific for B and T-lymphocyte attenuator reduced FO B cell levels, and increased regulatory B and T cells, resulting in reduced atherosclerosis.¹⁸⁶ The pro-atherogenic effects by FO B cells may also be caused by their capacity of presenting self-antigens to T cells, which mediates effector memory T cell responses and overpowers regulatory T cell responses.^{187,188} Indeed, *Ldlr*^{-/-} mice with B cell-specific MHCII deficiency developed less atherosclerosis.¹⁸⁵ On the other hand, B1a cell-derived innate response activator (IRA) B cells that produce GM-CSF, promote expansion of conventional dendritic cells which induced IFN- γ -producing Th1 cells, thereby aggravating atherosclerosis.¹⁸⁹ Another B cell subset described in atherosclerosis are regulatory B cells (Bregs), of which the ontogeny is unclear.¹⁹⁰ Like Tregs, Bregs can mediate immunosuppression by producing anti-inflammatory cytokines such as IL-10 and IL-35, or via signaling through co-inhibitory molecules, PD-L1, FAS ligand or ectoenzyme CD73.¹⁹¹⁻¹⁹⁵ A limited amount of studies investigated the atheroprotective potential in atherosclerosis models. Although a study showed that IL-10-producing Bregs were able to decrease atherosclerotic lesion development, others have shown no effect on atherosclerosis.¹⁹⁶⁻¹⁹⁸

AGING OF THE IMMUNE SYSTEM: IMMUNOSENESCENCE

Upon aging, many physiological systems undergo a functional decline, including the immune system. The term immunosenescence was introduced in 1964 by Roy Walford and currently refers to all age-related changes affecting the immune system, including degeneration and remodeling of the immune organ structures, proportional rearrangement of the innate and adaptive immune cell compartment, reduced vaccine outcomes and increased risk for infectious and age-related diseases.^{199,200}

A prominent feature of immunosenescence is the age-induced alteration of hematopoietic output from the bone marrow. Upon aging, hematopoietic stem cells (HSCs) have reduced regenerative capacity, which leads to skewed immune cell differentiation and impaired adaptive and innate immunity.²⁰¹ Furthermore, HSCs from aged (22-24 months) mice compared to young mice, showed decreased gene expression associated with lymphopoiesis, but upregulation of myeloid genes, resulting in skewing towards myeloid lineages.²⁰² This results in increased frequencies of myeloid cells monocytes, macrophages, neutrophils and dendritic cells, while the output of lymphoid cells, such as T and B cells, is reduced. Notably, myeloid skewing can also be promoted by hypercholesterolemia and hyperglycemia.²⁰³⁻²⁰⁵ Another hallmark of aging is clonal hematopoiesis of indeterminate potential (CHIP), which is the clonal expansion of HSCs containing somatic mutations that confer a competitive advantage in hematopoiesis over HSC without these mutations. Carriers of CHIP have a two-fold increase in cardiovascular risk compared to non-carriers, in which mutations of genes *DNMT3A*, *TET2*, *ASXL1* and *JAK2* mainly account for CHIP.²⁰⁶ Moreover, presence of CHIP is also associated with an increased inflammatory state that is associated with enhanced risk to develop atherosclerosis and poorer cardiovascular outcome.²⁰⁶ Furthermore, the thymus is subjected to age-induced changes.²⁰⁷ Stromal cells (mainly connective tissue cells) support the function of the thymus as a differentiation organ. With age, stromal cells degenerate and lead to involution of the thymus, and consequently aberrant maturation and selection of T cells, causing a decrease in naïve T cells and increase in autoreactive T cells. Moreover, lifelong exposure to pathogens and antigens results in the accumulation of antigen-experienced memory T cells. These processes contribute to the shift from functional naïve T cells to increased memory T cells and autoreactive T cells. Furthermore, aged T cells display a more extreme effector phenotype, as illustrated by enrichment of effector cytokine and cytotoxicity genes.²⁰⁸ As a consequence, aging is associated with an increased susceptibility for infections but also developing chronic inflammatory diseases and autoimmune diseases.^{200,209-211} Aging is also accompanied by the accumulation of senescent cells. Cellular senescence is characterized by cell cycle arrest, expression of senescent-cell specific surface proteins and acquisition of a senescence-associated secretory phenotype (SASP). The SASP is a secretome comprised of various cytokines, proteases and other mediators

that contribute to a chronic low-grade state of inflammation, also termed inflammaging,²¹² which is most likely caused by continuous antigen load and stress. Senescent cell generation is believed to be a beneficial mechanism to re-establish tissue homeostasis in response to stress. However, excessive accumulation and the insufficient elimination of senescent cells may contribute to the pathology of age-related diseases, such as atherosclerosis.²¹³

Using single-cell methods, studies have described reductions and accumulations of immune cell populations with specific phenotypes in various tissues of aged mice.²¹⁴ For instance, an overall increase in CD4⁺ and CD8⁺ T cells expressing co-inhibitory molecule PD-1 has been described in organs of aged mice, while NK cells and innate lymphoid cell levels are reduced.^{215–217} Organ-specific alterations are age-induced expansion of inflammatory CD38⁺ macrophages^{218,219} in the liver and fat, and increased activated Tregs in the spleen of aged mice.^{208,217,220} Also, aged mice showed accumulation of specific B cell populations such as splenic age-associated B cells and peritoneal cavity B1 cells, which presence have been associated with the pathology of several age-related and inflammatory diseases.^{214,217,221,222}

CELLULAR AGING AND ATHEROSCLEROSIS

The importance of inflammatory immune responses as contributing factor to atherosclerosis progression is evident by the tremendous number of studies available. However, the vast majority of experimental studies investigating immune responses and immunomodulating therapies has been conducted in relatively young animals, whereas cardiovascular disease patients receiving treatment are often of advanced aged and have an aged immune system, which limits translating experimental findings to the patient. It is therefore essential to take aging into consideration when investigating immune cells and their responses in atherosclerosis studies.

Previous studies have shown that CVD patients show accelerated aging features, such as reduced telomere length of leukocytes and T cells and elevated, potentially senescent, CD4⁺CD28^{null} T cells, compared to age-matched healthy subjects.^{53,223–225} Cellular senescence and therapeutic strategies targeting senescence in atherosclerosis, such as selective elimination of senescent cells, have gained significant attention the past few years^{226–229}. A study by Childs et al. showed that deletion of the senescent cell plaque macrophages, vascular smooth muscle cells (VSMCs) and endothelial cells reduces atherosclerosis in LDLr-deficient mice.²³⁰ However, the markers that they and others used to identify senescent cells (p16, SA-β-gal and SASP) are not restricted to senescent cells, and could also be markers for non-senescent inflammatory macrophages.²³¹ While most of these studies focus on vascular cells (e.g. VSMCs and endothelial cells) and

innate immune cells, limited experimental studies have investigated age-associated changes in atherosclerotic mice, particularly in the adaptive arm of the immune system.

Hu et al. reported age-associated reductions of total CD4⁺ and CD8⁺ T cells in renal lymph node (RLN), spleen and blood in aged *ApoE*^{-/-} mice.¹⁷⁴ A different study also showed altered T cell immunity in middle-aged compared to young *Ldlr*^{-/-} mice.²³² By employing PCSK9 overexpression and a Western-diet to promote atherosclerosis in aged (20 months) C57BL/6 mice, Tyrell et al. showed that aged mice exhibited increased inflammatory monocytosis but did not focus on the adaptive arm of the immune system.²³³ Recently, Zhang et al. mapped out the transcriptome of myeloid cells and T cells paired with their TCR profile from RLN, ATLOs and plaque of aged *ApoE*^{-/-} mice and compared this to the RLN of age-matched C57BL/6 mice, but not to tissues of young *ApoE*^{-/-} mice.¹⁵⁹ B cells were disregarded in most of these studies. Evidently, research investigating the influence of age-associated immunity, particularly B cells and T cells, in atherosclerosis is needed.

THERAPEUTIC STRATEGIES

As atherosclerosis was long considered as a disease solely driven by lipids, lipid-lowering therapies were applied in the clinic. To this day, lipid-lowering statins are still one of the first-line therapies in combatting cardiovascular disease, which have proven effective to prevent and treat atherosclerosis. Statins owe their beneficial effects to their inhibition of cholesterol synthesis and promotion of LDL uptake by the liver.¹ Nevertheless, at least 20% of patients that suffered from acute coronary heart disease and received high-dose statin treatment endure recurrent cardiovascular events.²³⁴ Besides lipid lowering, medical therapies include the use of antiplatelet, antithrombotic and antihypertensive agents.²³⁵ To treat acute cardiovascular events, surgical methods can be applied to attain revascularization, such as a bypass procedure, carotid endarterectomy, angioplasty and stenting. Unfortunately, perioperative complications such as restenosis and concomitant recurrent cardiovascular events often occur, particularly in aged individuals, largely owing to the endothelial damage and local inflammation inflicted by surgical procedures.^{236,237}

Despite these effective treatment strategies, a residual risk remains in approximately 30% of atherosclerotic CVD patients.²³⁸ This raised awareness for the role of immune responses in driving recurrent cardiovascular events, and led to the development of several therapeutic strategies targeting cardiovascular inflammation.²³⁹ A pioneering study that demonstrated improved cardiovascular outcome by immunotherapy was the CANTOS trial. The CANTOS trial investigated the effects of canakinumab, a monoclonal antibody against the pro-inflammatory cytokine interleukin 1-beta (IL-1 β), in patients that recently suffered a myocardial infarction.

A 150 mg dose of canakinumab significantly reduced the risk of cardiovascular events by 15%, albeit with an increased risk of infection due to systemic immunosuppression.²⁴⁰ After the CANTOS trial, other trials investigating immunomodulating therapies in CVD patients followed. The CIRT trial studied the effect of a low-dose of methotrexate, an inexpensive treatment used for treating inflammatory diseases including rheumatoid arthritis and psoriatic arthritis, but it did not have the desired anti-inflammatory effect nor did it result in decreased cardiovascular events.²⁴¹ Anti-inflammatory colchicine was administered to CVD patients in the COLCOT (patients with myocardial infarction), LoDoCo and LoDoCo2 (patients with stable coronary artery disease) trials, which significantly reduced the risk of ischemic cardiovascular events.^{242–244} IL-6 is a pro-inflammatory cytokine that stimulates production of C-reactive protein (CRP), of which high levels are highly associated with increased cardiovascular disease risk. Tocilizumab and ziltivekimab are monoclonal antibodies that target the IL-6/IL-6 receptor axis and have been shown to reduce CRP levels in a dose-dependent manner.^{245,246} A follow-up study (ZEUS trial) is currently performed to monitor the occurrence of cardiovascular events.²⁴⁷ Antimalarial drug hydroxychloroquine is used for treatment of inflammatory rheumatic diseases, including rheumatoid arthritis and systemic lupus erythematosus (SLE), and can reduce the production of pro-inflammatory cytokines. Observational studies showed great risk reduction of cardiovascular events in rheumatoid arthritis and SLE patients treated with hydroxychloroquine,^{248,249} although strong adherence to the dose regimen is required to obtain these strong beneficial effects.²⁵⁰ Ongoing clinical trials are testing the effect of hydroxychloroquine in CVD patients.²¹

These promising results from clinical trials further underline the undeniable contribution of inflammatory immune responses in atherosclerotic CVD. However, systemic immunosuppression in these trials raises the risk adverse effects such as fatal infections. Hence, a more tailored approach targeting specific immune cell populations is needed. In combination with the silver tsunami (e.g. rapidly increasing elderly population)²⁵¹, it is of utmost importance to investigate age-associated immunity in atherosclerosis to enhance disease etiology, discover novel biomarkers and identify therapeutic targets for the development of beneficial therapies to combat atherosclerotic CVD. A valuable tool to address these objectives is single-cell RNA sequencing.

Single-cell RNA sequencing to investigate the immune landscape of atherosclerosis

Heterogeneity of immune cells in the atherosclerotic plaque was originally studied by performing immunostainings in the 1980s²⁵², allowing staining of about two markers, which currently has evolved to staining about 16 markers simultaneously.²⁵³ Since its introduction in atherosclerosis research in 2006, flow cytometry became one of the main methods to phenotypically characterize heterogenous cell populations on a single-cell level. Although flow cytometry is

relatively cheap, fast and does not require a lot of reagents, the number of markers that can be measured within a single panel is limited by the colors that can be distinguished without excessive overlap or autofluorescence, which nowadays is up to 20 markers. With the advanced mass cytometry (CyTOF) technique first applied in atherosclerosis in 2009^{45,254}, about 50 markers can be reached which increases dimensionality. In the same year, single-cell RNA sequencing (scRNA-seq) was introduced.²⁵⁵ Whereas bulk transcriptomics is used to measure the average expression of genes across a population of cells or tissues, single-cell transcriptomics enables high resolution mRNA transcriptome quantification of each single cell. Moreover, scRNA-seq allows for unbiased analysis of the different cells within heterogenous tissues or systems, while flow cytometry and CyTOF require staining of pre-defined markers. Advances in single-cell technologies now allow the integration and simultaneous profiling of genome, epigenome, transcriptome, proteome, immune repertoire, metabolome, and other omics modalities to characterize cell type, state and activity.²⁵⁶

Although single-cell omics are still at their infancy in the cardiovascular disease field, studies using high dimensional multimodal single-cell technologies in atherosclerosis are emerging. In 2018, scRNA-seq analysis pioneered in this field by two studies that mapped the transcriptome of immune cells within the atherosclerotic plaque of *ApoE*^{-/-} and *Ldlr*^{-/-} mice that revealed presence of previously undefined TREM2^{hi} macrophages.^{44,45} From that moment onwards, many studies using single-cell modalities in murine and human atherosclerosis have followed. These single-cell approaches identified previously undescribed cell targets and yielded new insights into signaling pathways and molecular mechanisms underlying atherosclerosis disease progression and can be utilized as a valuable tool for enhancing our comprehension of age-associated immunity in atherosclerosis disease etiology.

THESIS OUTLINE

Healthy aging is one of the prime goals in today's society and atherosclerosis is among the greatest causes of morbidity in elderly. Cardiovascular disease patients receiving treatment are often of advanced age and have an aged immune system, which limits translating experimental findings to the patient. It is therefore essential to take aging into consideration when investigating immune cells and their responses in atherosclerosis studies. This thesis describes research exploring the impact of aging on the immunological landscape in atherosclerotic cardiovascular disease using single-cell profiling, including identification of potential novel biomarkers and therapeutic targets of atherosclerosis.

In **chapter 2**, we studied the effects of key risk factors for atherosclerosis development, specifically aging and smoking, on the human immune system. We aimed to identify biomarkers in the

blood collected from young healthy volunteers (18-25 years old), elderly healthy volunteers (>60 years), young smokers (18-25 years), heavy smokers (>45 years), and patients with stable coronary artery disease (>60 years). Aged volunteers and CAD patients showed reduced naïve T cell levels and increases in effector memory T cells and T cells expressing markers of senescence. Moreover, CAD patients had reduced levels of oxLDL-specific IgM. Smokers displayed a pronounced pro-inflammatory cellular phenotype. In **chapter 3**, we showed that aging promotes advanced atherosclerosis with increased calcification and cholesterol crystals and systemic immunosenescence in chow diet-fed aged *Ldlr*^{-/-} mice compared to chow diet and Western diet-fed young *Ldlr*^{-/-} mice. We performed integrative scRNA-seq analysis on atherosclerotic aortic arches isolated from chow diet and Western diet-fed young and chow diet-fed aged *Ldlr*^{-/-} mice. This allowed us to identify age-associated alterations in the immunological landscape of atherosclerotic plaques at transcriptome level, including the emergence of age-associated GzmK⁺CD8⁺ T cells and previously in atherosclerosis undefined CD11c and T-bet expressing age-associated B cells. We confirmed the presence of these age-associated T- and B cells in cardiovascular disease patients. In **chapter 4**, we describe distinct sex differences in the atherosclerotic plaque that were measured by histological analysis, integration of scRNA-seq data and confirming flow cytometry analysis of atherosclerotic plaques from male and female chow diet-fed aged *Ldlr*^{-/-} mice. We found female-specific accumulation of inflammatory *Il1b*⁺ M1-like macrophages and age-associated B cells (ABCs). We further characterized the role of ABCs in atherosclerosis in **chapter 5** by profiling the splenic B cell repertoire in young and aged *Ldlr*^{-/-} mice, which revealed large clonal expansion of CD21^{low} age-associated B cells that shared related clonotypes with plasma cells and displayed a gene signature associated with plasma cell differentiation. Adoptive transfer of ABCs in lymphocyte-deficient *Ldlr*^{-/-} mice indicated a pro-atherogenic role for ABCs. Importantly, CD21^{low} B cell levels increased with age in CVD patients and high counts of these cells were shown to predict future cardiovascular events. In **chapter 6**, we examined how aging effects mast cells in atherosclerosis and show that the aging microenvironment in the plaque drives mast cell activation in the atherosclerotic aorta and promotes antigen-presenting capacity of mast cells. Summary and discussion of the results in this thesis, including concluding remarks and future perspectives, will be described in **chapter 7**.

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