The aging B cell landscape in atherosclerosis

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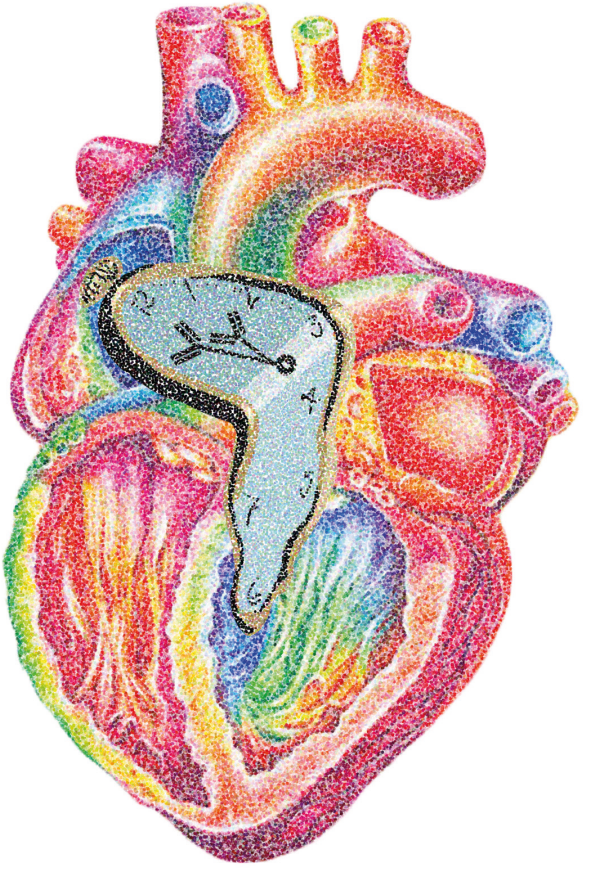

Chapter 1

General Introduction

CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) refers to all conditions affecting the heart and blood vessels, including ischemic heart disease, stroke, angina pectoris, and aortic aneurysm.[1] CVDs are the leading cause of death worldwide, responsible for approximately 20.5 million deaths each year.[2] The primary underlying pathology of most CVDs is atherosclerosis, a process marked by the progressive accumulation of lipid-rich plaques within the arterial wall.[3] Expansion of the plaque can lead to narrowing of the arteries, also known as stenosis, which impedes normal blood flow and manifests in various symptoms, including intermittent claudication. Plaques eventually can rupture, or erode, resulting in thrombus formation, which can subsequently cause severe cardiovascular events, such as heart attacks or strokes.[4]

Atherosclerosis has long been viewed as a predominantly lipid-driven condition and elevated cholesterol levels generally resulting from an unhealthy diet and sedentary lifestyle are among the well-established risk factors.[5] Over the past two decades, it has become evident that immune cells also play a significant role in the development of atherosclerosis and several clinical trials, including the CANTOS and LoDoCo trial, have highlighted the crucial role of immunotherapies in treating CVD.[6–8] In line with these findings, individuals with chronic inflammatory or autoimmune disorders, such as systemic lupus erythematosus and rheumatoid arthritis, are at increased risk of cardiovascular events.[9,10] In addition to chronic inflammation and genetic predisposition, unmodifiable risk factors include sex and aging. Although CVD is the primary cause of mortality in both men and women, there are notable sex differences in the prevalence and manifestation of CVD.[2,11,12] CVD tends to develop approximately 7 to 10 years later in women compared to men. This delay is mainly attributed to the protective effects of estrogen.[13] After menopause, women's risk of developing CVD increases, eventually catching up with or even surpassing the risk faced by men. Moreover, where men suffer from classical symptoms of chest pain, women generally experience atypical symptoms, complicating diagnosis. Once diagnosed, however, women show a poorer prognosis than men and have higher mortality rates following acute cardiovascular events. Since the mortality rate from cardiovascular disease in the 70+ age category is almost 7-fold higher than in the 50-69 year old age group[14], and even 84-fold higher compared to 15-49 year old individuals, aging is considered one of the most dominant risk factors for cardiovascular death. Upon aging, several physiological and biochemical changes occur, such as arterial stiffening and cellular senescence, which can increase CVD susceptibility.[15] Therefore, the ongoing demographic shift towards an older population drastically increases the global social and economic burden of CVD.

ATHEROSCLEROSIS

Healthy blood vessels are composed of three distinct layers: the intima, media, and adventitia. Each layer plays a crucial role in maintaining vascular function and integrity. The intima is the innermost layer that lines the lumen, the central space through which the blood flows. This layer is covered by a single layer of endothelial cells (ECs), which, under healthy conditions, forms a selective and protective barrier. Beneath the intima lies the media, which is primarily composed of vascular smooth muscle cells (VSMCs). These VSMCs are responsible for regulating blood pressure and flow. The outermost layer, the adventitia, is predominantly composed of fibrous connective tissue, which provides additional structural support. In atherosclerosis, the intimal endothelial cell layer becomes disrupted, enabling infiltration of lipids and immune cells and subsequent atherosclerotic plaque formation.

Initial atherosclerosis

At sites of endothelial dysfunction, atherosclerosis is initiated with the infiltration of lipid-rich lipoproteins, including chylomicrons, very low-density lipoproteins (VLDL) and low-density lipoprotein (LDL) particles into the intima of the arterial wall (**Figure 1**).[16] These particles all contain apoliprotein B on their surface, a protein which, upon transmigration, interacts with the extracellular matrix, resulting in the retention of lipoproteins in the intima. Subsequently, retained lipoproteins undergo pro-inflammatory chemical modifications[17], such as oxidation, which can induce upregulation of leukocyte adhesion molecules, including VCAM-1, ICAM-1, P- and E-selectin, on endothelial cells and VSMCs.[18] In addition, activated ECs and VSMCs secrete chemokines, which promote the recruitment of monocytes to the site of endothelial disruption. Consequently, integrins on recruited monocytes bind to the upregulated leukocyte adhesion molecules, allowing their transmigration into the intima. Under the influence of macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), secreted by endothelial cells, monocytes transdifferentiate into macrophages. Macrophages are phagocytic cells that express scavenger receptors, enabling them to engulf oxidized LDL particles.[19] The continuous uptake of oxLDL by macrophages leads to extensive lipid accumulation, a process also known as foam cell formation. These foam cells are central to the early development of atherosclerotic lesions, manifesting as fatty streaks within the arterial wall.

Advanced atherosclerosis

The presence of foam cells triggers a robust inflammatory response characterized by the release of cytokines, growth factors, and enzymes that exacerbate endothelial dysfunction and further contribute to the recruitment of inflammatory cells. Uptake of lipid particles by antigen-presenting immune cells also results in antigen presentation and subsequent T cell recruitment.[20,21] These T cells, in turn, release various cytokines and chemokines, thereby contributing to the inflammatory plaque environment. Chemokine signaling also orchestrates migration of additional immune cells, including neutrophils, mast cells and B cells, which further contributes to the development of advanced atherosclerotic lesions. Simultaneous to immune cell infiltration, VSMCs from the media layer migrate into the intima, proliferate, and secrete extracellular matrix components such as collagen.[22] These activities contribute to the formation of a fibrous cap over the lipid-rich core and accumulation of activated immune cells in the plaque. Like macrophages, VSMCs are capable of lipoprotein internalization, resulting in a VSMC-foam cell phenotype. As the plaque progresses, both VSMCs and macrophages accumulate excessive amounts of lipids, eventually surpassing the storage capacity of these cells, leading to cellular dysfunction and apoptosis. In the early stages, apoptotic cells are cleared efficiently by phagocytes through a process called efferocytosis.[23] Under normal circumstances, efferocytosis ensures that dying cells are rapidly cleared, preventing the release of their contents into the surrounding tissue, thereby maintaining plaque stability and preventing further inflammation. However, in advanced stages of plaque development, efferocytosis is impaired by several factors, including the increased volume of dying cells and reduced uptake capacity of phagocytes.[24] This leads to secondary necrosis, a process where the contents of the dying cells, including the accumulated lipids and cellular debris, are released into the atherosclerotic environment. This provokes additional inflammation and formation of a mass of dead cells, referred to as a necrotic core. Initially, the plaque is stabilized by a thick fibrous cap, which prevents interaction between the highly thrombogenic material within the plaque and the blood. In this

stage, patients often remain asymptomatic. However, as the inflammatory processes and necrotic core formation within the plaque continue, the integrity of the fibrous cap begins to deteriorate. Chronic inflammation leads to the activation of matrix metalloproteinases (MMPs), enzymes that degrade components of the fibrous cap. Moreover, plaque stability is further decreased by the development of calcified areas within the lesion, which introduce mechanical stress, increasing the risk of plaque erosion or rupture and subsequent clinical manifestations.

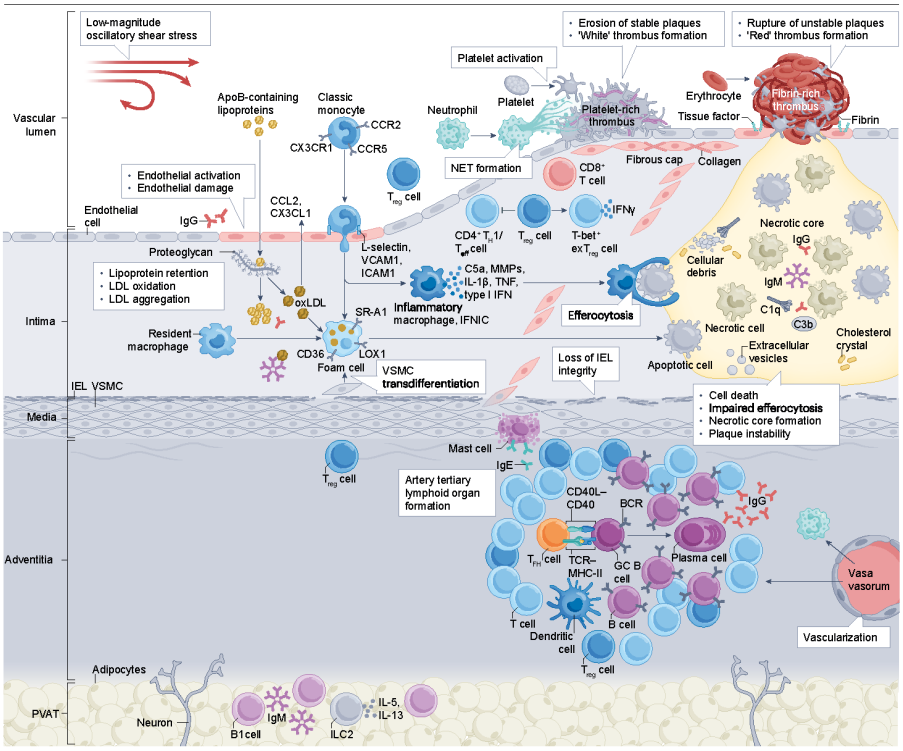

Figure 1. Atherosclerosis plaque development. Atherogenesis is initiated at sites of low shear stress, when apoB-containing lipoproteins (ApoB-LP) transmigrate through the disrupted endothelial cell layer. Consequently, ApoB-LPs are retained and modified, resulting in monocyte recruitment to the atherosclerosis-prone site. Monocytes migrate into the vessel wall and differentiate into macrophages, which can internalize ApoB-LPs. These macrophages differentiate into foam cells, which further attract immune cells of both the innate and adaptive immune system to the site of inflammation. Simultaneously, vascular smooth muscle cells (VSMCs) from the media migrate into the intima, where they can both internalize ApoB-LPs and form a stable fibrous cap over the lipid-rich core of the plaque. As the plaque progresses, excessive lipid uptake by macrophages and VSMCs causes apoptosis. Although apoptotic cells are initially cleared by phagocytosis, in later stages this process is impaired, causing secondary necrosis which leads to necrotic core formation within the lesion. As a result, more immune cells are attracted to the atherosclerosis-prone site, eventually forming artery tertiary lymphoid structures in the adventitia and aggravating inflammation. This chronic inflammation, in addition to necrotic core formation and calcification, reduces plaque stability, which can promote plaque erosion or rupture. *Adapted with permission from Porsch and Binder. (2024)Nat. Rev. Cardiol 21(11):780-807.*[25]

Experimental models of atherosclerosis

Although significant progress has been made in the development of *in vitro* microvasculature models that can replicate aspects of the healthy vasculature and shear stress in atherosclerosis, these models have not yet reached the level of accuracy necessary to mimic the complex etiology of atherosclerotic plaque formation.[26,27] Experimental animal models, therefore, remain essential for studying the multifactorial nature of plaque progression, including lipid accumulation, immune cell infiltration,

and the dynamic interactions between different cell types and extracellular matrix components. Atherosclerosis has been investigated in a variety of species, including zebrafish, rats, rabbits, pigs, and non-human primates. However, the mouse remains the most widely used animal model for atherosclerosis research, due to its rapid reproduction and short lifespan, cost-effective maintenance, ease of genetic manipulation and well-characterized genetics and physiology.[28] It must be noted that mice show a distinct lipoprotein profile compared to humans. Unlike humans, who transport most of their cholesterol in atherogenic LDL particles, wildtype C57Bl/6 mice primarily transport cholesterol in anti-atherogenic high-density lipoprotein (HDL) particles, making them naturally resistant to the development of atherosclerosis. To overcome these differences, mouse models with genetic and dietary modifications that better mimic human atherosclerosis are available. The two most commonly used genetically modified strains are the LDL receptor knockout (*Ldlr*$^{-/-}$) and the apolipoprotein E knockout (*Apoe*$^{-/-}$) mice, which exhibit significantly elevated levels of total plasma cholesterol, particularly in the form of VLDL and LDL, leading to the development of atherosclerotic plaques.[29]

Deficiency in the *Apoe* gene results in plasma cholesterol levels between 400 and 600 mg/dL, allowing accelerated atherosclerosis progression upon a standard laboratory chow diet (containing 4-6% fat and <0.03% cholesterol) in *Apoe*$^{-/-}$ mice.[30] When fed a Western-type diet (containing 21% fat and 0.15% cholesterol), these mice develop plasma cholesterol levels around ~1500 mg/dL, promoting atherosclerotic lesion development even more rapidly. Besides dyslipidemia, *Apoe* deficiency exacerbates inflammation. In the absence of *Apoe*, which is normally produced by macrophages, lipid uptake is increased and efferocytosis impaired[31], contributing to a more pro-inflammatory macrophage state.[32] Moreover, *Apoe* prevents excessive T and B cell activation, and inhibits the production of pro-inflammatory cytokines, such as TNFα and IL-6.[33,34] In contrast, *Ldlr* deficient mice show less pronounced immune cell alterations. These mice lack the receptor necessary for clearing LDL particles from the bloodstream, resulting in mild hypercholesteremia, with cholesterol levels around 200-300 mg/dL, when fed a standard diet.[28] To induce more severe hypercholesteremia and accelerate atherosclerosis development in young *Ldlr*$^{-/-}$ mice, feeding a pro-inflammatory Western-type diet is necessary. Notably, this accelerated atherosclerosis induction may not fully capture the complexity of advanced plaques, which gradually develop during the lifetime of humans. In addition to these traditional models, the overexpression of proprotein convertase subtilisin/kexin type 9 (PCSK9) in C57Bl/6 mice has emerged as a powerful model for studying atherosclerosis. PCSK9 is a protein that regulates cholesterol metabolism by promoting the degradation of LDL receptors on hepatocytes, thereby reducing the clearance of LDL cholesterol from the bloodstream.[35] Overexpression of PCSK9 in mice, achieved through viral vector-mediated gene transfer, complemented by a Western-type diet, leads to marked hypercholesterolemia and rapid development of atherosclerotic lesions, similar to atherosclerosis development in *Ldlr*$^{-/-}$ mice.[36]

THE IMMUNE SYSTEM IN ATHEROSCLEROSIS

The immune system, which can be divided in the innate and adaptive immune system, is a crucial defense mechanism that protects the body against infections and tissue damage. Immune cells mostly arise from hematopoietic stem cells in the bone marrow, which differentiate into myeloid and lymphoid lineages.[37] The myeloid lineage gives rise to innate immune cells, including neutrophils, monocytes,

macrophages, mast cells and dendritic cells. These cells are the first line of defense and recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) of invading and endogenous danger.[38] Certain innate immune cells, in particular macrophages and dendritic cells, can activate lymphoid cells of the adaptive immune system via antigen presentation. The adaptive immune system comprises of T and B cells, and although its activation upon first antigen encounter is slow, this arm has the unique ability to develop immunological memory.[39] Both innate and adaptive immune cells play pivotal roles in the development and progression of atherosclerosis.

INNATE IMMUNITY

Monocytes and macrophages

During homeostasis, monocytes circulate in the blood and can either return to the bone marrow or migrate into tissues. There are two main monocyte types: classical monocytes ($CCR2^{+}Ly6C^{high}$ in mice, $CD14^{+}CD16^{-}$ in humans) and non-classical monocytes ($CCR2^{-}Ly6C^{low}$ in mice, $CD14^{low}CD16^{+}$ in humans).[21] Classical monocytes are pro-inflammatory, producing high levels of cytokines and chemokines, and play a key role in atherosclerosis by being recruited to the vessel wall, entering the intimal layer, and differentiating into macrophages.[40] Non-classical monocytes patrol the endothelium and are generally atheroprotective, though higher levels have been linked to carotid intima-media thickening in men in a multicenter, population-based cohort.[41]

Macrophages are central to atherosclerosis development, with functions like phagocytosis, efferocytosis, antigen presentation, and lipid processing. Traditionally classified into pro-inflammatory M1 and anti-inflammatory M2[42], macrophages in atherosclerosis are now known to have a broader range of subtypes, including resident-like macrophages, foamy $TREM2^{+}$ macrophages, interferon-inducible macrophages, and inflammatory macrophages.[43–47] Tissue resident-like macrophages are already present in healthy aortas, where they regulate collagen production by VSMCs and clear apoptotic cells. Although resident-like macrophages can take up cholesterol, thereby initiating foam cell formation during early atherogenesis, it has recently been shown that tissue resident-like macrophages exert atheroprotective properties.[48] Foamy macrophages, however, rapidly outnumber the tissue resident-like macrophages. These macrophages engulf large amounts of LDL, leading to their characteristic 'foamy' appearance. Recent studies have indicated that a large subset of foamy macrophages express TREM2[43,49–51], a surface receptor involved in lipid uptake and metabolism.[52] Lipid-associated TREM2-expressing macrophages initially perform a protective role by processing and storing lipids. Furthermore, $TREM2^{hi}$ macrophages limit necrotic core formation and show a low inflammatory profile, which is associated with the traditional anti-inflammatory M2 phenotype.[53,54] Recent evidence shows that, upon toll-like receptor 2 signaling, intraplaque $TREM2^{hi}$ macrophages can transform into more inflammatory lipid-associated $PLIN2^{hi}/TREM1^{hi}$ macrophages.[55] These $PLIN2^{hi}/TREM1^{hi}$ macrophages contribute to plaque progression by sustaining local inflammation and are associated with cerebrovascular events. Interferon-inducible macrophages also stimulate chronic inflammation by secreting high levels of pro-inflammatory cytokines, including IL-1β, TNFα and CXCL2.[51,56,57] These macrophages also express markers associated with the inflammasome and resemble a more M1-like macrophage phenotype. Their presence is particularly pronounced in advanced plaques, where they contribute to plaque instability by promoting the degradation of the extracellular matrix and thinning the fibrous cap, increasing the risk of plaque rupture and subsequent thrombus formation.

Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) that bridge the innate and adaptive immune system. In atherosclerosis, DCs are found in the intima and adventitia of arteries, where they capture and process antigens derived from modified lipoproteins, apoptotic cells, and other sources.[58,59] Upon antigen internalization, activated DCs travel to draining lymph nodes, where these processed antigens are presented to T cells, leading to their activation, migration and differentiation. The role of DCs in atherosclerosis is complex, as they can drive both pro-atherogenic and protective immune responses depending on the context. For instance, DCs can promote the activation of pro-inflammatory T cells, contributing to plaque progression, while they also have the capacity to induce regulatory T cells (Tregs) that may help to suppress inflammation and stabilize plaques.

Mast cells

Mast cells, traditionally associated with allergic responses, also contribute to the inflammatory milieu in atherosclerosis.[60] They are found in the adventitia and perivascular tissue around atherosclerotic plaques, where they secrete a variety of pro-inflammatory mediators, including histamine, cytokines, proteases, and growth factors. These mediators can degrade the extracellular matrix, promote lipid uptake by macrophages, and enhance the recruitment of additional immune cells to the plaque. The proteases released by mast cells, such as chymase and tryptase, can also weaken the fibrous cap of the plaque, increasing the risk of rupture and subsequent thrombosis.[61]

ADAPTIVE IMMUNITY

T cell development

T cells are a critical component of the adaptive immune response in atherosclerosis. Their development starts with the migration of common lymphoid progenitors (CLPs) from the bone marrow to the thymus, where these cells commit to the T cell lineage under the influence of thymic stromal cells and cytokines such as IL-7.[62] In the early stages of T cell development, known as the double negative (DN) phase, thymocytes lack the CD4 and CD8 co-receptors. These DN thymocytes go through different differentiation stages in which the T cell receptor (TCR) is developed with either α and β or γ and δ chains.[63] At the end of the DN phase, αβ thymocytes transition to double positive (DP) cells, expressing both CD4 and CD8. In this stage, a complete and unique TCR is developed, which is then tested for its ability to recognize self-antigens.[64] TCRs with moderate affinity are positively selected, while those with high affinity receive pro-apoptosis signals. Dependent on the affinity for either major histocompatibility complex (MHC) class I or II molecules, these cells are committed to the $CD8^+$ or $CD4^+$ T cell lineage, respectively. These mature T cells exit the thymus and enter the peripheral circulation, where they contribute to immune surveillance and responses against pathogens.

Naïve T cells become activated primarily in secondary lymphoid organs, such as the lymph nodes and spleen, where they encounter their specific antigen, presented by APCs.[65] Antigen recognition, in combination with co-stimulatory signals from the APC, results in T cell activation. This activation prompts the T cell to proliferate and differentiate into effector T cells, which then leave the lymphoid organ to travel to sites of infection or inflammation to perform their immune functions.

In the context of atherosclerosis, both CD4⁺ and CD8⁺ T cells that recognize modified self-antigens, such as oxLDL, escape the thymic selection process. Upon activation, these autoreactive T cells then migrate to atherosclerotic lesions, contributing to the auto-immune like response.[66]

CD4⁺ T cells

CD4⁺ T cells, also known as helper T (Th) cells, are one of the most extensively studied immune cell types in atherosclerosis. They can differentiate into various subsets, including Th1, Th2, Th9, Th17, Th22, follicular T helper (T_{FH}) cells, and regulatory T cells (Tregs), each playing distinct roles in disease progression (**Figure 2**).

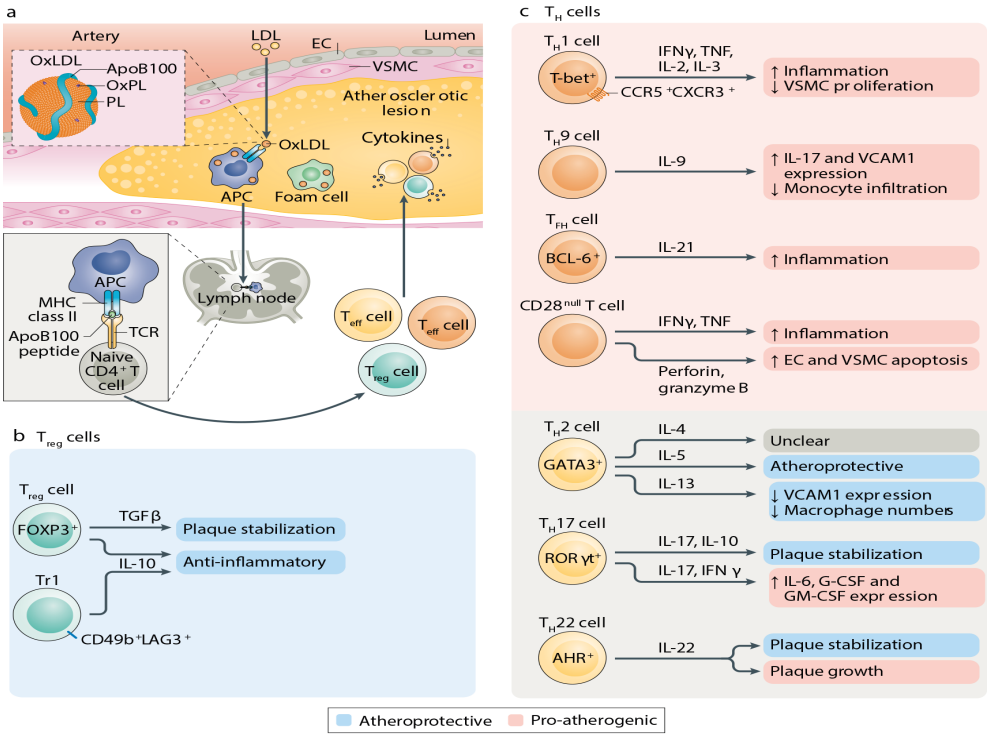

Figure 2. CD4⁺ T cell subsets in the pathology of atherosclerosis. a. Naïve CD4⁺ T cells are activated by presentation of oxLDL peptides on APCs in lymph nodes, after which they differentiate into effector CD4⁺ or regulatory T cells and migrate towards the atherosclerotic lesion. **b.** Regulatory T cells (FoxP3⁺) secrete anti-inflammatory cytokines and promote plaque stabilization. **c.** Effector CD4⁺ T cells can have distinct phenotypes. Atherogenic Th1 (T-bet⁺) cells are the most abundant in the plaque and promote inflammation. Th9 cells secrete IL-9 and stimulate leukocyte infiltration. T_{FH} (BCL-6⁺) cells secrete IL-21 and promote inflammation. CD28^{null} T cells promote inflammation and plaque destabilization. The role of Th2 (GATA-3⁺) cells is less clear, but they mainly exert atheroprotective functions. Th17 (RORγT⁺) promote plaque stabilization but also aggravate inflammation. IL-22, secreted by Th22 (AHR⁺) cells, increases plaque growth and stabilization. *Adapted with permission from Saigusa, Winkels and Ley (2020) Nat. Rev. Cardiol 17(7):387-401.*

Th1

Th1 cells, characterized by the transcription factor TBX21 (T-bet), are the most abundant CD4⁺ T cell subset in the human atherosclerotic plaque.[46,67] Upon stimulation, Th1 cells secrete the pro-inflammatory cytokines IFNγ, IL-2 and TNFα, which promote macrophage activation and stimulate

inflammation. Deficiency in T-bet or IFNγ both resulted in reduced atherosclerosis development[68,69], underlining the pro-atherogenic nature of this subset.

Th2

In contrast, Th2 cells, defined by the transcription factor GATA-3, are generally associated with anti-inflammatory responses.[70] Th2 cells produce IL-4, IL-5 and IL-13, which can counterbalance the pro-atherogenic actions of Th1 cells. For instance, IL-4 can inhibit macrophage activation and reduce the production of IFNγ by Th1 cells.[71,72] Deficiency of IL-5 and IL-13 accelerated atherosclerosis lesion development, further suggesting an atheroprotective role.[73,74] An excessive Th2 response, however, can also lead to increased fibrosis, which might contribute to the thickening of the vessel wall and complicate the disease.[75]

Th9

Although the function of Th9 cells in atherosclerosis remains to be elucidated, recent studies have reported pro-atherogenic effects.[76,77] Th9 cells are the primary source of the cytokine IL-9, which is associated with increased IL-17 secretion and mast cell recruitment, thereby aggravating atherosclerosis progression.

Th17

Similar to Th2 and Th9 cells, the nature of Th17 cells in atherosclerosis development is not completely understood. Th17 cells are characterized by the transcription factor RORγT and a major source of IL-17. Studies have shown that IL-17 can exert pro-atherogenic functions by exacerbating immune cell infiltration and chemokine secretion[78–80], but might also increase plaque stability by promoting collagen production.[81,82]

Th22

In atherosclerosis, the role of Th22 cells is complex, as they might help in tissue repair and stability in some contexts, while promoting inflammation and plaque progression in others.[83,84] Th22 express the transcription factor AHR and secrete IL-22, which is involved in vascular repair.

T_{FH}

T_{FH} cells are a subset of pro-atherogenic $CD4^+$ T cells that express the transcription factor BCL-6 and chemokine receptor CXCR5.[85] Under the influence of CXCL13, T_{FH} cells home towards B cell follicles in lymphoid organs, where they are crucial for the formation of germinal centers. Upon interaction with CD40 and IL-6 or IL-21 signals, T_{FH} cells help B cells undergo affinity maturation and class-switch recombination to produce high-affinity antibodies. In the pathogenesis of atherosclerosis, T_{FH}cell expansion has been identified in the circulation of patients and tertiary lymphoid organs in mice.[86,87] Dysregulation of the T_{FH}- germinal center B cell axis in atherosclerosis can drive the production of pro-inflammatory autoantibodies against oxLDL, which can form immune complexes that exacerbate inflammation and contribute to the progression of the disease.[88] Moreover, T_{FH} cells can indirectly contribute to the chronic inflammation characteristic of atherosclerosis by supporting detrimental B cell responses and the formation of aortic TLOs in mice.[87] Pro-inflammatory T_{FH} cells can be counteracted

by regulatory follicular helper T cells (T_{FR}), which inhibit T_{FH} differentiation and promote regulatory B cell expansion.[89] Adoptive transfer of these T_{FR} cells in atherosclerotic mice decreased atherosclerotic plaque burden and the infiltration of pro-inflammatory macrophages.

$CD28^{null}$ T cells

Unlike conventional $CD4^+$ T cells, $CD28^{null}$ T cells lack the CD28 co-stimulatory molecule, which is essential for typical T cell activation and survival. This loss of CD28 is associated with a senescent-like phenotype, characterized by reduced proliferative capacity and altered function.[90,91] Notably, $CD28^{null}$ T cells exhibit increased production of pro-inflammatory cytokines, such as IFNγ and TNFα, contributing to vascular inflammation.[92] They also express cytotoxic molecules like perforin and granzymes, enabling them to induce apoptosis in ECs and VSCMCs, thereby destabilizing atherosclerotic plaques. Additionally, these cells demonstrate resistance to apoptosis due to the downregulation of death receptors like Fas and pro-apoptotic proteins such as Bim and Bax, leading to their accumulation in atherosclerotic lesions.[93] The presence of $CD28^{null}$ T cells has been correlated with increased risk of myocardial infarction and poor cardiovascular outcomes.[94,95]

Tregs

Tregs, defined by their FoxP3 and CD25 expression, are essential for maintaining immune tolerance and preventing excessive immune responses by producing the anti-inflammatory cytokines IL-10 and TGFβ.[96] These cytokines suppress the activation and proliferation of pro-inflammatory T cells, including Th1 and Th17 cells, and reduce the activation of macrophages. Although the number of Tregs in the atherosclerotic lesion is limited[97], depletion of Tregs resulted in accelerated atherosclerosis progression[98,99], underlining the atheroprotective function of this subset. Furthermore, Treg expansion studies have shown that Tregs support the stabilization of plaques by enhancing collagen production and inhibiting the degradation of the extracellular matrix[100–102], highlighting the therapeutic potential of promoting Treg responses.[103,104]

$CD8^+$ T cells

Compared to $CD4^+$ T cells, the role of cytotoxic $CD8^+$ T cells in atherosclerosis is less well characterized. Nevertheless, emerging evidence suggests a multifaceted and context-dependent role in plaque development and stability.[105] $CD8^+$ T cells can recognize and kill cells presenting MHC class I antigens. In the early stages of atherosclerosis, this cytotoxic activity, mediated by the release of perforin and granzyme B, contributes to endothelial dysfunction, thereby increasing vascular permeability and lipid infiltration.[106] Moreover, $CD8^+$ T cells secrete pro-inflammatory cytokines, such as IFNγ and TNFα, which further amplify the inflammatory response in atherosclerosis.[107] As the plaque progresses, their continued cytotoxic activity and production of pro-inflammatory cytokines can lead to the apoptosis of smooth muscle cells and degradation of the extracellular matrix, promoting plaque rupture. Nevertheless, atheroprotective functions of $CD8^+$ T cells, mostly exerted by regulatory $CD8^+$ T cells[108], have also been described. Depletion of $CD8^+$ T cells in advanced stages of atherosclerosis resulted in an increased pro-inflammatory Th1 response and plaque destabilization and immunization-induced $CD8^+$ T cell expansion reduced atherosclerotic lesion development.[109,110] The distinct effects exerted by $CD8^+$ T cells might be caused by different subpopulations identified in atherosclerotic lesions.[46,66,67,111,112]

γδ T cells

γδ T cells represent a small subset of T cells that express a distinct TCR composed of γ and δ chains.[113,114] Unlike conventional αβ T cells, γδ T cells do not require antigen presentation by MHC molecules and can respond rapidly to stress signals, making them an important part of the innate-like immune response in atherosclerosis. Albeit in small numbers, γδ T cells are found in atherosclerotic plaques, where they can produce pro-inflammatory cytokines like IL-17, contributing to local inflammation and plaque progression. Additionally, γδ T cells may influence the activity of other immune cells, such as macrophages and DCs, further modulating the inflammatory environment within the plaque.

B cells

Next to T cells, the adaptive immune system consists of B lymphocytes, which are traditionally known for their role in humoral immunity and antibody production. B cells also arise from common lymphoid progenitors. In the bone marrow, CLP differentiate into pre-pro, pro- and pre-B cells, during which a unique B cell receptor (BCR) is developed.[115] Similar to TCRs, BCRs undergo selection procedures before immature B cells are approved to leave the bone marrow, after which they enter the peripheral circulation as transitional B cells. Transitional B cells migrate to secondary lymphoid organs, such as the spleen and lymph nodes, where they further mature and undergo additional selection processes to ensure tolerance. Transitional B cells that pass through the selection processes become mature, naive B cells.[116] Mature B cells express both IgM and IgD on their surface and circulate through the blood and lymphoid tissues, ready to encounter their specific antigen. Follicular (FO) B cells are the most common subset of mature B cells, which reside in the follicles of lymph nodes and the spleen.[117] They participate in T cell-dependent immune responses, where they can be activated by $CD4^+$ T cells upon encountering their specific antigen. Located mainly in the spleen, marginal zone (MZ) B cells respond rapidly to blood-borne pathogens and are involved in T cell-independent immune responses.[118] Predominantly found in the peritoneal and pleural cavities, B1 cells are a unique subset that arise from distinct B cell progenitors, produce natural antibodies and play a role in early defense against pathogens.[119–121] B1 cells are also involved in T cell-independent responses and can self-renew, maintaining a steady state of natural antibodies in the body. Upon activation, mature B cells can differentiate into plasma cells, which are specialized for antibody production and secretion.[122] Some activated B cells differentiate into memory B cells, which persist in lymphoid organs to provide a rapid and robust immune response upon subsequent encounter with the same antigen.

Different B cell subsets contribute differently to atherosclerosis.[123] B1 cells, which in mice can be subdivided in the B1a and B1b subpopulations, are generally considered protective in atherosclerosis. The natural IgM antibodies they produce can recognize and bind to oxLDL-specific epitopes (OSEs) and apoptotic cells, facilitating their clearance through opsonization and promoting their uptake by phagocytes.[124] This process helps prevent the formation and progression of atherosclerotic plaques. Studies have shown that adoptive transfer of B1 cells or their IgM antibodies decreased susceptibility to atherosclerosis.[125,126] Other protective B cell subsets include marginal zone B cells, which exert atheroprotective functions by the production of natural IgM and the inhibition of pro-inflammatory Tfh responses[127,128], and regulatory B cells (Bregs), which secrete the anti-inflammatory IL-10 cytokine.[129] However, the majority of mature B cells comprise the FO B cell subset. Multiple studies demonstrated

that FO B cells and FO B cell-derived plasma cells aggravate atherosclerosis by their production of IgG and release of pro-inflammatory cytokines, such as IL-6.[88,130,131]

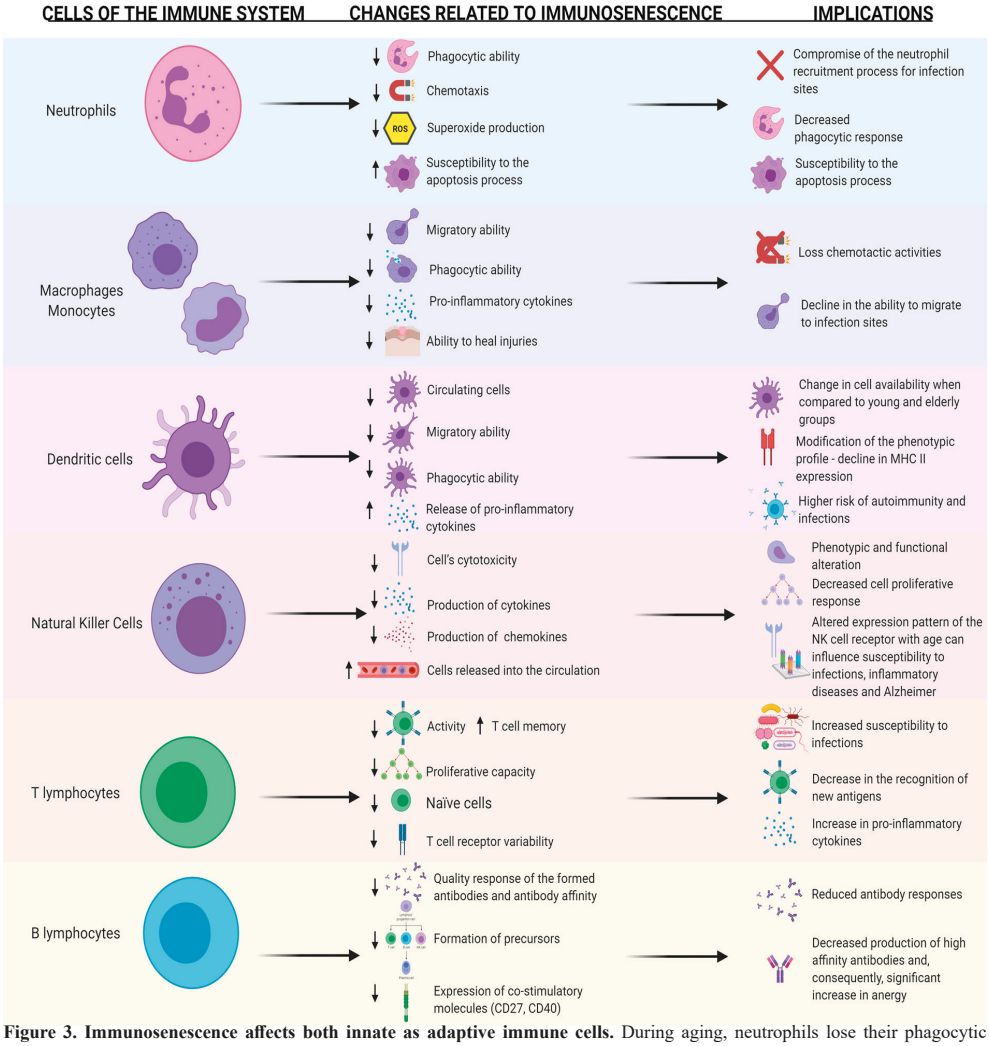

Figure 3. Immunosenescence affects both innate as adaptive immune cells. During aging, neutrophils lose their phagocytic and chemotactic ability, resulting in compromised neutrophil recruitment and increased neutrophil apoptosis. Monocytes and macrophages also show reduced migratory and phagocytic capacities, leading to a decline in chemotaxis. In addition to reduced migration and phagocytosis, dendritic cells also become more pro-inflammatory upon aging. Natural Killer cells become less cytotoxic, thereby increasing susceptibility to inflammatory diseases. T lymphocytes show reduced activity, receptor variability and proliferative capacity, resulting in a decreased recognition of new antigens. Moreover, B lymphocyte, and subsequent antibody, production is dysfunctional. *Adapted from Rodrigues et al (2021) Cytokine Growth Factor Rev. 59:9-21.*[132]

AGING OF THE IMMUNE SYSTEM: IMMUNOSENESCENCE

Aging profoundly impacts the immune system, leading to a decline in immune function, increased susceptibility to infections, and a higher risk of chronic inflammatory diseases such as atherosclerosis. This phenomenon, referred to as immunosenescence, involves changes in both the innate and adaptive arms of the immune system (**Figure 3**).[132] Additionally, aging is associated with inflammaging, a chronic

low-grade inflammation that further contributes to age-related pathologies. Key processes involved in aging of the immune system include myeloid skewing in the bone marrow, thymic involution, and the development of the senescence-associated secretory phenotype (SASP).[133]

Hematopoietic stem cells (HSCs) in the bone marrow give rise to all blood cells, including immune cells. In young individuals, HSCs can differentiate into both myeloid and lymphoid lineages, maintaining a balance between innate and adaptive immune cells. However, aging disrupts this balance, leading to a phenomenon known as myeloid skewing.[134] The increased production of myeloid cells, such as neutrophils, monocytes, and macrophages, occurs at the expense of lymphoid cells, particularly B and T cells. This shift results in a reduced output of lymphoid progenitors and a decline in the diversity and function of adaptive immune cells. Moreover, the diversity of T cells is declined upon aging due to thymic involution[135], leading to a reliance on memory T cells. This shift impairs the ability to respond to novel infections and may contribute to the accumulation of dysfunctional, pro-inflammatory, senescent T cells. Besides diminished T cell responses, B cells in the aged immune system produce antibodies with lower affinity, less diversity, and impaired class-switching, thereby reducing the effectiveness of humoral immunity.[132] Although the number of myeloid cells generally increases upon aging, the chemotactic, cytotoxic and phagocytotic capability of neutrophils, macrophages, dendritic cells and natural killer cells, is declined.

In addition to structural and functional changes, aging leads to an accumulation of senescent cells. Cellular senescence is a state of irreversible cell cycle arrest, marked by the high expression of cell cycle suppressors p16, p21 and p53, that occurs in response to various stressors, including DNA damage, oxidative stress, and telomere shortening.[136] Although senescence prevents the proliferation of damaged cells, the upregulation of the pro-survival kinase mTOR and anti-apoptotic Bcl-2 proteins promote senescent cells to remain metabolically active and develop resistance to apoptosis.[137] Senescent cells develop a distinct phenotype characterized by the secretion of a variety of pro-inflammatory cytokines, chemokines, growth factors, and proteases, collectively known as the SASP.[138] Chronic SASP expression contributes to tissue dysfunction, inflammation, and the progression of age-related diseases.

CELLULAR AGING AND ATHEROSCLEROSIS

It has become clear that aging is a major risk factor for atherosclerosis through various mechanisms at the cellular level. In addition to immunosenescence, studies have demonstrated that aging influences endothelial cells, VSMCs, and other components of the vascular system.[139] With age, endothelial cells lose their ability to produce nitric oxide[140], a molecule that helps dilate blood vessels and maintain a healthy vascular tone. This leads to reduced vasodilation, increased oxidative stress, and heightened vulnerability to damage from circulating lipids and immune cells. Moreover, aged endothelial cells exhibit increased permeability[141], allowing for easier infiltration of LDL and increased atherogenesis.[142] In response to aging, VSMCs become more prone to proliferation and migration into the intima.[143] This shift is associated with changes in the production of extracellular matrix components, such as increased collagen deposition and reduced elastin, resulting in arterial stiffness. This stiffening increases blood pressure, which can further damage the endothelium and promote atherosclerosis. In contrast, when VSMCs become senescent, they lose their ability to proliferate and produce matrix components.[144]

Senescent VSMCs may also undergo osteogenic differentiation, leading to vascular calcification, a hallmark of advanced atherosclerotic lesions. Calcified plaques are more rigid and prone to rupture, which can augment acute cardiovascular events. Like endothelial and immune cells, senescent VSMCs also exhibit the pro-inflammatory SASP, thereby further aggravating atherosclerosis progression.

Most experimental research on atherosclerosis therapies has been conducted in relatively young animals, which correspond to a human equivalent age around 20-30 years. This presents a challenge, as cardiovascular disease patients who are in need for such treatments are often of advanced age with an aged vascular and immune system, making it difficult to directly apply these findings to clinical settings. As evidenced by Simo *et al.* aging is accompanied with a decreased expression of membrane cholesterol transporters on macrophages, resulting in cholesterol accumulation and increased susceptibility to atherosclerosis progression.[145] Furthermore, multiple studies demonstrated that age-associated mitochondrial and vascular dysfunction promote inflammation and aggravate atherosclerosis development in mice.[142,146,147] In addition, senescent cells have been identified in the atherosclerotic plaque, and their presence has been associated with accelerated atherosclerosis progression and increased inflammation.[148] Incorporating aging in atherosclerosis studies is therefore crucial to better understand immune cell behavior and responses in the cardiovascular disease patient. Moreover, recent studies have demonstrated that CVD patients exhibit signs of accelerated biological aging, including shortened telomere length in leukocytes and increased T cell senescence compared to healthy age-matched individuals.[149,150] Therapeutic anti-aging strategies and the elimination of senescent cells have therefore emerged as promising anti-atherosclerotic approaches.[151–153] Senolytics selectively induce apoptosis in senescent cells, whereas senomorphics suppress SASP production.[154] Various classes of senolytics have been developed and senolytic mechanisms of action include disruption of anti-apoptotic proteins Bcl-2 and Bcl-x, inhibition of mTOR-induced cell survival, nuclear exclusion of p53, PI3K inhibition and proteasomal degradation.[155–158]

SINGLE-CELL APPROACHES TO STUDY THE CELLULAR LANDSCAPE IN ATHEROSCLEROSIS

In order to develop new anti-atherosclerotic immune therapies for the aged CVD patient, it is important to gain more insights into immune cell heterogeneity, crosstalk and function during atherosclerosis progression. Historically, immune cell diversity within plaques was explored using immunostaining techniques in the 1980s[159], which allowed researchers to detect and visualize only two markers simultaneously. Over time, this approach approved, and by combining various staining methods, it is now possible to examine up to 16 markers concurrently.[160,161] However, these methods are limited in scope and provide only a partial view of the complex cellular environment within plaques. When flow cytometry was introduced into atherosclerosis research, it quickly became a powerful tool for studying heterogenous immune cell populations at the single-cell level. Flow cytometry offers significant advantages, including its speed, cost-effectiveness, and ability to analyze large numbers of cells.[162] Moreover, it provides insights into immune cell phenotypes by allowing the simultaneous analysis of multiple markers, keeping it still a widely used method in atherosclerosis research today. Limitations in flow cytometry stem from the overlap of fluorescence signals, making it nowadays still difficult to analyze more than 20 markers without signal interference or autofluorescence. Spectral

flow cytometry technology reduces signal interference and autofluorescence, extending the number of markers that can be distinguished simultaneously to >40. The introduction of mass cytometry, or Cytometry by Time of Flight (CyTOF), with heavy metal-tagged antibodies in 2009 allowed to detect up to 50 markers simultaneously, far surpassing the capabilities of traditional flow cytometry.[163] This increased dimensionality offered a more detailed and comprehensive view of the cellular landscape in atherosclerotic plaques.

In parallel, single-cell RNA sequencing (scRNA-seq) was introduced, bringing an entirely new level of resolution to study gene expression in individual cells. Unlike bulk transcriptomics, which averages gene expression across large populations of cells or tissues, scRNA-seq enabled measuring the gene expression profiles of individual cells, allowing for the identification of distinct cellular subpopulations without prior knowledge of specific markers. Single-cell transcriptomics thus provides an unbiased method to characterize the diversity of cells in heterogeneous environments, offering insights into the molecular mechanisms driving disease progression. Although single-cell omics are still relatively new in cardiovascular research, the rapid development of these technologies has enabled the integration of multiple omics layers, including genome, epigenome, transcriptome, proteome, metabolome, and immune repertoire, thereby already providing invaluable insights into cellular function and identity in atherosclerosis. In 2018, the immune transcriptome in young *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice was mapped using single-cell RNA sequencing analysis, revealing heterogeneity in macrophage subsets and the abundance of T cells inside the atherosclerotic lesion.[43,49] This murine data was followed by the single-cell immune landscape in human carotid plaques, confirming the high percentage of lesional T cells.[46,67] In the past years, additional transcriptomic data of the atherosclerotic lesion has become available, allowing the integration of different atherosclerosis models and further identification of myeloid and lymphoid subsets.[164,165] Nevertheless, the effects of aging on the immune transcriptome in atherosclerosis remain to be investigated.

THESIS OUTLINE

In this thesis, single-cell approaches were applied to characterize the impact of aging on the immunological B cell landscape in different models of atherosclerotic cardiovascular disease, with the goal to identify and study new B cell-associated biomarkers and therapeutic targets to halt atherosclerosis progression.

In **chapter 2** we provide an overview of the aged B cell in health and disease. We describe how aging affects the proliferation and function of distinct B cell subsets, and their possible effect in atherosclerosis development. In **chapter 3**, we characterized the impact of aging on atherosclerosis progression in *Ldlr*$^{-/-}$ mice and illustrated that aging promotes more advanced atherosclerotic lesions, enriched in collagen, cholesterol crystals, and calcification. In addition, we identified age-associated immune cells, such as age-associated B cells (ABCs), and revealed increased immunosenescence in the aged atherosclerotic environment, using single-cell RNA sequencing and flow cytometry. We compared the morphology and immune landscape of atherosclerotic lesions between aged female and aged male *Ldlr*$^{-/-}$ mice in **chapter 4**, and show that age-associated B cells are more pronounced in females and that female B cells exhibit a more activated phenotype. In **chapter 5**, we induced atherosclerosis in non-atherosclerotic aged C57Bl/6 mice, providing insight into how immunosenescence influences disease development.

We demonstrate that the immune system of aged mice shows immunosenescent features, including the emergence of ABCs and increased antibody production, which was accompanied with aggravated atherosclerosis development in aged mice compared to young mice. To investigate the role of age-associated B cells in atherosclerosis development, we further characterized these cells in **chapter 6**. We show that ABCs predict coronary events in humans and are clonally expanded in aged atherosclerotic mice. Upon adoptive transfer, we reveal that ABCs differentiate into plasma cells, thereby exacerbating lesion development. In **chapter 7**, we identified a new anti-atherosclerotic strategy with IFNγ-stimulated B cells by significant upregulation of the inhibitory ligand PD-L1. We demonstrated that these cells inhibit follicular T helper cell responses and halt atherosclerosis progression. To further explore anti-atherogenic therapies in the aged CVD patient, we aimed to reduce advanced atherosclerosis with rapamycin treatment in **chapter 8**. We show that rapamycin was able to restrict inflammation in the aged atherosclerotic lesion and reduced the frequency of ABCs, which might stabilize the atherosclerotic lesion and reduce the risk for a cardiovascular event. **Chapter 9** provides a summary and discussion of all the data presented in this thesis, along with concluding remarks and future perspectives.

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